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Abstract: The most significant accomplishment of the last year was the first demonstration of mechanochemical coupling (chemomechanical transduction) in synthetic polypeptides. The demonstration was achieved, not by copy of a known contractile protein but rather by a design based on a new principle of contraction which was derived independently of a known contractile protein. The new principle, described as chemical modulation of an inverse temperature transition, was derived from studies of the polypentapeptide of elastin, poly(VPGVG), and analogs. Chemical modulation of the temperature of an inverse temperature transition and the resulting contraction and relaxation has been achieved in two distinctly different ways:

In the solvent-based mode, increases in concentrations of salts such as NaCl lower the temperature of a transition of γ-irradiation cross-linked poly(VPGVG) which results in thermomechanical transduction at the lower temperature. Therefore isothermally at an intermediate temperature, an increase in salt concentration causes contraction and a decrease in salt concentration causes relaxation. This provides a new process and a new material for desalination.
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19. Abstract - Continued

In the polymer-based mode an amino acid with a functional side chain is introduced into the polypentapeptide at the level of a few residues per 100 residues of polypeptide. In the example demonstrated poly[4(VPGVG),(VPGEG)] was prepared such that the glutamic acid residue (E) provides the carboxyl (COOH)/carboxylate(COO^-) chemical couple. At neutral pH the cross-linked polypentapeptide is relaxed, and on lowering the pH the elastomeric matrix contracts. In principle this approach should work with any functional group and by any chemical processes which reversibly changes the polarity of the functional group.

The molecular process is considered to be due to an aqueous mediated apolar-polar interaction free energy and is considered to be of fundamental importance in protein folding. It is proposed for example, that phosphorylation/dephosphorylation modulate protein structure and function in this manner.

ANNUAL REPORT

Contract Title: Development of Elastomeric Polypeptide Biomaterials

Contract Number: N00014-86-0402

I. BRIEF SUMMARY OF PROJECT GOALS: (Same as Previously)

To design, prepare and characterize novel elastomeric polymers comprised of repeating peptide sequences, primarily the elastin pentamer and analogs of it alone and combined with repeating related hexapeptides and/or tetrapeptides. The purpose is to develop polymers with different elastic moduli and increased extension limits, polymers with different temperature ranges for their inverse temperature transitions over which elastomeric force dramatically changes, polymers in which different heat changes effect the large changes in elastomeric force, polymers with different intensities and frequencies of their dielectric relaxations and polymers with wider temperature ranges over which they function as nearly ideal elastomers. In the elastomer design, the dominant repeat units will be pentamers and tetramers. Hexamers and alanine-rich, lysine-containing cross-linking sequences will be used to fine-tune properties.

II. SUMMARY OF ACCOMPLISHMENTS IN THE SECOND YEAR

Since the last report, 8 papers have been published, 4 additional manuscripts are in press, and two patent applications have been filed. In what follows are the abstracts from the publications and manuscripts followed by a brief summary of the progress toward the microbial synthesis of the polypentapeptide.

A. *Publications*

1. D.K. Chang and D.W. Urry, "Molecular Dynamics Calculations on Relaxed and Extended States of the Polypentapeptide of Elastin", Chem. Phys. Lets., In press.

Abstract: Reported are the first molecular dynamics calculations on the elastomeric polypentapeptide of elastin as $(VPGVG)_7$. The salient points are that 1) there is little change in internal energy on extension; 2) a trajectory of 50 ps is insufficient to reflect the primary structural periodicity in RMS displacements of torsion angles, but does show librational processes and their damping on extension; and 3) the recurring β-turn structure is retained.

2. D.W. Urry, "Free Energy (Chemomechanical) Transduction in Elastomeric Polypeptides by Chemical potential Modulation of an Inverse Temperature Transition.", Intl. J. Quantum Chem.: Quantum Biol. Symp. 15, in press.

Abstract: Data and analyses are presented on the first synthetic polypeptide system to exhibit mechanochemical coupling; the mechanochemical coupling can also be demonstrated to be both polymer-based and solvent-based with respect to where the result of the change in the chemical potential is focused. Both polymer-based and solvent-based processes are the result of chemomechanical transduction in which the change in chemical potential results in a change in temperature at which an inverse temperature transition occurs. In the polymer-based process, the contraction/relaxation occurs due to a change in the chemical nature of the polypeptide; in the solvent-based process there is no change in the chemical nature of the polypeptide on contraction or relaxation, but rather the change in chemical potential changes the state of hydration of the polypeptide.

The new mechanochemical system provides an experimental system with which to clarify and to quantitate what may be called aqueous mediated apolar-polar interaction energies in polypeptides and proteins with hydrophobic groups that may be variously exposed to the aqueous solution or buried within the folded polypeptide

or protein. Furthermore, it is noted that any conformational change exhibited by a polypeptide or protein that is the result of a binding of a chemical moiety, the change in chemical nature of a bound moiety or the change in chemical potential of the medium can be viewed in terms of mechanochemical coupling or chemomechanical transduction.

3. D.W. Urry, R.D. Harris and K.U. Prasad, "Chemical Potential Driven Contraction and Relaxation by Ionic Strength Modulation of an Inverse Temperature Transition," J. Am. Chem. Soc., 110. 3303-3305, 1988..

Abstract: The sequential polypentapeptide of elastin, (L-Val^1-L-Pro^2-Gly^3-L Val^4-Gly^5)$_n$ when cross-linked by γ-irradiation and when in equilibrium with water undergoes a reversible contraction on raising the temperature from 20°C to 40°C. That this is the result of an inverse temperature transition has been shown by many physical characterizations and is also evidenced by observing in water that analogues which are more hydrophobic undergo the transition at lower temperatures, whereas less hydrophobic analogues undergo the transition at higher temperatures. In this communication, it is demonstrated that a change in salt concentration causes a shift in the temperature of the inverse temperature transition and in particular that contraction and relaxation can be achieved by such changes in ionic strength. To our knowledge, this is the first demonstration that changes in chemical potential can produce contraction and relaxation in a neutral polymer and in particular in a synthetic polypeptide containing only aliphatic (Val and Pro) or no (Gly) side chains where the process is one of ionic strength modulation of an inverse temperature transition.

4. D.W. Urry, B. Haynes, H. Zhang, R.D. Harris and K.U. Prasad, "Mechanochemical Coupling in Synthetic Polypeptides by Modulation of an Inverse Temperature Transition," Proc. Natl. Acad. Sci., USA, in press.

Abstract: For the polypentapeptide of elastin, (L-Val-L-Pro-Gly-L-Val-Gly)$_n$, and appropriate analogs when suitably cross-linked, it has been previously

demonstrated that development of elastomeric force at fixed length and length changes at fixed load occur as the result of an inverse temperature transition, with the temperature of the transition being inversely dependent on the hydrophobicity of the polypeptide. This suggests that at fixed temperature a chemical means of reversibly changing the hydrophobicity could be used for mechanochemical coupling. Evidence for this mechanism of mechanochemical coupling is given here with a 4%-Glu-polypentapeptide, in which the valine in position 4 is replaced in 1 out of 5 pentamers by a glutamic acid residue. Before cross-linking, the temperature for aggregation of 4%-Glu-polypentapeptide is remarkably sensitive to pH, shifting from 25°C at pH 2 to 70°C at pH 7.4 in phosphate-buffered saline (PBS). At 37°C, the cross-linked 4%-Glu-polypentapeptide matrix in PBS undergoes a pH-modulated contraction and relaxation with a change from pH 4.3 to 3.3 and back. The mean distance between carboxylates at pH 4.3 in the elastomeric matrix is greater than 40 Å, twice the mean distance between negatively charged species in PBS. Accordingly, charge-charge repulsion is expected to make little or no contribution to the coupling. Mechanochemical coupling is demonstrated at fixed load by monitoring pH dependence of length and at constant length by monitoring pH dependence of force. To our knowledge, this is the first demonstration of mechanochemical coupling in a synthetic polypeptide and the first system to provide a test of the recent proposal that chemical modulation of an inverse temperature transition can be a mechanism for mechanochemical coupling. It is suggested that phosphorylation and dephosphorylation may modulate structure and forces in proteins by locally shifting the temperatures of inverse temperature transitions.

5. D.W. Urry, "Entropic Elastic Processes in Protein Mechanism. Part 2. Simple (Passive) and Coupled (Active) Development of Elastic Forces," J. Protein Chem., 7(2), 81-114, 1988.

Abstract: The first part of this review on entropic elastic processes in protein mechanisms demonstrated with the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ that elastic structure develops as the result of an inverse temperature transition and that entropic elasticity is due to internal chain dynamics in a regular nonrandom structure. This demonstration is contrary to the pervasive perspective of entropic protein elasticity of the past three decades wherein a network of random chains has been considered the necessary structural consequence of the occurrence of dominantly entropic elastomeric force. That this is not the case provides a new opportunity for understanding the occurrence and role of entropic elastic processes in protein mechanisms. Entropic elastic processes are considered in two classes: passive and active. The development of elastomeric force of a chemical process shifting the temperature of a transition of elastomeric force as the result of a chemical process shifting the temperature of a transition is class II (active). Examples of class I are elastin, the elastic filament of muscle, elastic force changes in enzyme catalysis resulting from binding processes and resulting in the straining of a scissile bond, and in the turning on and off of channels due to changes in transmembrane potential. Demonstration of the consequences of elastomeric force developing as the result of an inverse temperature transition are seen in elastin, where elastic recoil is lost on oxidation, i.e., on decreasing the hydrophobicity of the chain and shifting the temperature for the development of elastomeric force to temperatures greater than physiological. This is relevant in general to loss of elasticity on aging and more specifically to the development of pulmonary emphysema. Since random chain networks are not the products of inverse temperature transitions and the temperature at which an inverse temperature transition occurs depends on the hydrophobicity of the polypeptide chain, it now becomes possible to consider

chemical processes for turning elastomeric force on and off by reversibly changing the hydrophobicity of the polypeptide chain. This is herein called mechanochemical coupling of the first kind; this is the chemical modulation of the temperature for the transition from a less-ordered less elastic state to a more-ordered more elastic state. In the usual considerations to date, development of elastomeric force is the result of a standard transition from a more-ordered less elastic state to a less-ordered more elastic state. When this is chemically modulated, it is herein called mechanochemical coupling of the second kind. For elastin and the polypentapeptide of elastin, since entropic elastomeric force results on formation of a regular nonrandom structure and thermal randomization of chains results in loss of elastic modulus to levels of limited use in protein mechanisms, consideration of regular spiral-like structures rather than random chain networks or random coils are proposed for mechanochemical coupling of the second kind. Chemical processes to effect mechanochemical coupling in biological systems are most obviously phosphorylation-dephosphorylation and changes in calcium ion activity but also changes in pH. These issues are considered in the events attending parturition in muscle contraction and in cell motility.

6. D.W. Urry, "Entropic Elastic Processes in Protein Mechanisms. Part 1. Elastic Structure due to an Inverse Temperature Transition and Elasticity due to Internal Chain Dynamics," J. Protein Chem., 7(1), 1-34, 1988.

Abstract: Numerous physical characterizations clearly demonstrate that the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ in water undergoes an inverse temperature transition. Increase in order occurs both intermolecularly and intramolecularly on raising the temperature from 20 to 40°C. The physical characterizations used to demonstrate the inverse temperature transition include microscopy, light scattering, circular dichroism, the nuclear Overhauser effect, temperature dependence of composition, nuclear magnetic resonance (NMR) relaxation, dielectric relaxation, and temperature dependence of elastomer length. At

fixed extension of the cross-linked polypentapeptide elastomer, the development of elastomeric force is seen to correlate with increase in intramolecular order, that is, with the inverse temperature transition. Reversible thermal denaturation of the ordered polypentapeptide is observed with composition and circular dichroism studies, and thermal denaturation of the cross-linked elastomer is also observed with loss of elastomeric force and elastic modulus. Thus, elastomeric force is lost when the polypeptide chains are randomized due to heating at high temperature. Clearly, elastomeric force is due to nonrandom polypeptide structure. In spite of this, elastomeric force is demonstrated to be dominantly entropic in origin. The source of the entropic elastomeric force is demonstrated to be the result of internal chain dynamics, and the mechanism is called the librational entropy mechanism of elasticity. There is significant application to the finding that elastomeric force develops due to an inverse temperature transition. By changing the hydrophobicity of the polypeptide, the temperature range for the inverse temperature transition can be changed in a predictable way, and the temperature range for the development of elastomeric force follows. Thus, elastomers have been prepared where the development of elastomeric force is shifted over a 40°C temperature range from a midpoint temperature of 30°C for the polypentapeptide to 10°C by increasing hydrophobicity with addition of a single CH_2 moiety per pentamer and to 50°C by decreasing hydrophobicity. The implications of these findings to elastic processes in protein mechanisms are (1) When elastic processes are observed in proteins, it is unnecessary, and possibly incorrect, to attempt description in terms of random chain networks and random coils; (2) rather than requiring a random chain network characterized by a random distribution of end-to-end chain lengths, entropic elastomeric force can be exhibited by a single, short peptide segment; (3) perhaps of greatest significance, whether occurring in a short peptide segment or in a fibrillar protein, it should be possible reversibly to turn elastomeric force on and off by

reversibly changing the hydrophobicity of the polypeptide. Phosphorylation and dephosphorylation would be the most obvious means of changing the hydrophobicity of a polypeptide. These considerations are treated in Part 2: Simple (Passive) and Coupled (Active) Development of Elastic Forces.

7. D.W. Urry, "Of Molecules, Motion, Man and Machines," Ala. J. Med. Sci., in press.

8. D.W. Urry, "Bioelastics: A New Dimension in Biomaterials, Applications", Research & Development Magazine, in press.

Abstract: Certain polypeptides comprised of repeating sequences are the basis of a new class of biomaterials. These elastomeric polypeptides are life-like biomaterials. They can be made to match the compliance of natural biological tissues and when useful can be modified to elicit desirable tissue reactions at the cellular and enzymatic levels. They can be designed to contract and to relax and to do work as the result of a change in chemical potential in a manner analogous to the production of motion in all living organisms. They are functional in an aqueous environment and can be optimized for physiological temperatures making biomedical applications all the more appropriate.

In terms of industrial applications these biomaterials have the properties to function as sensors based on demonstrated physical properties of thermomechanical and chemomechanical transduction. With this class of biomaterials, contraction can be achieved by reversible chemical modulation of an inverse temperature transition such that stretching becomes the free energy input for reversing the chemical process, and in one design the biomaterial would become a mechanochemical engine which on driving in reverse would achieve desalination. Among the biomedical applications under development and consideration are synthetic arteries, a material to prevent post-surgical and post-trauma adhesions, burn cover, synthetic ligaments and targeted drug delivery.

9. R. Buchet, C.-H. Luan, K.U. Prasad, R.D. Harris and D.W. Urry, "Dielectric Relaxation Studies on Analogs of the Polypentapeptide of Elastin," J. Phys. Chem. 92(2), 511-517, 1988.

Abstract: Dielectric measurements of the complex permittivity of coacervate concentrations of two analogues of the polypentapeptide of elastin, $(Xxx^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_m$ where Xxx is Val for the elastin polypentapeptide and Ile and Leu for the two analogues, were taken over the frequency range 1-1000 MHz and over the temperature range 0-60°C. Two relaxation processes were observed in each polypentapeptide. One relaxation has a frequency centered in the low megahertz frequency range, which has been attributed to a low-frequency librational mode within the polypeptide. The other relaxation is located near the gigahertz frequency range. The magnitude of the dielectric increment, $\Delta\varepsilon$, of the librational mode of each polypentapeptide analogue increases with increasing temperature from near zero at 0°C to approximately 40 at 60°C, showing an inverse temperature transition to a more ordered structure. Conversely, the magnitudes of the dielectric increment of the high-frequency relaxation decrease with increasing temperature and differ in approximate proportion to the hydrophobicity of the pentamer for the polypentapeptide of elastin and the two analogues at temperatures below the inverse temperature transition. It is suggested that clathrate-like water surrounding hydrophobic side chains contributes to the high-frequency relaxation.

10. D.W. Urry, "Elastic Molecular Machines and a New Motive Force in Protein Mechanisms," Materials Biotechnology Symposium Proceedings (David L. Kaplan, ed.), U.S. Army Natick Research, Development and Engineering Center, Technical Report Natick/TR-88/033, 25-42, 1988

Abstract: It is demonstrated that the polypentapeptide, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ when γ-irradiation cross-linked, can perform work on raising the temperature from 20° to 40°C. This is due to an inverse temperature transition leading to a regular helical structure called a dynamic β-spiral, which exhibits entropic elastomeric force. Processes which alter the hydrophobicity of a peptide

segment can shift the temperature of an inverse temperature transition. When the hydrophobicity is changed reversibly as is possible with 20% Glu^4-polypentapeptide, the temperature for the onset of the inverse temperature transition can be reversibly shifted from being initiated at 37°C at pH 2 (COOH) to being initiated at 50°C at pH 7 (COO^-). Presumably therefore once a synthetic elastomeric matrix is formed from 20% Glu^4-polypentapeptide, it should be possible at 50°C to turn "on" elastomeric force by changing the pH from 7 to 2 and to turn "off" elastomeric force by returning the pH to 7. This is called mechanochemical coupling of the first kind, and, in addition to ionization and deionization, it should be possible similarly to turn off and on elastomeric force by phosphorylation and dephosphorylation, respectively.

When the elastomeric state is arrived at by means of a regular transition from a more ordered state (e.g., α-helix) to a less ordered state (e.g., a spiral) on raising the temperature and a chemical process can change the temperature of the transition, this is referred to as mechanochemical coupling of the second kind. Mechanochemical coupling on-going from an ordered state to a disordered state has often been considered. The studies on the polypentapeptide bring consideration of an inverse temperature transition for mechanochemical coupling of the first kind and of a less-ordered but nonrandom state for mechanochemical coupling of the second kind. It is proposed that these new considerations are relevant to mechanisms for the turning on and off of elastic forces in protein mechanisms as varied as those of enzymes and muscle contraction.

11. K.U. Prasad, M. Iqbal and D.W. Urry, "Synthesis of Two Component Models of Elastin", Proceedings of the Tenth American Peptide Symposium, 1987, Peptides-Chemistry & Biology (G.R. Marshall, ed.) Escom Beiden, 399-403, 1988.

Abstract: The sequence data between the cross-linking regions of tropoelastin, obtained by tryptic digestion, is almost completely determined. The largest tryptic

peptide is about 80 AA long and contains 11 (in pig) or 13 (in chick) pentamer (VPGVG) repeats in a continuous sequence. The polypentapeptide, $(VPGVG)_n$, on γ-irradiation cross-linking has been shown to be an entropic elastomer. Two natural cross-linking sequences frequently observed are AAAAKAAKY(F)GA (XL-1) and AAKAAAKAA (XL-2). Two chains of tropoelastin having such sequences cross-link enzymatically by lysyloxidase to form the native elastin. The cross-links utilize four lysines in the formation of desmosine and isodesmosine structures which are substituted pyridiniums. One of the four lysines, the one preceding Y or F, contributes to the nitrogen atom of the heterocyclic ring having been protected from oxidization by lysyloxidase. To test the cross-linking hypothesis and possibly to obtain a biomaterial suitable as a substitute for native elastin, we have prepared polypentapeptide attached to each of the cross-linking sequences (XL-1 and XL-2). Here we present the synthesis of two chains of 86 and 84 AA long, XL-1-$(VPGVG)_{15}$ and XL-2-$(VPGVG)_{15}$, by the solid phase methodology. After characterization by amino acid analysis and NMR, they are activated as p-nitrophenyl esters and polymerized to yield Poly [XL-1-$(VPGVG)_{15}$]-OH and Poly[XL-2-$(VPGVG)_{15}$]-OH with molecular weights demonstrated by dialysis to be greater than 50 kD. This is the first demonstration that monomeric chains of such length (86 or 84 AA) could be polymerized to such a degree. These two polymers are then subjected to lysyloxidase individually and as a mixture to investigate the formation of desmosine and isodesmosine cross-links.

12. D.W. Urry, "Entropic Elastomeric Force in Protein Structure/Function," Int. J. Quantum Chem.: Quantum Biol. Symp., 14, 261-280, 1987.

B. Microbial Synthesis of the Polypentapeptide

Over the past year we have continued to make progress towards expression of elastin polypentapeptide sequences in a microbial system. In the previous report we described the synthesis of overlapping oligonucleotides that, after hybridization and enzymatic extension, yielded a 150 base pair synthetic gene. Two different nucleotide sequence variants of the basic coding sequence were made in order to account for potential problems in prokaryotic codon usage - in particular the preferential use of a single proline codon by bacteria.

The double stranded synthetic genes were initially cloned into the single-stranded phage vector M13mp18; the size of the inserts were confirmed by gel electrophoresis and nucleotide sequence analysis, and the genes were subcloned into the double stranded inducible expression vector pUC118 by transferring an EzaR1-Pst (190bp) fragment. The presence of the polypentapeptide coding sequence was confirmed by restriction endonuclease mapping and by Maxam-Gilbert sequencing of the insert. Since bacteria containing the pUC118 plasmids with either the JG1/JG2 or JG3/JG4 synthetic genes yielded blue colonies in the presence of 1PTG and XGal, it was concluded that both genes could be translated as polypentamer -α-subunit -β-galactosidase fusion proteins. To investigate the size and abundance of the fusion protein (which would be predicted to be 13-14kd), cells were grown overnight in normal medium containing glucose, diluted 1:100 in B-broth and grown to an O.D. of 0.6 prior to induction with 1PTG. These initial experiments yielded faint, poorly reproducible bands of the expected molecular weight although in some experiments diffuse bands at a M.W. of approximately 40kd were seen in cell extracts of bacteria carrying the JG3/JG4 insert. Given the unusually high proline content of the polypentapeptide sequence (20%) abnormal migration on SDS-polyacrylamide gels is high likely. These experiments clearly dimenstrate that the highly reiterated polypentapeptide sequence can be propagated in a stable fashion in bacterial plasmids and the products consistent with those expected from a polypentapeptide-β-Gal fusion could be observed. However, despite these promising results it became clear that the pUC expression system would not be appropriate for the long term

goals of this project. First: the levels of fusion protein seen after induction were insufficient for scale-up; second the construction yielded a fusion protein too small for easy detection and characterization.

In an effort to overcome these problems we have attempted to develop expression of the polypentapeptide sequence using two additional expression vector systems. In addition, in order to produce biological products that approach the sizes of those obtained by chemical synthesis we have constructed dimer and trimer inserts of the polypentamer sequences. The ability to synthesize in bacteria sequences of 50, 100, and 150 amino acids should facilitate biophysical studies on the microbial-derived product. In order to generate these multimeric inserts the overlapping oligonucleotides were hybridized and extended as before, sized on a polyacrylamide gel, phosphorylated and then ligated at moderate concentrations. The preligated inserts were then blunt-end ligated into the SmaI site of pUC18. Plasmids containing suitably sized B inserts were selected and the inserts subjected to sequence analysis either by the Maxam-Gilbert chemical degradation method or by the Sanger dideoxy-method. Initial sequencing results indicated that monomer, dimer and trimer sequences capable of encoding polypentapeptide of 50, 100 and 150 amino acids had been obtained and that these could be stably propagated in E. coli. In colloration with Dr. Casey Morrow, Department of Microbiology, the monomer, dimer and trimer inserts were removed from the pUC plasmid by EcoRi/Pst I digests and inserted into the pATH3 vector of Koorner et al. This vector utilized the highly efficient inducible tryprophan operator-promoter system for expression of foreign genes. Thus bacteria can be grown to high density then depleted of tryptophan and induced to express by addition of the tryp. competitor indole acrylic acid (1AA). The pfTH vectors have the advantage that the inserted gene is preceded by TrpE coding sequences that encode a 34kd protein, and followed immediately by a termination codon. Thus the presence of foreign coding sequences can be assayed by a mobility shift of the TrpE-polypentamer fusion protein. In addition to providing a method for assesing the size of the translated protein sequences, the large size of the product makes identification by gel

electrophoresis and immunological blotting procedures much more straightforward. The vector also has the advantage that, once expression has been confirmed, the bulk of the TrpE coding sequencing can be removed by cutting the plasmid with unique restriction enzymes. The synthetic gene insert can then be expressed with just a minimal leader sequence. Initial experiments with this vector in which we cloned the monomer-polypentamer sequence are promising with a larger polypeptide being observed, however the dimer and trimer inserts appeared to generate molecules that were rapidly degraded. We therefore re-analysed the sequence of these multimeric inserts to confirm their coding potential and have found that for both dimer molecules a single nucleotide deletion has occured at the start of the coding sequence. Whether this reflects a selection against expression of the long polypentapeptide, or merely mutations missed in our initial sequence analysis is currently under study. However, the pATH vector system appears to be useful for expression of the monomer-insert and we are currently carrying out experiments to confirm the authenticity of this product.

C. Patent Applications

The Development of Entropic Motive Force in Protein Systems and Molecular Machines Using the Same (Ser. #07/062,557, Pending) Filed 06/15/87

Reversible Mechanochemical Engines Comprised of Bioelastomers Capable of Modulable Inverse Temperature Transitions for the Interconversion of Chemical and Mechanical Work (Pending), Filed 03/02/88

III. PLANS FOR YEAR THREE

The primary objectives of the 03 year in the chemical syntheses are to introduce phenylalanine (F) residues into poly(VPGVG) and poly(VPGG) at decreasing percentages in positions 1 and 4 of the polypentapeptide and position 1 of the polytetrapeptide. To date poly(FPGVG), poly(VPGFG) and poly(VPGVGVPGFG) have been synthesized, but in each case the hydrophobicity is so great that the temperature of the inverse temperature transition is below 0°C. This means that solubility in water and the viscoelastic state required for

preparation of the elastomer have not been achieved. The amount of phenylalanine will be further decreased in the polypentapeptide until solubility is achieved in the 0° to 25°C temperature range. Amounts of phenylalanine in the polypeptide will be found that will stepwise lower the transition temperature from 45°C for poly(VPGG) to near 0°C. The approach will initially be to prepare the random sequential polypeptides; poly[n(VPGVG),(VPGFG)], poly[n(VPGVG),(FPGVG)] and poly[n(VPGG),(FPGG)], that will give the desired transition temperatures. Subsequently the fixed sequential polypeptides; poly[$(VPGVG)_n$(VPGFG)], poly[$(VPGVG)_n$(FVGVG)] and poly[$(VPGG)_n(FPGG)_m$] will be prepared. The values of n and m will be chosen from the properties found for the random sequential polypeptides. In each case optimal ranges of γ-irradiation cross-linking will be determined.

With regard to the microbial preparations, once adequate quantities of poly(VPGVG) have been expressed then particular analogs will be prepared.

All of the materials prepared will be physically characterized as has been done in the last two years.

The primary objectives of the 03 year in the microbial synthesis of the polypentapeptide are threefold.

1. Scale up and expression of multimeric polypentapeptide genes for the pATH vector system. Following authentication of the TrpE-PP chimeric protein, we will remove the TrpE coding sequences and confirm expression of the polypentamer alone. This will be facilitated by immunological probes and we will be initiating production of anti-polypentapeptide antibodies in the next few weeks. Additional dimer and trimer inserts will be inserted into the pATH vector system after complete sequence characterization and the insert in the pATH vector will again be sequenced using double-strand sequencing protocols currently in use in this laboratory. Successful insertion and expression of longer inserts will allow larger and biologically more interesting molecules to be made.

2. Utilization of novel expression system. Recent collaborative studies between Dr. Casey Morrow and Dr. Charles Turnbough in the Department of Microbiology, UAB, have yielded a novel bacterial expression system based on the Pyr-B promoter-operator system. This vector has allowed 5-10 times higher levels of expression of the human immunodeficiency virus polymerase than from pATH vectors and we believe it could provide a significant advantage for these studies. Dr. Morrow will collaborate with us on these studies.

3. We will start to synthesize elastin hexamer and poly-hexamer coding sequences in order to construct genes that contain hybrid sequences. These will be particularly important in defining the biophysical properties of these molecules.

MOLECULAR DYNAMICS CALCULATIONS ON RELAXED AND EXTENDED STATES OF THE POLYPENTAPEPTIDE OF ELASTIN

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ABSTRACT

Reported are the first molecular dynamics calculations on the elastomeric polypentapeptide of elastin as $(VPGVG)_7$. The salient points are that 1) there is little change in internal energy on extension; 2) a trajectory of 50 ps is insufficient to reflect the primary structural periodicity in RMS displacements of torsion angles, but does show librational processes and their damping on extension; and 3) the recurring β-turn structure is retained.

INTRODUCTION

The study of the structure and mechanism of elasticity of biological elastin has been an active, albeit controversial, area of research in the past decades (1-8). One of the structural characteristics of bovine and porcine elastin is that it has a long repeating pentapeptide sequence, i.e., $(L\text{-}Val^1\text{-}L\text{-}Pro^2\text{-}Gly^3\text{-}L\text{-}Val^4\text{-}Gly^5)_n$ with n = 11 (9). As a result, physical properties of the synthetic polypeptide, poly(VPGVG), have been extensively investigated. From experiments on the thermoelasticity of elastin at constant length and volume, Hoeve and Flory concluded that the internal energy component of force accounts for a negligible fraction of the total elastomeric force. Recently, Urry and coworkers studied the thermoelastic behavior of γ-irradiation cross-linked poly(VPGVG) and reached the same conclusion, that is, that the entropy change of the polypeptide with increasing length is the major source of the retractive force. The origin of the entropic mechanism for the elastic peptide is different, however, according to these two groups. Flory and collaborators consider that the entropy decrease on stretching is due to the change in the distribution of end-to-end distance between cross-linking points of peptide chains. The internal degrees of freedom, such as changes in bond angles and torsion angles, are taken into account only in the context of end-to-end distance of peptide chains. A damping of the amplitudes of correlated motions of torsion angles (librations) is considered to be a major source of the entropy decrease on elongation of the polypeptide elastic fiber according to Urry and collaborators,that is, the damping of backbone librational processes of peptide chains upon stretching is taken to be the primary source of entropy decrease. The

primary differences between these two theories are (1) each individual internal degree of freedom (torsion angles, in particular) is taken into consideration by Urry, et al., and (2) the distribution of end-to-end distance of a collection of chains is not central to the mechanism proposed by Urry, et al. This means that entropic elastic force can be generated by a single peptide chain segment and can occur in a regular, nonrandom, albeit dynamic, structure. The helically recurring β-turn structure of poly(VPGVG) is called a β-spiral.

Stereo pair plots of the β-spiral of poly(VPGVG) for seven pentamers with 2.7 pentamers per turn is given in Figure 1A (axis view) and 1B (side view), and in Figure 1C (relaxed) and 1D (extended) are three pentamers, approximately one turn, with a central pentamer unit showing the range of librational processes that can occur within a 2 kcal/mole-residue cut-off energy. In these conformational energy calculations there is seen a damping of the amplitude of librations on extension (6). The calculation was done on a pentadecapeptide, $(VPGVG)_3$, and the damping of libration upon 130% elongation was observed in the (suspended) segment containing the correlated torsion angle pairs $\psi(Val^4)$-$\phi(Gly^5)$ and $\psi(Gly^5)$-$\phi(Val^1)$. The number of accessible states was enumerated by using different cut-off energies above the minimized energy conformation. The entropy change was found to be essentially independent of the value of the cut-off energy. Experimentally, a dielectric relaxation near 10 MHz for the PPP coacervate was observed and interpreted as arising from peptide backbone libration (10).

Since the aforementioned computation was static in nature, i.e., the structure was minimized without explicitly taking account of atomic thermal energy, in the present effort it is of interest to apply molecular dynamics simulation to the polypentapeptide and to address the problem of correlation (or relaxation) time for the librational processes observed in the MHz frequency range, to assess the presence of structural features and to determine the effect of extension on internal energy .

METHODS

The software package, CHARMm (Version 20.3), developed by Karplus and coworkers and adapted by Polygen, Inc., was used in the calculation. The potential energy functions and parameters were as described by Brooks, et al. (11). The harmonic approximation was employed in the energy terms of bond lengths, bond angles and improper torsional potentials. $E_\phi = k_\phi [1 + \cos(n\phi-\delta)]$ was used for the dihedral (torsion) angle energy term and the Lennard-Jones 6-12 potential was used for van der Waals interactions. Since intramolecular interturn interactions are considered to be important in the formation of the β-spiral structure as the result of an inverse temperature transition (12), a polypeptide with seven repeating pentamer units, $(VPGVG)_7$, was used in the molecular dynamics (MD) simulation. For both relaxed and extended states of the β-spiral, a structure with 2.7 pentamer units per turn was used. The axial rise was 9.45 Å per turn in the relaxed state and 21.6 Å per turn in the extended state, corresponding to 130% extension. In the simulation, the amide nitrogen atom of residue one, i.e., Val^1, and the carbonyl carbon atom of residue 35, i.e., Gly^{35}, were fixed in space. Each step in the simulation corresponds to 10^{-15} sec. Both structures were thermalized to 300°K with 0.05°C rise per step. The structures were then equilibrated for 25 ps (picosecond). Finally, the systems were allowed to undergo 50 ps of a molecular dynamics production stage. The algorithm used for integrating the equations of motion adopted in CHARMm was proposed by Verlet (14). The root-mean-square (RMS) fluctuations of backbone torsion angles as well as internal energy (sum of kinetic and potential energy) were noted during the production period.

RESULTS AND DISCUSSION

The average values of total energy and various potential energy terms are presented in Table I, for 50 ps of molecular dynamics production time for both relaxed and extended states of $(VPGVP)_7$. The total energy is lower by 15 kcal/mole for the relaxed state than for the extended state. The primary source for the difference is obviously the van der Waals (VDW) term, which is lower by a similar 17 kcal/mole for the relaxed state. The difference arises

from the interactions between the side chains of peptide residues which are absent or much weaker in the extended state. It has been reported that the extension of elastin fiber immersed in water is an exothermic process with its magnitude four times as large as the elastic work performed on the elastin sample (3). Since the internal energy of the peptide is lower for the relaxed state, it can be deduced from our results that the heat of hydration, or heat of hydrophobic interaction due to the exposure of hydrophobic groups to the the solvent upon elongation, overcomes the loss in VDW interactions. It is also interesting to note that the heat generated upon extension of the elastin fiber decreases as the polarity of the solvent decreases (3). For example, the heat decreases to zero in the 3:7 ethylene glycol/water mixed solvent. The heat also decreases when solvent is changed from pure water to 2 M Methanol to 2 M ethanol to 2 M propanol to pure formamide. This can be rationalized by noting that the heat of solvation is less for the less polar solvents which more closely compensate for the loss of VDW interactions of the peptide chains when they are stretched.

Accordingly for such an elastic system where the chains become solvated on extension, when examining results of an *in vacuo* calculation for the change in internal energy on extension, it is perhaps more appropriate to delete the van der Waals term. In this regard it is seen that the change in internal energy due to changes in bond lengths, bond angles, dihedral angles and improper torsional angles are equal in magnitude but opposite in sign to the change in the electrostatic interaction terms. The perspective therefore for the polypentapeptide β-spiral becomes one of little or no change in internal energy on extension when in an appropriate solvent.

Table 2 gives the 50 ps MD simulation of $(VPGVG)_7$ in terms of RMS displacements of backbone torsion angles in both relaxed and extended states. The polypeptide chain end effects are evident from the magnitude of $\Delta\psi_1^2$ and $\Delta\phi_{35}^2$. A significant point is the lack of pentamer periodicity of RMS displacements for the torsion angles in the same position in each pentapeptide, i.e. $\Delta\phi_l^2 \neq \Delta\phi_{l+5}^2$ or $\Delta\psi_l^2 \neq \Delta\psi_{l+5}^2$. This is most likely due to the fact that the 50 ps of molecular dynamics production time used in the present study is too short because the

experimental correlation time is in the range of 10 ns as determined by both nuclear magnetic resonance (13) and dielectric relaxation measurements (10). In the latter case the measured correlation time is not an average of many different correlation times, but rather it is a discrete relaxation of the Debye-type indicating that the net dipole moment of each pentamer is rocking at the same frequency; the relaxation is due to a pentameric peptide librational mode.

Table 2 is also characterized by the correlated RMS fluctuation values for adjacent torsion angles, i.e., when $\Delta\psi_i^2$ is large, $\Delta\phi_{i+1}^2$ or $\Delta\phi_i^2$ is correspondingly large. For example, $\Delta\psi_{15}^2$, $\Delta\phi_{16}^2$ and $\Delta\psi_{20}^2$, $\Delta\phi_{21}^2$ in the relaxed state; $\Delta\phi_6^2$, $\Delta\psi_6^2$ and $\Delta\phi_{31}^2$, $\Delta\psi_{31}^2$ in the extended state. This corresponds to localized peptide librational or rocking segmental motion. In general, the amplitude of librational motion is larger for suspended segments in the β-spiral structure. In a 35 residue helical structure with 2.7 pentamer repeats per turn, the true internal sequence not subject to end effects involves residues 14 through 21. This sequence is highlighted in Table 2 and includes two putative suspended segments which are the two sets of four torsion angles ψ^{14}, ϕ^{15}, ψ^{15}, ϕ^{16} and ψ^{19}, ϕ^{20}, ψ^{20}, ϕ^{21}. It is apparent that these suspended segments experience the largest amplitude desplacements in both relaxed and extended states and that the displacements are damped on extension.

While the trend of damping of librational motion upon elongation can be observed, it will be necessary to achieve trajectories with times of the order of magnitude of the 10 nsec correlation time for peptide libration as measured from the dielectric relaxation studies and to do so in water before a truly satisfactory molecular dynamics characterization is achieved. Nevertheless this initial effort clearly provides much valuable insight.

It is also significant to note that the β-turns of $(VPGVG)_7$ are retained after bringing the temperature to 300°K, after 25 ps of equilibration period, and after 50 ps of molecular dynamics production time both for relaxed and for extended states of polypentapeptide. Specifically, for the relaxed state, the average C_i-N_{i+3} distance is equal to 4.05 ± 0.10 Å for the static minimum energy structure before equilibration, 4.30 ± 0.15 Å after equilibration and 4.30 ± 0.3 Å after 50 ps of production time. For the extended state,the average C_i-N_{i+3}

distance is equal to 4.20 ± 0.90 Å before equilibration, 4.45 ± 0.90 Å after equilibration and 4.25 ± 1.2Å after 50 ps of production time. Furthermore using the pentadecapeptide the β-turns were maintained after 0.12 nsec of trajectory time. Therefore the recurrng β-turn, from which the β-spiral derives its name, is a structural feature of the elastomeric polypentapeptide of elastin in both the earlier molecular mechanics and now the molecular dynamics characterizations. As circular dichroism (15), nuclear magnetic resonance (17) and Raman spectroscopic studies (18) demonstrate the recurring β-turn in solution, the *in vacuo* calculations demonstrating the same structural feature are demonstrably of relevance to the solution state.

This elastomeric polypeptide system takes an added significance with recent studies. When high molecular weight polypentapeptide, i.e., n > 120, is synthesized and γ-irradiation cross-linked, the elastomeric matrix exhibits mechanochemical coupling in response to changes in salt concentration (19). Furthermore when there are four glutamic acid residues included per 100 residues of polypeptide in place of Val^4, the elastomeric matrix exhibits mechanochemical coupling in response to changes in pH (20) This is the first synthetic polypeptide system to exhibit mechanochemical coupling and the process involves a newly described mechanism of chemical modulation of an inverse temperature transition (21).

SUMMARY

In this first report of molecular dynamics simulations of the elastomeric polypentapeptide of elastin represented by $(L\text{-}Val^1\text{-}L\text{-}Pro^2\text{-}Gly^3\text{-}L\text{-}Val^4\text{-}Gly^5)_7$, it is demonstrated that 1) when the *in vacuo* van der Waals interaction terms are neglected, the change in internal energy on extension is small as required for a dominantly entropic elastomer, 2) the RMS displacements for the backbone torsion angles do not reflect the periodicity of the primary structure indicating that 50 ps is an insufficient trajectory time, 3) the magnitudes of the RMS displacements for the backbone torsion angles tend to be greater in the suspended segments defined as the peptide segment from the Val^4 α-carbon of the i^{th} repeat

to the Val^1 α-carbon of the i + 1^{st} repeat, 4) the RMS displacements of the suspended segments are damped on extension, 5) there is a correlation in the magnitude of the RMS displacements of the torsion angles on each side of a peptide moiety reflecting librational processes, and 6) the β-turn, defined as the Val_i C···$NVal_{i+3}$ distance, within a pentamer remains throughout the molecular dynamics simulation both in the relaxed and extended states.

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Table I Internal Energy Components of $(VPGVG)_7$ in Relaxed and Extended States Resulting From a 50 ps Molecular Dynamics Simulation [a,b]

	Relaxed	Extended	Δ(Extended-Relaxed)
Total E	352	367	+15
Total Potential Energy	37	56	+19
van der Waals	-56	-39	+17
Bond length	68	68	0
Bond angle	209	206	-3
Dihedral angle	88	86	-2
Improper torsional angle	22	21	-1
Electrostatic terms	-295	-287	+8

[a] Values are rounded off to the nearest kcal/mole.

[b] The potential energies listed are relative to the minimum value of the potential functions with respect to the given internal coordinates. Total energy is defined as the sum of total potential energy and total kinetic energy, with 0°K used as the reference for zero atomic kinetic energy.

Table 2. Root mean square (RMS) fluctuation of torsion angles (ϕ and ψ) of $(VPGVG)_7$ after 25 ps of equilibration time and 50 ps of molecular dynamics simulation.

Torsion Angle	Relaxed	Extended
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
ϕ4	9.2	9.7
ψ4	10.1	10.9
ϕ5	7.7	8.8
ψ5	8.2	11.8
ϕ6	11.2	26.2
ψ6	9.7	22.2
ϕ7	9.5	6.8
ψ7	9.7	17.4
ϕ8	12.4	10.4
ψ8	10.1	28.2
ϕ9	9.9	9.4
ψ9	15.3	10.8
ϕ10	14.3	7.8
ψ10	9.6	11.1
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
ϕ12	10.0	6.9
ψ12	[illegible]	[illegible]

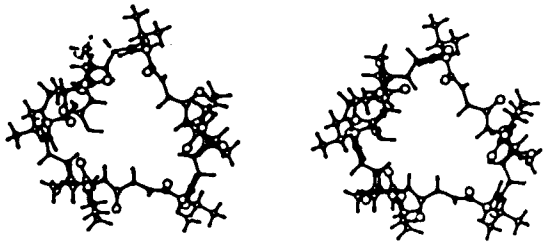
Torsion Angle	Relaxed	Extended
ϕ13	6.6	8.2
ψ13	10.1	8.5
ϕ14	8.9	10.7
suspended segment: ψ14	10.1	10.0
suspended segment: ϕ15	15.6	14.3
suspended segment: ψ15	12.5	10.6
suspended segment: ϕ16	9.7	12.2
ψ16	7.2	9.7
ϕ17	7.3	8.8
ψ17	9.6	10.1
ϕ18	8.4	8.0
ψ18	9.5	7.8
ϕ19	9.0	7.1
suspended segment: ψ19	9.1	8.7
suspended segment: ϕ20	11.7	12.2
suspended segment: ψ20	40.0	11.8
suspended segment: ϕ21	23.7	11.8
ψ21	11.2	7.4
ϕ22	16.2	8.6
ψ22	14.7	13.2
ϕ23	9.7	10.0
ψ23	9.2	10.7
ϕ24	9.6	7.9

Torsion Angle	Relaxed	Extended
ψ24	10.5	8.9
ϕ25	8.6	9.2
ψ25	9.8	8.9
ϕ26	13.4	29.2
ψ26	13.1	10.9
ϕ27	11.5	11.1
ψ27	36.0	12.7
ϕ28	53.8	8.4
ψ28	37.3	12.7
ϕ29	9.1	8.5
ψ29	11.4	12.3
ϕ30	16.5	8.5
ψ30	11.0	12.7
ϕ31	13.1	23.3
ψ31	10.5	17.5
ϕ32	9.5	6.0
ψ32	30.0	11.6
ϕ33	36.4	10.2
ψ33	10.3	27.8
ϕ34	6.9	19.4
ψ34	17.1	82.6
ϕ35	78.8	58.5

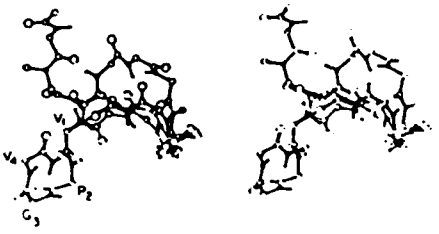
FIGURE LEGEND

Figure 1 β-spiral of the polypentapeptide of elastin, $(VPGVG)_n$, with n = 7 in A and B and with n = 3 for C in the relaxed and for D in a state at 130% extension.

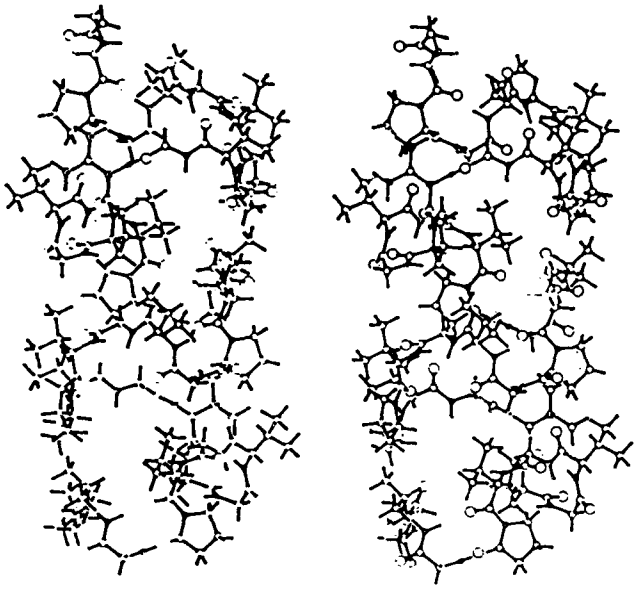
β-Spiral of the Polypentapeptide of Elastin



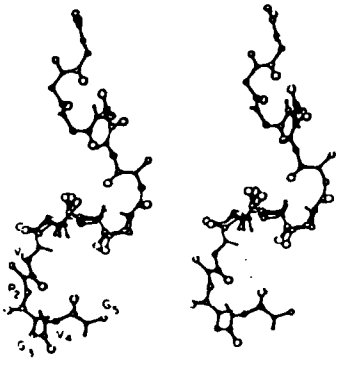
A. axis view



C. relaxed



B. side view



D. extended

FIGURE 1

FREE ENERGY (CHEMOMECHANICAL) TRANSDUCTION IN ELASTOMERIC POLYPEPTIDES BY CHEMICAL POTENTIAL MODULATION OF AN INVERSE TEMPERATURE TRANSITION

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"The isothermal conversion of chemical metabolic energy into mechanical work underlies the motility of all living organisms."

A. Katchalsky

FREE ENERGY (CHEMOMECHANICAL) TRANSDUCTION IN ELASTOMERIC POLYPEPTIDES BY CHEMICAL POTENTIAL MODULATION OF AN INVERSE TEMPERATURE TRANSITION

OUTLINE

I. INTRODUCTION

II. MECHANISMS OF MECHANOCHEMICAL COUPLING

- A. The Charge-Charge Repulsion Mechanism of Mechanochemical Coupling
- B. An Apolar-Polar Repulsion Mechanism of Mechanochemical Coupling in Water

III. CARBOXYL/CARBOXYLATE MODULATION OF CONTRACTION/RELAXATION: The 4% Glu-Polypentapeptides

- A. A Hydrophobicity-Induced Shift in pK_a
- B. A Stretch-Induced Shift in pK_a

IV. IONIC STRENGTH MODULATION OF CONTRACTION/RELAXATION

V. GENERALIZATION AND EXTRAPOLATIONS

ABSTRACT:

Data and analyses are presented on the first synthetic polypeptide system to exhibit mechanochemical coupling; the mechanochemical coupling can also be demonstrated to be both polymer-based and solvent-based with respect to where the result of the change in the chemical potential is focused. Both polymer-based and solvent-based processes are the result of chemomechanical transduction in which the change in chemical potential results in a change in the temperature at which an inverse temperature transition occurs. In the polymer-based process, the contraction/relaxation occurs due to a change in the chemical nature of the polypeptide; in the solvent-based process there is no change in the chemical nature of the polypeptide on contraction or relaxation, but rather the change in chemical potential changes the state of hydration of the polypeptide.

The new mechanochemical system provides an experimental system with which to clarify and to quantitate what may be called aqueous mediated apolar-polar interaction energies in polypeptides and proteins with hydrophobic groups that may be variously exposed to the aqueous solution or buried within the folded polypeptide or protein. Furthermore, it is noted that any conformational change exhibited by a polypeptide or protein that is the result of a binding of a chemical moiety, the change in chemical nature of a bound moiety or the change in chemical potential of the medium can be viewed in terms of mechanochemical coupling or chemomechanical transduction.

I. INTRODUCTION

One year ago at the 1987 Sanibel Symposium was presented the concept that it should be possible to achieve mechanochemical coupling by chemical modulation of the hydrophobicity of elastomeric polypeptides that undergo reversible, thermally induced contraction at transition temperatures that could be varied by changing the hydrophobicity of the polypeptide (1). The parent elastomeric polypeptide was the polypentapeptide of elastin, $(L\text{-}Val^1 - L\text{-}Pro^2 - Gly^3 - L\text{-}Val^4 - Gly^5)_n$ also called PPP, which, when γ-irradiation cross-linked with n>120 form elastomeric matrices in water which contract to less than one-half the 20°C length on raising the temperature to 40°C. When the polypentapeptide was made more hydrophobic as in $(L\text{-}Ile^1 - L\text{-}Pro^2 - Gly^3 - L\text{-}Val^4 - Gly^5)_n$ also called Ile^1-PPP and γ-irradiation cross-linked, the contraction occurred between 0 and 20°C (2). When the sequential polypeptide was made less hydrophobic by removal of the Val^4 residue to give $(L\text{-}Val^1 - L\text{-}Pro^2 - Gly^3 - Gly^4)_n$, also called PTP, and similarly cross-linked, the contraction occurred between 40° and 60°C (3). These data are schematically shown in Figure 1. Therefore, the proposal was that if the sequential polypentapeptide were made with inclusion of a residue containing a functional side chain that could, by a reversible chemical process, be made more and less hydrophobic, that is, more or less polar, then it should be possible by changes in chemical potential to achieve contraction and relaxation (1). This has been achieved by the simple expediency of including four glutamic acid residues per 100 residues of polypentapeptide with the substitution being at residue four (4). The formal description is poly [(VPGVG), (VPGEG); 4:1] where V,P,G and E stand for Val, Pro, Gly and Glu, respectively. The structure is referred to as 4%Glu-PPP and when 20 Mrad γ-irradiation cross-linked, the elastomeric matrix is labelled X^{20}-4%Glu-PPP. When in phosphate buffered saline (PBS), which is 0.15 N NaCl and 0.01 M phosphate and when at 37°C, the elastomeric matrix contracts on lowering the pH and relaxes near neutral pH; this is shown in Figure 2. The elastomeric matrix is capable of lifting and setting down weights that are 1000 times its own dry weight. This represents the first synthetic polypeptide or model protein system to exhibit mechanochemical coupling and the mechanism, chemical modulation of

an inverse temperature transition, represents a new process for achieving mechanochemical coupling. The following constitutes a brief review of the past year's progress in achieving and characterizing this new mechanism, which is viewed as being relevant to protein mechanisms in general. It will begin, however, by describing and delineating between the two presently demonstrated mechanisms for mechanochemical coupling: charge-charge repulsion and chemical modulation of an inverse temperature transition.

II MECHANISMS OF MECHANOCHEMICAL COUPLING

In polymer-based mechanochemical coupling a chemical couple is utilized. It can be two states of a functional side chain of an amino acid residue or of a covalently attached functional moiety, or the couple could be an empty and an occupied binding site. In the present analysis, the carboxyl/carboxylate anion chemical couple will be initially considered. In mechanochemical coupling in general, a change in chemical potential brings about either a change in length, or a change in force, or both in a cross-linked elastic polymer. In the example of the carboxyl/carboxylate couple, the change in chemical potential is a change in proton potential which changes the ratio of [COOH] to [COO^-]. Of particular interest in delineating mechanisms, is the sign of the partial differential of chemical potential with respect to force at constant polymer composition, which could be stated as at constant degree of ionization, i.e., $(\partial\mu_i/\partial f)_{n_j}$ where μ_i and f are the chemical potential and force, respectively, and the partial differential is at constant composition, n_j. The sign of the partial differential is reversed depending on whether the mechanism for the carboxyl/carboxylate couple involves charge-charge repulsion or a new mechanism described as chemical modulation of an inverse temperature transition which may, with some license, be termed apolar-polar repulsion in an aqueous system. This will be briefly analyzed in what immediately follows and experimental data for the latter new mechanism will be given in the subsequent section.

A. The Charge-Charge Repulsion Mechanism of Mechanochemical Coupling

The charge-charge repulsion mechanism of mechanochemical coupling was first described by Katchalsky and Kuhn and colleagues (5,6), using the polymer polymethacrylic acid, $\left[- \underset{COOH}{\overset{CH_3}{C}} CH_2 - \right]_n$. When this mechanochemical system is approximately 20% ionized, it is largely contracted to an extent limited by the repulsion between anions. On stretching, due to an increase in applied force, there is an increase in the mean distance between anions which relieves the repulsion between anions and allows more carboxyls to release their protons in the formation of more anions (7). There is an increase in the acidity of the surrounding solution due to a decrease in the pK_a of the chemical couple. Equivalently, if the solution is made more acidic by an increase in the chemical potential of protons or addition of HCl, i.e. $\Delta\mu_i$ is positive, then the carboxylate anions become protonated lowering the charge-charge repulsion and the elastomer will contract with the development of force such that Δf is also positive. Accordingly, $(\partial\mu_i/\partial f)_{n_i}$ is positive (6).

B. An Apolar-Polar Repulsion Mechanism of Mechanochemical Coupling in Water.

The present polypeptide mechanism is not based on repulsion of like charges but rather it may be described as a water mediated apolar-polar repulsion. It is based on the antagonism in an aqueous system of polar and nonpolar (hydrophobic) chemical moieties; it is a particular expression of the well-known salt effect on the solubility of hydrophobic solutes (8,9), and it has its expression in polypeptides or proteins as the chemical modulation of the temperature of inverse temperature transitions. An inverse temperature transition involves, of course, the folding of a polypeptide or protein in such a way as to maximize hydrophobic contacts, that is, to remove hydrophobic groups from aqueous interaction. Its basis is the hydrophobic effect (10-12). If the mechanochemical system involves an elastic matrix of a folded relatively hydrophobic polypeptide with occasional glutamic acid residues, e.g. 2 to 4 per 100 residues, then stretching the elastomer results in exposure of hydrophobic side chains and increases the free energy of carboxylate anions with the result of a shift toward protonation. On stretching, therefore, such a matrix would take up protons rather than release protons and the pK_a would increase as the result of the increase in force of stretching. An increase in pK_a means a

decrease in proton potential. In terms of the interesting partial differential there is a decrease in the chemical potential of protons with an increase in force, that is $(\partial\mu_i/\partial f)_{n_i}$ is negative. Using the same chemical couple, the sign is exactly opposite for a mechanism involving apolar-polar repulsion than for a mechanism of charge-charge repulsion. This provides a diagnostic means for determining the dominant mechanism in a contractile polypeptide or protein as will be shown below for the carboxyl/carboxylate anion couple.

It should be noted for a chemical couple involving a protonated cationic species as in the imidazole/imidazolium couple of histidine that the sign would be reversed for both mechanisms with respect to the chemical potential of protons. It should also be appreciated that the chemical modulation of the temperature of an inverse temperature transition, i.e., the so-called apolar-polar repulsion mechanism, can utilize the entire polarity scale from the most hydrophobic to the most hydrophilic.

III. CARBOXYL/CARBOXYLATE MODULATION OF CONTRACTION/RELAXATION: The 4%Glu-Polypentapeptides.

When the sequential polypeptide, poly[(VPGVG), (VPGEG); 4:1], is synthesized, there are four Glu residues per 100 residues of polypentapeptide. The most ready characterization of pH dependence of the temperature of the inverse temperature transition is to scan temperature while following solution turbidity starting from low temperature where the polypeptide is soluble in PBS (phosphate buffered saline: 0.15 N NaCl, 0.01 M phosphate). A set of such scans, called temperature profiles of turbidity formation or TPτ's, are given in Figure 3A. A remarkable pH dependence is seen for this measure of the temperatures at which the inverse temperature transitions occur; the temperature for the onset of the transition shifts from a low of 25°C at low pH (2.5) to a high of 70°C at high pH (7.0). The curves of Figure 3A approximate, after the polypentapeptide is cross-linked, for a given pH the temperature over which contraction occurs (4).

A. A Hydrophobicity-Induced Shift in pK_a.

When the more hydrophobic sequential polypeptide, poly[(IPGVG), (IPGEG); 4:1] where I is Ile, is prepared, the temperature for the inverse temperature transition for a given pH, as expected for a more hydrophobic polypeptide, is shifted to a lower temperature (see Figure 3B). Also, very significantly for understanding mechanism, as is most readily seen from the inserts of Figures 3A and B, the plots of temperature of the inverse temperature transition versus pH are displaced for the Val^1 and Ile^1 4%Glu-polypentapeptides. Using the midpoint of the curve to estimate pK_a, for 4%Glu-PPP the pK_a is 4.4, whereas, for the more hydrophobic 4%Glu- Ile^1- PPP the pK_a is 5.4. For the more hydrophobic polypentapeptide the pK_a is raised by one pH unit. When the study utilizes the γ-irradiation cross-linked elastomers and measures, at a similar force per cross-sectional area for each of the elastomers, the length versus pH, the same result is obtained (see Figure 4A). The pK_a is raised for the more hydrophobic elastomer by approximately one pH unit (13). Therefore, either the carboxyl moiety is more stabilized or the carboxylate anion is destabilized in the more hydrophobic elastomer.

Both polypentapeptides have the same number of Glu residues, per 100 residues of polypeptide, and the two dimensional nuclear Overhauser enhancement spectroscopy (2D-NOESY) data for both show no differences in conformation (13). Also, both parent polypentapeptides exhibit the same circular dichroism pattern before the transition and, though the CD patterns change after the transition, they are again the same for both polypentapeptides (2). Therefore, the pK_a shift is not due to some difference in hydrogen bonding of the Glu side chain to the polypeptide backbone. It appears necessary to seek an explanation for the pK_a shift in terms of the change in hydrophobicity per se. This perspective will be confirmed below where the evidence is for an increase in the free energy of the more polar species in situations where there is more expression of hydrophobicity by the polypeptide.

B. A Stretch-Induced Shift in pK_a.

The contracted elastomer results from the optimization of hydrophobic interactions within the matrix both intramolecularly and intermolecularly. Thus on stretching the elastomer, there is necessarily an exposure of hydrophobic groups to the aqueous medium. The hydrophobic side chains become solvated in an exothermic reaction resulting in clathrate-like water surrounding the hydrophobic groups. The question being addressed here is whether or not this exposure of hydrophobicity and formation of clathrate-like water affects the pK_a of the Glu residues in the matrix. The data is given in Figure 4B. With a small load on the elastomer of 1.2×10^4 dynes/cm^2, the apparent pK_a is 4.1. When the load is increased to 1.3×10^5 dynes/cm^2, the apparent pK_a becomes 4.6. Therefore, in the same X^{20}-4%Glu-PPP matrix the pK_a increases on stretching. This is exactly the opposite of what happens in the polymethacrylic acid system where charge-charge repulsion is the mechanism and reflects what may, by analogy, be called an apolar-polar repulsion exhibited in an aqueous system. In particular, from the data in Figure 4B $(\partial\mu_i/\partial f)_{n_i}$ is negative and is approximately -1×10^7 cm/mole (15). In what follows it will be shown that the apolar-polar repulsion can be described as an increase in the free energy of ionic species on increasing the expression of hydrophobicity of the elastomeric polypeptide system.

IV. IONIC STRENGTH MODULATION OF CONTRACTION/RELAXATION

When the parent polypentapeptide, $(VPGVG)_n$, is γ-irradiation cross-linked to form simply X^{20}-PPP, it has been shown that the temperature for the inverse temperature transition is lowered on going from pure water to PBS as schematically shown with the thermoelasticity curves of Figure 5 (16). This means that increased salt solution shifts the equilibrium to the folded state where expression of hydrophobicity is minimized. At an intermediate temperature say 25°C, the elastic matrix relaxes and lengthens in the presence of pure water and it contracts on addition of the salt solution. Thus, there is a solvent-based mechanochemical coupling which demonstrates that the structural transition can be entirely

solvent mediated; in this case there is no change in the chemical nature of the polymer, but there is a change in the free energy of solvation. Most significantly, the result directly demonstrates the antagonism between the presence of polar ions and the expression of hydrophobicity. Therefore, it can be concluded that the pK_a shifts, which are observed on increasing the hydrophobicity of the matrix either by Val^1 replacement by Ile^1 or by stretching, are due to an increase in the free energy of the carboxylate anion.

V. GENERALIZATION AND EXTRAPOLATIONS:

General Considerations: The elastomeric strips that can now be prepared and are now being characterized provide an experimental system with which to clarify and to quantify what are here referred to as apolar-polar interaction energies in an aqueous environment. Thus by changing the hydrophobicity of the polypeptide the free energy of the anionic species changes as can be measured by the change in pK_a with $\mu = 2.3\ RTpK_a$. Analogously, by applying a force to the elastomer and measuring the shift in pK_a, it becomes possible to assess the hydrophobicity change. (In fact the polymers provide the opportunity to develop a hydrophobicity scale covering essentially the complete polarity scale). This data supplemented with direct calorimetric measurements of the heat and temperatures of the transitions along with temperature dependence of pK_a for relaxed and loaded states will provide a thermodynamic characterization of these mechanochemical systems. Also, of great interest, will be the equivalent Carnot cycle in the force vs. length plane, for example, where it will be possible to determine the efficiency with which these elastomeric strips may function as reversible mechanochemical engines.

Of further significance is that these mechanochemical systems may be operated using functional groups ranging in polarity from the most hydrophobic to the most hydrophilic. In place of the carboxyl/carboxylate couple could be any acid/base couple. More generally, it could be any chemical couple which involved a change in hydrophobicity such as, for example, a redox couple. The chemical potential change could be used to drive a mechanochemical engine, or

by putting in the mechanical energy of stretching the mechanochemical system could be used to develop concentration gradients, to achieve separations of the two states of the couple and to prepare chemical species.

Biological Relevance: In general proteins tend to fold with their hydrophobic side chains in the interior away from exposure to the aqueous milieu at physiological temperatures. Whether a particular polypeptide chain is so folded depends on the mean hydrophobicity of the interacting moieties and on the binding of any chemical species that would be antagonistic (more polar) or synergistic (less polar) to that folding. Phosphorylation would force an unfolding whereas dephosphorylation would force the folding. The reduction (making less polar) of a bound co-factor or prosthetic group would enhance the folded state, whereas oxidation would favor the unfolded state. The binding of any chemical species which altered the mean hydrophobicity would shift the equilibrium in a predictable way if the hydrophobicities were well calibrated. (Incidentally, these elastomeric sequential polypeptides provide an excellent system with which to achieve the calibration). Accordingly this apolar-polar repulsion mechanism in aqueous systems is proposed to be a fundamental driving force in protein mechanisms.

It has already been demonstrated that the oxidation of elastin causes the elastin fibers to unfold or unwind, increasing their length and causing loss of elastic recoil (17). This chemically irreversible step is proposed to be part of the pathology of the elastic fiber central to the development of pulmonary emphysema. Phosphorylation of muscle proteins whether it be of myosin, actin, titin, etc., by this mechanism can be expected to cause relaxation of force and, where relevant, to bring about disassembly. These are but a few of the examples of relevance to biology.

SUMMARIZING REMARKS

This manuscript reports on the first demonstration of mechanochemical coupling in synthetic polypeptides. The demonstration was achieved not by copy of a known contractile

protein, but rather by a design based on a principle of contraction derived independent of a known contractile protein. The principle is one in which a change in chemical potential shifts the temperature of an inverse temperature transition. The new mechanism was demonstrated using the carboxyl/carboxylate couple and has been experimentally delineated from a polymethacrylic acid, $\left[- \underset{COOH}{\overset{CH_3}{C}} CH_2 - \right]_n$, mechanochemical system where contraction was due to a mechanism in which protonation relieved a charge-charge repulsion, that is, in which $(\partial\mu_i/\partial f)_{n_i} > 0$. By contrast, for the polypeptide mechanochemical system, $(\partial\mu/\partial f)_{n_i} < 0$. Accordingly, the mechanism is called an aqueous mediated apolar-polar repulsion. On applying force to the elastomer comprised of polymethacrylic acid, the pK_a of the carboxyl/carboxylate couple decreases whereas for the Glu containing hydrophobic polypeptide the application of force causes an increase in the pK_a. Importantly the apolar-polar repulsion mechanism does not require ionic species but rather can utilize the entire polarity scale from the most hydrophobic to the most hydrophilic.

The aqueous mediated apolar-polar repulsion mechanism is considered relevant to modulation of protein structure and function, in general. The elastomeric polypeptides under study provide an opportunity to quantitate the apolar-polar repulsion energies. Furthermore, any conformational change can be viewed as an expression of mechanochemical coupling when it is induced by 1) the binding of a chemical moiety, 2) the change in polarity of an attached chemical moiety, or 3) a change in chemical potential of the medium that alters the thermodynamics of clathrate-like water formation. In general in biology, free energy transduction (18) could be viewed as conformational changes altering function as the result of either chemomechanical or electromechanical transduction. And these may be viewed as modulations of temperatures for conformational changes that maybe called thermomechanical transduction.

Finally the point should be made that these elastomeric polypeptides provide the basis for numerous reversible mechanochemical and possibly electromechanical engines that could by means of chemical or electrical work achieve mechanical work for example to power a rotary

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FIGURE LEGENDS

Figure 1 Schematic representation of the development of force/T(°K) at constant length or the decrease in length/T(°K) at constant force as a function of temperature (°C). Curves a, b and c are representative of Ile^1 -PPP, PPP and PTP, respectively. See text for the structural formulae. The temperature of the inverse temperature transition giving rise to force development or contraction is inversely dependent on the hydrophobicity of the polypeptide with curve a for the more hydrophobic, curve b for an intermediate hydrophobicity and curve c for the less hydrophobic.

Figure 2 Mechanochemical coupling of X^{20}-4%Glu-PPP shown by a pH elicited contraction on addition of a phosphate buffered saline (PBS) solution at pH 2.5 and a relaxation on addition of a PBS solution at pH 7. The load on the sample was 0.13kg/cm^2 and the length changed from 4.2mm (contracted) to 8.7mm (relaxed).

Figure 3 Temperature dependence of the inverse temperature transition for 4%Glu-polypentapeptides as followed by the turbidity development on aggregation. Part A is for 4%Glu-PPP and part B is for the more hydrophobic 4%Glu- Ile^1 - PPP. There is a dramatic increase in the temperature of the transition on increasing the pH. The pK_a values can be estimated from the insets giving transition temperature versus pH; the values are 4.4 for 4%Glu-PPP and 5.4 for 4%Glu- Ile^1 - PPP. Increased hydrophobicity causes an increase in the pK_a of the Glu side chain carboxyl/carboxylate couple and, of course, the transition temperatures for the more hydrophobic polypentapeptide are shifted to lower temperatures.

drive as per Katchalsky and colleagues (19). As the processes are reversible it becomes possible, by means of mechanical work, to achieve chemical work as for example to develop concentration gradients of diverse nature. Practical applications could be desalination, the development of proton gradients, the production of the reduced component of a redox couple, etc.

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Figure 4 Dependence of pK_a on the expression of elastomeric matrix hydrophobicity.

A. The more hydrophobic elastomeric matrix, X^{20}-4%Glu-Ile^1 -PPP, exhibits a higher pK_a for the pH elicited contraction than does X^{20}-4%Glu-PPP even though both have the same number of Glu residues per total number of residues.

B. On stretching the elastomeric matrix, X^{20}-4%Glu-PPP, there is an increase in the pK_a of the pH elicited contraction due to the increased exposure of hydrophobic groups on extension.

Figure 5 Schematic representation of the increase in force at constant length of X^{20}-PPP for both a pure water medium and for phosphate buffered saline. Phosphate buffered saline (PBS) is seen to shift the transition to lower temperatures; this shift is independent of the pH of the buffer. By choosing an intermediate temperature such as 25°C, PBS can be shown to cause a contraction and changing to water causes relaxation. Thus, mechanochemical coupling is demonstrated without any change in the chemical nature of the polypeptide chain; the mechanism is due to an antagonism between ions in solution and the expression of hydrophobicity by the elastomeric matrix. This was also demonstrated in Figure 4 A and B where a more hydrophobic elastic matrix exhibited a raised pK_a and where stretching, which results in the exposure of hydrophobic groups, causes an increase in pK_a. What is observed in all three cases is an aqueous mediated apolar-polar repulsion mechanism.

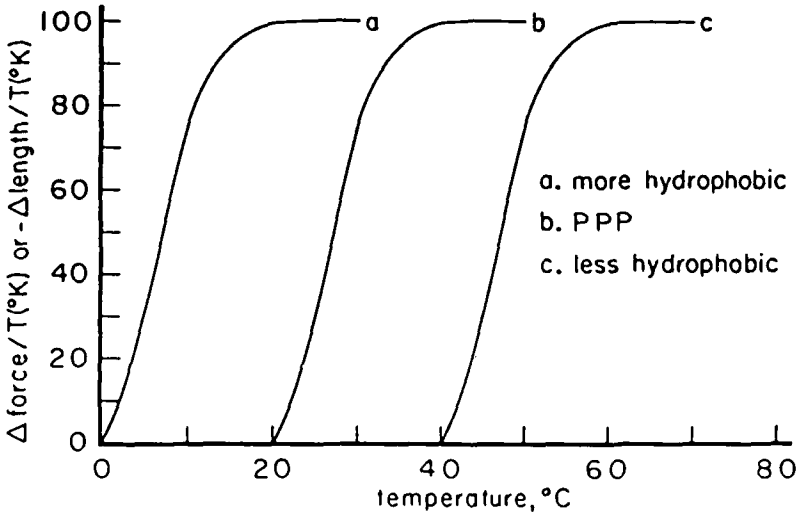

100
80
60
40
20
0
Δ force/T(°K) or -Δlength/T(°K)
0
20
40
60
80
temperature, °C
a
b
c
a. more hydrophobic
b. PPP
c. less hydrophobic

FIGURE 1

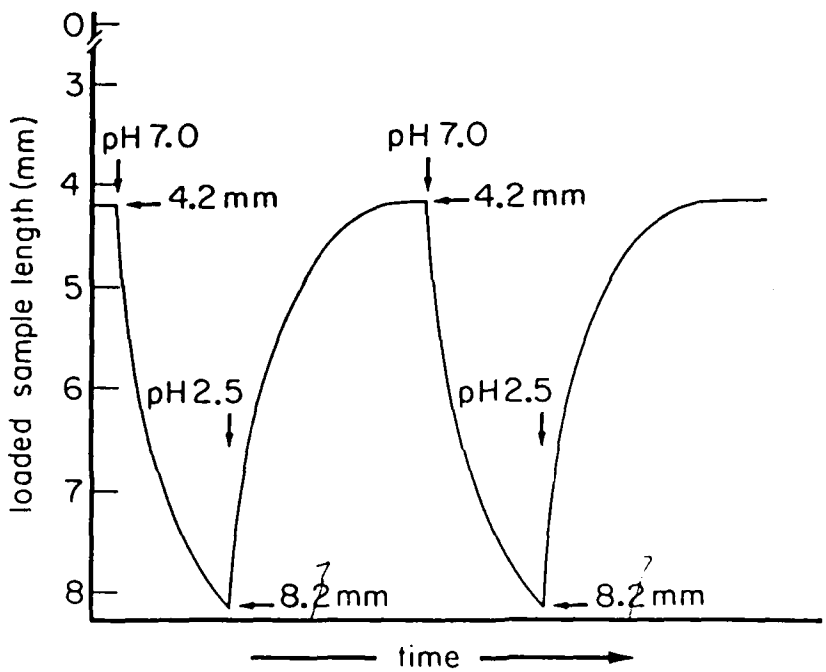
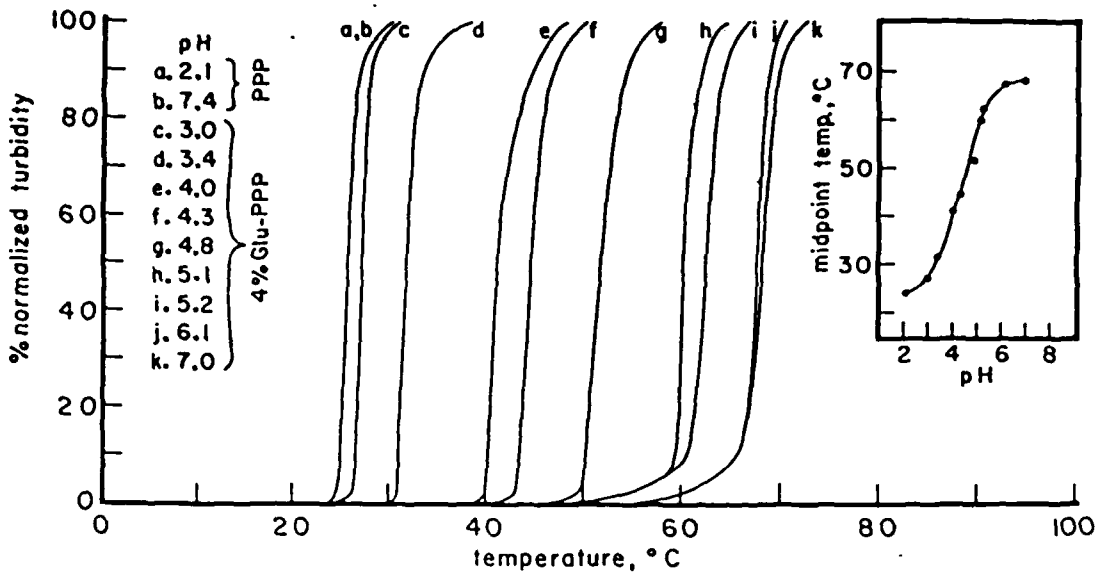

0
3
4
5
6
7
8
loaded sample length (mm)
pH 7.0
4.2 mm
pH 2.5
8.2 mm
pH 7.0
4.2 mm
pH 2.5
8.2 mm
time

FIGURE 2


pH
a. 2.1
b. 7.4
PPP
c. 3.0
d. 3.4
e. 4.0
f. 4.3
g. 4.8
h. 5.1
i. 5.2
j. 6.1
k. 7.0
4% Glu-PPP
% normalized turbidity
temperature, °C
midpoint temp., °C
pH

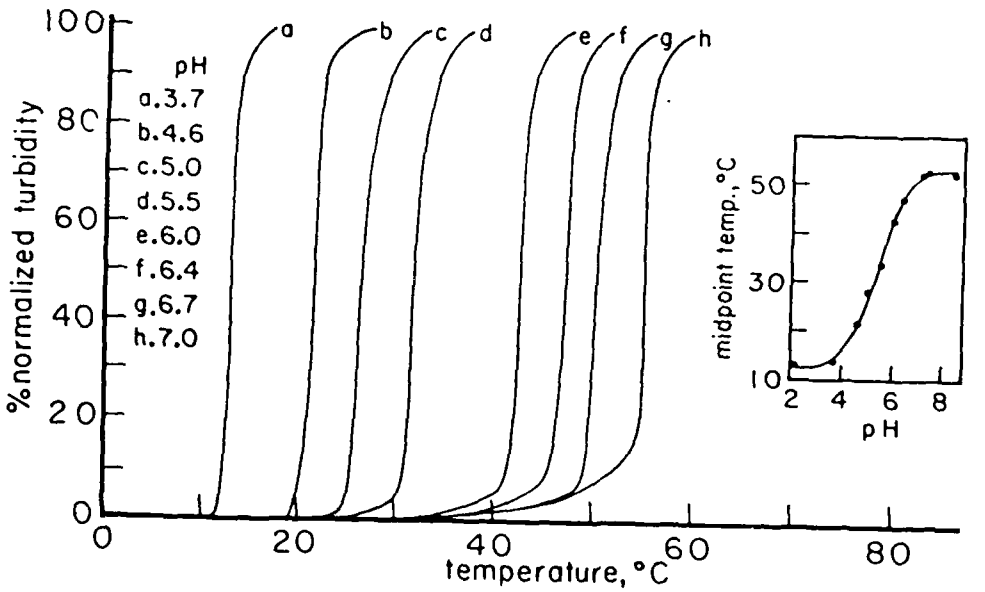

pH
a. 3.7
b. 4.6
c. 5.0
d. 5.5
e. 6.0
f. 6.4
g. 6.7
h. 7.0
% normalized turbidity
temperature, °C
midpoint temp., °C
pH

FIGURE 3

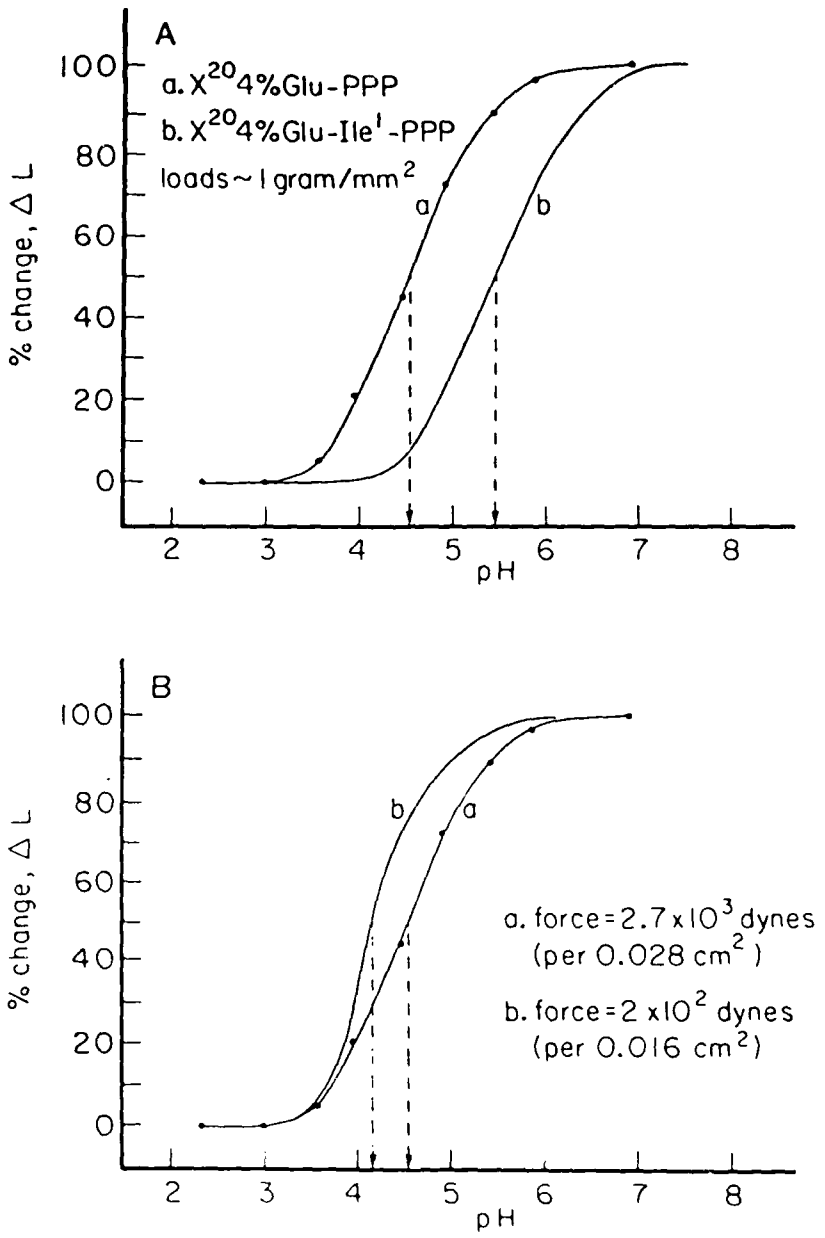

A
a. X20 4%Glu-PPP
b. X20 4%Glu-Ile1-PPP
loads ~ 1 gram/mm2
a
b
% change, Δ L
100
80
60
40
20
0
2
3
4
5
6
7
8
pH
B
b
a
a. force = 2.7 x 10^3 dynes
(per 0.028 cm^2)
b. force = 2 x 10^2 dynes
(per 0.016 cm^2)
% change, Δ L
100
80
60
40
20
0
2
3
4
5
6
7
8
pH

FIGURE 4

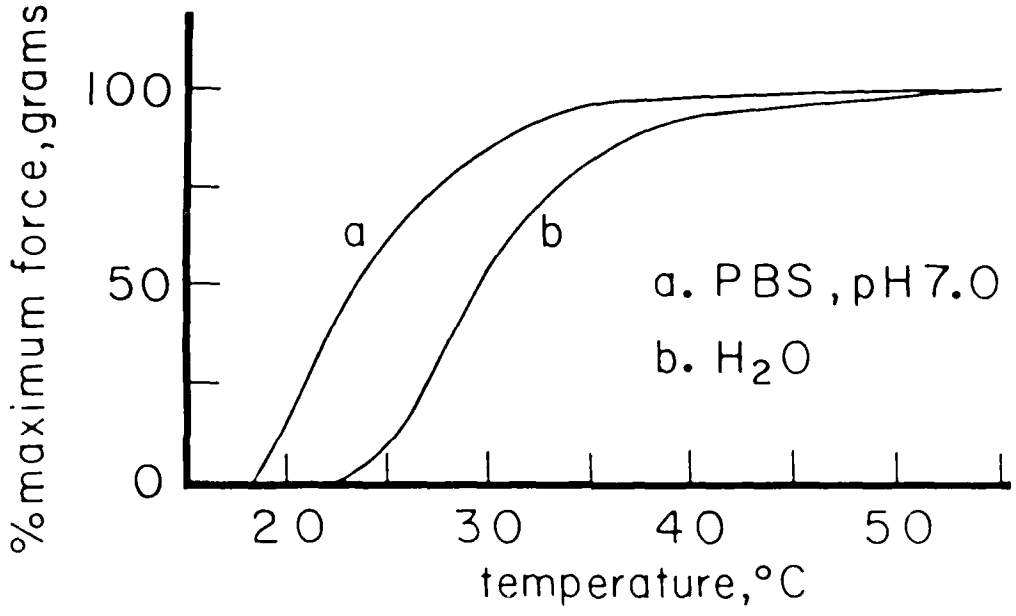

% maximum force, grams
100
50
0
20
30
40
50
temperature, °C
a
b
a. PBS, pH 7.0
b. H_2O

FIGURE 5

Chemical Potential Driven Contraction and Relaxation by Ionic Strength Modulation of an Inverse Temperature Transition

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The sequential polypentapeptide of elastin,[1,2] (L-Val[1]-L-Pro[2]-Gly[3]-L-Val[4]-Gly[5])$_n$, when cross-linked by γ-irradiation and when in equilibrium with water undergoes a reversible contraction on raising the temperature from 20 °C to 40 °C.[3] That this is the result of an inverse temperature transition has been shown by many physical characterizations[4] and is also evidenced by observing in water that analogues which are more hydrophobic undergo the transition at lower temperatures,[5] whereas less hydrophobic analogues undergo the transition at higher temperatures.[6] In this communication, it is demonstrated that a change in salt concentration causes a shift in the temperature of the inverse temperature transition and in particular that contraction and relaxation can be achieved by such changes in ionic strength. To our knowledge, this is the first demonstration that changes in chemical potential can produce contraction and relaxation in a neutral polymer and in particular in a synthetic polypeptide containing only aliphatic (Val and Pro) or no (Gly) side chains where the process is one of ionic strength modulation of an inverse temperature transition.

The polypentapeptide was synthesized as previously described.[7,8] This material is soluble in water in all proportions below 25 °C but on raising the temperature aggregation occurs.[9] Aggregation may be monitored by following the temperature dependence of solution turbidity as shown in Figure 1A for water and for phosphate buffered saline (0.15 N NaCl, 0.01 M phosphate) which is the physiological buffer system. In Figure 1A it is seen that phosphate buffered saline (PBS) causes aggregation to begin at a lower temperature while the effect of pH, curves a and b Figure 1A, is minimal, almost within the reproducibility of the data. The aggregates settle to form a more dense phase called the coacervate which in water is 38% peptide and 62% water by weight at 40 °C.[9] The coacervate is a viscoelastic phase which can be formed

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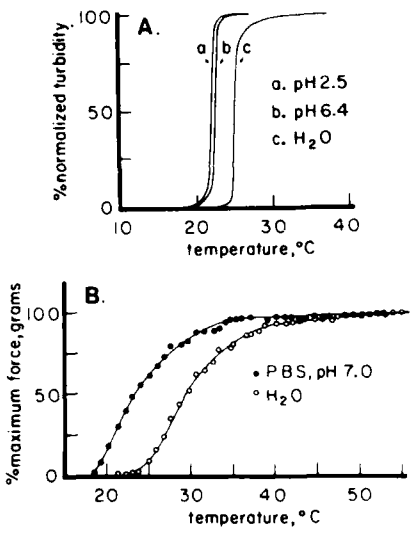

Figure 1. A. Temperature profiles for turbidity formation (aggregation) for 40 mg polypentapeptide/mL as the temperature is scanned from low to high temperatures at a rate of 30 °C/h. In phosphate buffered saline (PBS) the curves were determined at low (2.5) and near neutral (6.4) pH values to demonstrate the minimal effect of pH on the temperature of the transition. B. Thermoelasticity curves for an elastomeric band of cross-linked polypentapeptide. The sample in water of dimensions 0.76 × 4.06 × 5.44 mm^3 at 37 °C and zero load was stretched to 40% extension at 37 °C, and at this fixed length of 7.62 mm the temperature was lowered to below 20 °C for overnight equilibration, and then the temperature was raised at a rate of 1 °C per hour while monitoring force. The medium was then changed to PBS (pH 7); the sample was equilibrated at low temperature, and again the force was monitored as the temperature was raised. The forces at 37 °C were 3.2 g for water and 2.88 g for the subsequent PBS run.

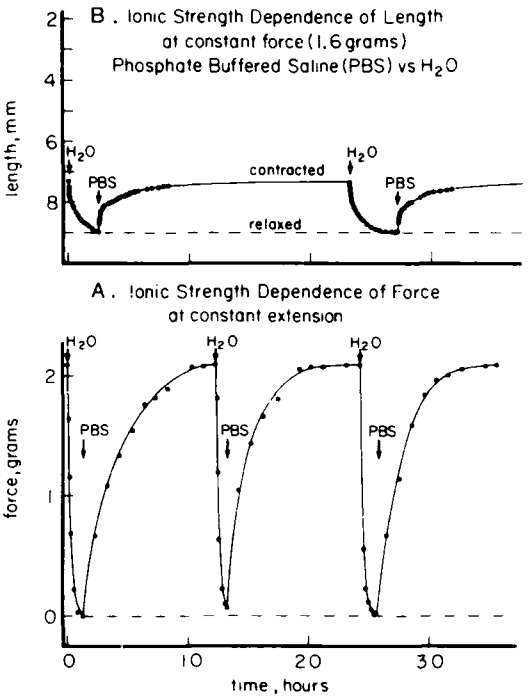

Figure 2. A. Force changes at constant extension due to changing the medium between PBS (pH 7) and water at 25 °C with reference to 28% extension above the zero load length in PBS at 25 °C. The sample dimensions at 25 °C in PBS at zero load were 0.7 × 3.56 × 5.72 mm^3. A slow irreversible swelling of the sample occurred during the time course of the experiment from an initial length of 5.72 mm to a final length of 6.06 mm as determined at zero load. B. Length changes at constant force (1.6 g obtained at 20% extension in PBS at 25 °C) due to changing the medium between PBS and water. The sample dimensions at 25 °C in PBS at zero load were 0.7 × 3.76 × 6.06 mm^3. This experiment immediately followed that of part A. The dry weight of the sample was approximately 5 mg; the sample can develop forces and pick up weights 1000 times its own weight.

in any desired shape and then γ-irradiation cross-linked at 20 Mrad (20 × 10^6 radiation absorbed dose) to form an elastomeric matrix.[7] Within the limits of nuclear magnetic resonance detectability for carbon-13 and nitrogen-15 enriched polypentapeptide, the coacervate is essentially indistinguishable from the cross-linked elastomeric matrix[10,11] demonstrating that γ-irradiation results in no NMR detectable breakdown of the polypentapeptide. Elastomeric bands so prepared can be characterized by stress/strain and thermoelasticity studies in a previously described apparatus.[12] When the sample is equilibrated in solution at 37 °C and stretched to 40% extension and the force is monitored as a function of temperature at the fixed extension, the thermoelasticity curves of Figure 1B are obtained for water and for PBS where elastomeric force is seen to develop abruptly at temperatures which approximately correspond with the temperature profiles of turbidity formation of Figure 1A. As the presence of PBS causes the development of elastomeric force to occur at lower temperature, it should be possible to remain at a fixed intermediate temperature, for example, at 25 °C, and to achieve contraction and relaxation by changing between water and PBS solutions. This is shown in Figure 2A for conditions of a fixed extension (28% extension at 25 °C in PBS) while monitoring force and in Figure 2B for conditions of a fixed force (1.6 grams obtained at 20% extension in PBS at 25 °C). Thus the polypentapeptide of elastin is seen to exhibit mechanochemical coupling in response to changes in ionic strength of the medium. Furthermore as seen in Figure 2, there is essentially complete reversibility of relaxation and contraction.

That the transition is an inverse temperature transition with increase in order within the polypeptide part of the system on increasing temperature has been demonstrated by numerous physical characterizations.[4] For example, by light and electron microscopy the aggregation process has been seen to be a process of self-assembly into anisotropic fibers comprised of parallel aligned filaments;[4] by circular dichroism spectroscopy the polypeptide backbone is seen to go from a less-ordered to a more-ordered state on raising the temperature through the transition;[9] dielectric relaxation studies on increasing the temperature through the transition show the development of an intense localized relaxation near 10 MHz[13] indicating the development of the same motional process in each of the pentamers, and, by nuclear magnetic resonance relaxation studies on increasing the temperaure of the coacervate concentration, the backbone mobility decreases as the temperature is raised through the transition.[10,11] By nuclear magnetic resonance,[14] circular dichroism,[9] and Raman[15] spectroscopies and X-ray crystallography[16] of cyclo(VPGVG)$_3$ and poly(VPGVG), the conformation of the polypentapeptide has been shown to be that of a recurring Pro^2-Gly^3 Type II β-turn with a Val^1C-O···HNVal^4 hydrogen bond. Furthermore a slow thermal denaturation is seen above 60 °C.[4] A description of inverse temperature transitions in water began with the early general work of Frank and Evans[17] which was extended to proteins by Kauz-

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mann[18] and to biological membranes by Tanford[19] and which has more recently been treated in general by Ben Naim[20]. The understanding of the inverse temperature transition is that more-ordered clathrate-like water surrounding the hydrophobic side chains below the transition becomes less-ordered bulk water above the transition as intramolecular and intermolecular contacts involving hydrophobic side chains occur. The decrease in length, which is from the contraction due to the inverse temperature transition at zero load and 40 °C, is to 45% of the 20 °C length,[3] i.e., the length changes by greater than a factor of 2.[21]

Studies on collagen by Katchalsky and colleagues[22,23] are particularly relevant to the present report. Collagen fibers undergo a melting or denaturation of structure with shrinkage in length on raising the temperature, and increasing certain salt concentrations such as LiBr, KSCN, and urea[22] lowers the temperature of the transition. Attending the melting is a contraction to almost one-half of the native length. By using these properties, Katchalsky and co-workers[23] devised a mechanochemical engine which could be driven by a pair of baths, one containing 11.25 M LiBr and the other containing water or dilute (0.3 M) LiBr. This followed work on the contraction of polyelectrolyte (polymethacrylate) fibers where decreased charge–charge repulsion was the mechanism of contraction[24-27] with 50% ionization being required to get the extended state. In the present demonstration, mechanochemical coupling is achieved with a polypeptide containing no charges, and it is the temperature of an inverse temperature transition rather than a regular transition that is being shifted by the charge in chemical potential of the salt solution.

Experimentally modulation of an inverse temperature transition is achievable with smaller changes in chemical potential (less chemical work) which is consistent with the small endothermic heat of the inverse temperature transition, i.e., the heat of polypentapeptide coacervation is about 2 cal/gram (unpublished data). Accordingly, this provides a particularly favorable type of system for free-energy transduction. In principle, of course, desalination could be achieved by driving such a polypentapeptide-based mechanochemical engine backwards. Since the more favored ionic interaction with the polypentapeptide would be cationic interaction with carbonyl oxygens and since this would lead to charge accumulation on the polypeptide with the consequence of charge–charge repulsion, increased cation interaction with polypeptide on raising salt concentration would not seem to be the mechanism with which to bring about contraction. As demonstrated, by carbon-13 NMR spectra, neither does the presence of salt alter the very small amount of Val-Pro cis peptide bond. If the effect of increasing NaCl concentration is not to bind polypeptide as a means of favoring the contracted state of the polymer, then it would seem necessary to consider the effect of increasing ionic strength on the structure of the clathrate-like water which would be to lower the temperature of the transition by destabilizing the clathrate-like water structure of the low-temperature relaxed state.[28]

Acknowledgment. This work was supported in part by National Institutes of Health Grant HL 29578 and the Department of Navy, Office of Naval Research contract N00014-86-K-0402. We also acknowledge the technical assistance of Bryant Haynes and John Jaggard.

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(21) In relation to structure, it is of interest to note in Figure 2B that the loss of force on addition of water is a faster process than the development of force on the addition of PBS. A possible explanation for this is that the process is not simply one of solvation swelling and desolvation shrinking, but rather it is an unfolding of structure with solvation while releasing load, and it is a desolvation with folding into the energetically favored helically recurring β-turn structure while developing load. The latter folding can be expected to be slowed by the multiple local minima problem which plagues molecular mechanics and dynamics simulations of protein folding.
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(28) While this proposed ionic strength modulation of an inverse temperature transition reasonably arises from the well-known salt effect on solubility of hydrophobic solutes,[29,30] the situation with these elastomeric hydrophobic polypeptides is more involved as mechanochemical coupling has recently been demonstrated with cross-linked polypentapeptide, in which there was included four Glu residues per 100 residues at position four. In this case, converting from COOH to ionic COO^- raises the temperature of the inverse temperature transition such that contraction occurs at low pH and relaxation occurs on conversion to the ionic COO^- side chains.[31] The pH driven mechanochemical coupling is polymer-based due to change of polypeptide structure. The mechanochemical coupling demonstrated here may be referred to as solvent-based, as it is achieved without any chemical change in the polypeptide structure.
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Mechanochemical coupling in synthetic polypeptides by modulation of an inverse temperature transition

(elastin polypentapeptide/hydrophobic effect)

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ABSTRACT **For the polypentapeptide of elastin, (L-Val-L-Pro-Gly-L-Val-Gly)$_n$, and appropriate analogs when suitably cross-linked, it has been previously demonstrated that development of elastomeric force at fixed length and length changes at fixed load occur as the result of an inverse temperature transition, with the temperature of the transition being inversely dependent on the hydrophobicity of the polypeptide. This suggests that at fixed temperature a chemical means of reversibly changing the hydrophobicity could be used for mechanochemical coupling. Evidence for this mechanism of mechanochemical coupling is given here with a 4%-Glu-polypentapeptide, in which the valine in position 4 is replaced in 1 out of 5 pentamers by a glutamic acid residue. Before cross-linking, the temperature for aggregation of 4%-Glu-polypentapeptide is remarkably sensitive to pH, shifting from 25°C at pH 2 to 70°C at pH 7.4 in phosphate-buffered saline (PBS). At 37°C, the cross-linked 4%-Glu-polypentapeptide matrix in PBS undergoes a pH-modulated contraction and relaxation with a change from pH 4.3 to 3.3 and back. The mean distance between carboxylates at pH 4.3 in the elastomeric matrix is greater than 40 Å, twice the mean distance between negatively charged species in PBS. Accordingly, charge–charge repulsion is expected to make little or no contribution to the coupling. Mechanochemical coupling is demonstrated at fixed load by monitoring pH dependence of length and at constant length by monitoring pH dependence of force. To our knowledge, this is the first demonstration of mechanochemical coupling in a synthetic polypeptide and the first system to provide a test of the recent proposal that chemical modulation of an inverse temperature transition can be a mechanism for mechanochemical coupling. It is suggested that phosphorylation and dephosphorylation may modulate structure and forces in proteins by locally shifting the temperatures of inverse temperature transitions.**

The polypeptide of interest is the polypentapeptide of elastin, (L-Val-L-Pro-Gly-L-Val-Gly)$_n$, discovered in porcine elastin (1, 2). In bovine elastin, the longest sequence between lysine residues, which can form the cross-links, is 72 residues; for a continuous and unsubstituted sequence of 57 residues, this is the polypentapeptide (3). The synthetic polypentapeptide is soluble in water in all proportions below 25°C, but when the temperature is raised above 25°C, aggregation occurs, followed by settling and phase separation. At 40°C, the more dense viscoelastic phase is 38% peptide and 62% water by weight (4). On γ-irradiation cross-linking at a dose of 20 Mrad (1 rad = 0.01 Gy), this viscoelastic phase, called a coacervate, forms an elastomer that exhibits dominantly entropic elastomeric force in the 40–60°C temperature range (5). Numerous physical characterizations* have demonstrated this transition to be an inverse temperature transition, in which the order in the polypeptide part of this two-component system increases as the temperature is raised through the transition. Those physical characterizations include (*i*) self-assembly studies, in which the polypeptide is found to assemble into several-micrometer-diameter fibers composed of fibrils that in turn are composed of approximately 50-Å-diameter filaments (8–10); (*ii*) circular dichroism and Raman spectroscopy, in which the polypeptide chains within the coacervate are seen to have a repeating β-turn conformation (11, 12); (*iii*) nuclear Overhauser effect studies, which demonstrate the specific hydrophobic side chain associations (ref. 13, D.W.U., D. K. Chang, R. Krishna, D. H. Huang, T. L. Trapane, and K.U.P., unpublished data); (*iv*) NMR studies, which show a decrease in backbone mobility as the temperature is raised through the transition under conditions of constant composition (14, 15); (*v*) dielectric relaxation studies, which show the development of an intense, localized, Debye-type relaxation near 10 MHz requiring the development of a regular dynamic backbone conformation (16); (*vi*) elastomer length studies, in which the elastomer shortens to less than 45% of its low-temperature length when the temperature is raised from 20°C to 40°C (17); and (*vii*) studies of slow thermal denaturation at 80°C in which the circular dichroism indicates the reversible loss of order (4), composition studies show a dramatic and reversible expulsion of water to a new composition of 68% peptide and 32% water by weight (4), and, for the elastomer, there is a decrease in length and loss of elastic modulus (ref. 18 and references therein).

In thermoelasticity studies when the synthetic elastomer is stretched and held at fixed length and the temperature is raised through the transition range from 20°C to 40°C, there is a dramatic increase in elastomeric force (5). Above 40°C, however, in a plot of ln(elastomeric force/temperature) versus temperature the slope is nearly 0 (5); these data, along with composition studies that show a near constant coacervate volume and composition in the 40–60°C temperature range (4), provide one basis for indicating that the elastomeric force is dominantly entropic in origin. A most instructive demonstration that this transition, centered near 30°C, in which elastomeric force develops is an inverse temperature transition is given when the more hydrophobic polypentapeptide (L-Ile-L-Pro-Gly-L-Val-Gly)$_n$—i.e., the [Ile^1]polypentapeptide—is similarly cross-linked and studied (18). For this more hydrophobic elastomeric matrix, the temperature of the transition for development of elastomeric force in a thermoelasticity study is 10°C rather than 30°C for the parent

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Abbreviations: 4%-Glu-PPP, elastin polypentapeptide in which 4% of the residues are glutamic acid; X^{50}-, crosslinked at 20 Mrad γ-irradiation.

*With the exception of two reports from one other laboratory (6, 7), to our knowledge, the only synthesis and physical characterizations of the sequential polypeptides of elastin and their analogs are due to the work of, and collaborations involving, this laboratory. Because of this, an unseemly high proportion of the references in this article will necessarily be to our own publications.

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polypentapeptide. A less hydrophobic elastomeric matrix has also been made with the des-Val[4]-polypentapeptido—i.e., (L-Val-L-Pro-Gly-Gly)$_n$: in a thermoelasticity study this cross-linked matrix develops elastomeric force with the midpoint of the transition at 50°C (18). These studies of changes in hydrophobicity must be done with an understanding of the structure and mechanism of elasticity. For example, the [Ile[1]]polypentapeptide analog represents the insertion of a CH_2 moiety on the side chain of residue 1; if the CH_2 moiety is inserted instead at residue 5, as in [L-Ala[5]]polypentapeptide, there is a total loss of elastic properties (18).

The above findings, that appropriately changing the hydrophobicity of the polypeptide chain can change the temperature range at which elastomeric force develops in a thermoelasticity study, provide the basis for a mechanism of mechanochemical coupling: it should be possible to turn "on" and "off" elastomeric force at a fixed temperature by reversibly changing the hydrophobicity of the polypeptide chain by means of changing the chemical potential. This can be achieved by including in the polypeptide an occasional residue in which a change in chemical potential would change the hydrophobicity of the residue. In the present report, the polypentapeptide is the copolymer of L-Val-L-Pro-Gly-L-Val-Gly and L-Val-L-Pro-Gly-L-Glu-Gly[5] at a mole ratio of 4:1. This gives a polypentapeptide matrix in which there are 4 Glu residues per 100 residues of polypentapeptide (abbreviated 4%-Glu-PPP). The change in chemical potential to be used is a change in pH. On ionization of the Glu side chains the polypentapeptide chain becomes less hydrophobic and, as reported here, the transition shifts to higher temperature and the elastomeric force turns off. To our knowledge, this report represents the first demonstration of mechanochemical coupling in a synthetic polypeptide and one in which the principle is to vary reversibly the hydrophobicity of a polypeptide to shift the temperature of an inverse temperature transition.

MATERIALS AND METHODS

Peptide Synthesis and Product Verification. For brevity, the details of peptide synthesis and product verification will be presented elsewhere. Here it is noted that the synthesis was by mixtures of two monomers, Boc-Gly-Glu(OMe)-Gly-Val-Pro-ONp and Boc-Gly-Val-Gly-Val-Pro-ONp (19, 20), which were mixed in a 1:4 ratio with polymerization initiated on removal of the Boc group by trifluoroacetic acid treatment. The purity of the intermediates and the final products was checked by thin layer chromatography, elemental analysis, amino acid analysis and C-13 NMR spectroscopy.

Temperature Profiles for Aggregation. Profiles for aggregation as a function of temperature were obtained by observing the turbidity of 40-mg/ml samples at 300 nm in a Cary 14 spectrophotometer with a 300-Hz vibrator to prevent settling. Also referred to as temperature profiles for coacervation, these data provide a means of monitoring the temperature of the inverse temperature transition, and the midpoints of the profiles are given in Fig. 1 as a function of pH for the polypeptide dissolved in phosphate-buffered saline (PBS; 0.15 N NaCl/0.01 M sodium phosphate); the titration curve shows a pK_a of 4.5. The curves for the parent polypentapeptide at pH 2.1 and 7.4 show no pH dependence.

Cross-Linking. The 4%-Glu-PPP, 400 mg/ml in distilled water, was formed in the bottom of a cryotube, and a pestle in which a channel had been turned was inserted, causing the viscoelastic material to flow into and to fill the circular channel. The sample was then γ-irradiated at a radiation absorbed dose of 20 Mrad. After cross-linking at 20 Mrad, the sample is designated by the prefix X^{20}-. This dose causes no significant changes in the carbon-13 and nitrogen-15 NMR spectra of isotopically enriched samples (14, 15), but it results in an insoluble matrix with an initial elastic modulus of 1×10^6 dynes/cm^2 (15).

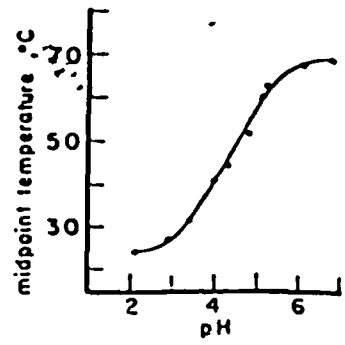

FIG. 1. pH dependence of the inverse temperature transition as determined from the midpoint of the temperature profiles for aggregation for 4%-Glu-PPP before cross-linking. The temperature of the inverse temperature transition shifts by a remarkable 45°C, from 25°C to 70°C, with an apparent pK_a of 4.5.

Stress/Strain Apparatus and Thermoelasticity Experiments. The custom-built stress/strain apparatus was as previously described (17, 19). Elastomers were equilibrated in PBS: 37°C for X^{20}-polypentapeptide at pH 7.4, 37°C for X^{20}-4%-Glu-PPP at pH 2.1, and 50°C for X^{20}-4%-Glu-PPP at pH 4.5. The samples were gripped; stress–strain data were taken; then samples were equilibrated below 15°C; the temperature was increased in 1°C increments, and the force was monitored to constancy.

Force Measurements at Constant Length. At 37°C, at constant length and at the completion of a pH 2.1 PBS thermoelasticity experiment involving X^{20}-4%-Glu-PPP, the changes in force due to changing the pH to 7.4 and then back to 2.1 were monitored with time.

Length Measurements at Constant Force. At 37°C, at constant force and at the end of a pH 2.1 thermoelasticity experiment to 35°C, the X^{20}-4%-Glu-PPP elastomer was placed under constant load of 1.5 and the pH was varied between 3.3 and 4.3 with monitoring of the length change resulting from the pH change.

RESULTS

Thermoelasticity Studies. The temperature dependence of force development for elastomers held at a fixed extension are given in Fig. 2 for pH 2.1 and pH 4.5. The choice of pH 2.1 gives the state of essentially complete protonation—i.e., 4%-Glu(OH)-polypentapeptide. The choice of pH 4.5 derives from the data in Fig. 1—i.e., only two carboxylates per 100 residues are expected to shift the transition sufficiently such that the turn on of elastomeric force would begin above 37°C at pH 4.5. This is verified in Fig. 2. Elastic force development is seen to begin just above 40°C. As irreversible loss of elastomeric force occurs for X^{20}-polypentapeptide in distilled water above 60°C, this temperature was not exceeded (18). When the pH was lowered to 2.1 and the temperature was equilibrated below 20°C before the temperature increase was started, the development of elastomeric force was seen to begin at about 20°C. Thus the introduction of two charged groups (two carboxylates) per 100 residues causes the inverse temperature transition to shift by about 20°C to higher temperatures. Therefore, it should be possible to hold the temperature constant at 37°C and turn the elastomeric force on by lowering the pH to 2.1 and to turn it off by raising the pH to a value of 4.5 or above.

Mechanochemical Coupling at Fixed Force. The sample was clamped in a pH 3.3 solution at a length of 5.3 mm and

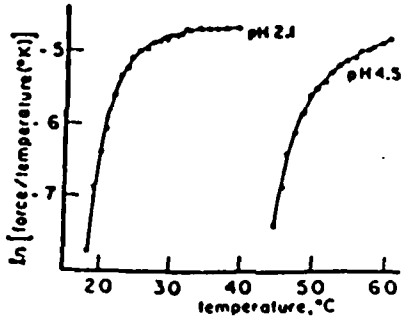

FIG. 2. Thermoelasticity studies on the γ-irradiation cross-linked 4%-Glu-PPP in PBS. For the left-hand curve, the sample had been equilibrated at 37°C and pH 2.1 and stretched to 60% then the temperature was increased from below 20°C. For the right-hand curve, the sample was equilibrated at 50°C and extended to 50%; the development of elastomeric force is seen to begin about 45°C. The temperature was not raised above 60°C as irreversible loss of elastomeric force occurs above this temperature. This is the sample used in the length study at constant force of Fig. 3*A*. The data were plotted as ln[force/temperature (K)] (with force in g; 1 g force = 9.8 mN) versus temperature such that the slope of the pH 2.1 curve could be shown to be essentially 0 in the temperature range of the 37°C studies, indicating with composition data on the host polypentapeptide that the elastomeric force is dominantly entropic in origin. The primary purpose of this data is to demonstrate that at 37°C it should be possible to turn force on by going to pH 2.1 and to turn it off by raising the pH to near 4.5 or greater.

extended at 37°C to a force value of 1.5 g which gave a length of 6.3 mm. The pH was then changed to 4.3 and the length was adjusted to maintain a constant force as the elastomer extended to 8.7 mm. When the pH was changed to 3.3, the sample contracted toward the 6.3 mm length. As plotted in Fig. 3*A*, the sample repeatedly relaxes as the pH is changed to 4.3 and contracts as the pH is changed to 3.3. The relaxations involve an increase in length of 35–40%, and the final contraction involved the raising of 1.5 g through a distance of 2.6 mm. This choice of pH values represents an effort to optimize the amount of work performed for the change in chemical potential and the number of groups protonated (see Eq. 6 below). These experiments demonstrate in a synthetic polypeptide system that a change in chemical potential can bring about a contraction in which work is performed.

Mechanochemical Coupling at Fixed Length. With the temperature fixed at 37°C and a force reading of 6.8 g, when the pH was changed from 2.1 to 7.4, the elastomeric force turned off. This is shown in Fig. 3*B*. When the pH was lowered to 2.1, the force turned back on to a value of 4.8 g. The force was again turned off and again turned on to recover the same force value and so on. Clearly, mechanochemical coupling is demonstrated in a synthetic polypeptide as the result of a change in pH.

DISCUSSION

Characterizations of the Inverse Temperature Transition. Understanding of inverse temperature transitions goes back to the work of Frank and Evans (21), which was then considered for proteins in the classic paper of Kauzmann (22) and was extensively developed with emphasis on membrane systems by Tanford (23) and in general by Ben-Naim (24). The basis of an inverse temperature transition may be referred to as the hydrophobic effect (23). As commonly stated, at temperatures below the transition the hydrophobic side chains are surrounded by clathrate-like water–i.e., by water that is more ordered than bulk water. As the temperature is raised through the transition, the clathrate-like water surrounding the hydrophobic side chains becomes less ordered bulk water as the hydrophobic side chains associate, increasing the order in the polypeptide part of the system. The transitions are endothermic with relatively small heat absorbed. For the polypentapeptide, the change in heat content is of the order of 1 cal/g (1 cal = 4.18 J) and, of course, there is a net increase in entropy for the complete system, polypentapeptide plus water. As detailed in the introduction, it is well established that the transition involved in the present studies is an inverse temperature transition.

Nature of Elastomeric Force at Temperatures Above the Inverse Temperature Transition. The data in Fig. 2 are plotted as ln[force/temperature (K)] versus temperature to demonstrate the entropic nature of the elastomeric force. The elastomeric force, f, may be written as

$$f = \left(\frac{\partial A}{\partial L}\right)_{V,T} = \left(\frac{\partial E}{\partial L}\right)_{V,T} - T\left(\frac{\partial S}{\partial L}\right)_{V,T}, \qquad [1]$$

where A, E, S, V, T, and L are the work function, internal energy, entropy, volume, temperature in K, and length, respectively, and the subscripts indicate the conditions of constant volume and temperature (25). The two terms on the right hand side of Eq. 1 are the internal energy and entropy components of force, f_e and f_s, respectively. An evaluation of the f_e/f ratio allows an estimate of the magnitude of f_s with the expression (26)

$$\frac{f_e}{f} = -T\left(\frac{\partial \ln(f/T)}{\partial T}\right)_{V,L,n}, \qquad [2]$$

when the experiment is carried out at constant volume, length, and composition, n. Composition studies on the polypentapeptide in water as a function of temperature have shown that above the temperature where the transition is complete and up to 60°C, above which temperature water is extruded, the volume and composition of the coacervate state are very nearly constant (4). Accordingly, Eq. 2 is applicable and a slope of 0 occurring in a plot of $\ln(f/T)$ versus T would indicate a 0 value for the f_e/f ratio—that is, the elastomeric force would be entirely entropic. In the pH 2.1 plot of Fig. 2, the near 0 slope in the 32–40°C temperature range calculates by Eq. 2 to have an f_e/f ratio of +0.104. In PBS at pH 7.4, the γ-irradiation-cross-linked polypentapeptide coacervate (X^{20}-polypentapeptide) at a 40% extension gave a calculated f_e/f ratio of −0.049 for the temperature range from 40°C to 55°C. Thus, these polypentapeptide elastomers appear to be dominantly entropic elastomers in the temperature interval in which the mechanochemical coupling studies were carried out.

Mechanochemical Coupling in Polyelectrolytes. The previous demonstration of mechanochemical coupling with a synthetic polymer was due to the fundamental work of Kuhn, Katchalsky, and co-workers, using polymethacrylic acid in water (27), where the mechanism was charge–charge repulsion and the charge density changes were very large. In considering mechanochemical coupling, Katchalsky *et al.* (28) began with the general expression

$$dE = TdS - PdV + fdl + \sum_i \mu_i dn_i, \qquad [3]$$

where dE, dS, dV, dL, and dn_i are the change in internal energy, entropy, volume, length, and the number of moles of the ith chemical species introduced, respectively; and P and μ_i are the pressure and chemical potential of the ith introduced species, respectively. In an isothermic mechanochemical process, it was written (28)

$$dE = 0,\ dS = 0,\ dV = 0, \qquad [4]$$

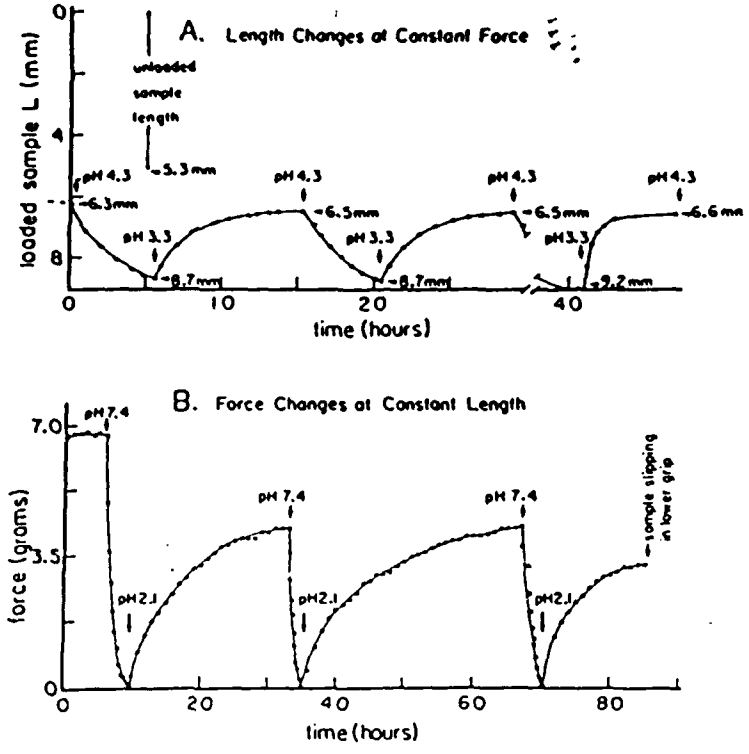

FIG. 3. Mechanochemical coupling exhibited by X^{20}-4%-Glu-PPP due to changes in pH at 37°C in PBS. (*A*) Length (*L*) changes at constant force. An elastomeric matrix 5.3 mm in length was loaded with 1.5 g and stretched to 6.3 mm at pH 3.3. When the pH was changed to 4.3, the sample elongated to 8.7 mm; returning the sample to pH 3.3 caused contraction to 6.5 mm with the lifting of the weight. This cycling was repeated. Finally, after waiting long enough, the limiting work is seen to be the shortening of the elastomer from 9.2 mm to 6.6 mm. There was a slow swelling as the sample was exposed for a prolonged period to the acid pH. (*B*) Force changes at constant length. An elastomeric matrix at pH 2.1 was stretched such that in a thermoelasticity study, the force of 6.8 g was reached at 37°C. When the pH was changed to 7.4, the force turned off; when the pH was returned to 2.1, the force turned on to 4.8 g; the force shifted between 4.8 g at pH 2.1 and 0 g at pH 7.4 until the sample began slipping in the lower grip, at which point the experiment was terminated.

such that the change in the Helmholtz free energy (i.e., the work function, *A*) becomes

$$dA = fdL = -\sum_i \mu_i dn_i. \qquad [5]$$

This provides a simple expression for analyzing mechanochemical coupling under isothermal conditions, but it is not appropriate for the mechanochemical coupling reported here, which is due to chemical modulation of an inverse temperature transition and involves a dominantly entropic elastomer such that dS cannot be taken as 0.

Charge Distribution in the X^{20}-4%-Glu-PPP Matrix. The concentration of polypentapeptide in water at 37°C is 400 mg/ml (4). With this concentration, with 4 carboxyls per 100 residues and with a mean residue weight of 83, there is a mean volume per carboxyl group of 8600 Å^3. In Fig. 2, the shift in transition temperature occurs with less than one-half of the carboxyls ionized, giving more than 17,200 Å^3 per charged side chain. This gives a mean distance between charges of greater than 26 Å in the contracted state. For the expanded state that is achieved by a pH of 4.3, where the volume is 4 times greater, the mean distance between charges would be greater than 40 Å. Such dilute concentrations of negative charge due to the polypeptide matrix, particularly in PBS, where the mean distance betwen negatively charged species is about 20 Å, are not expected to be capable of altering structure on the basis of charge–charge repulsion. Instead, as reviewed in the introduction, the mechanism is expected to be one in which a change in hydrophobicity of the polypeptide causes the shift in temperature of the inverse temperature transition. As the polypentapeptide becomes more hydrophilic, the temperature of the inverse temperature transition increases with remarkable sensitivity, as shown in Figs. 1 and 2.

Heats of Transition, Chemical Work and Mechanical Work. A satisfactory analysis of the thermodynamics of this mechanism of mechanochemical coupling will have to await appropriate equations and more complete calorimetry data. Nonetheless, some qualitative considerations may be noted here. The heat of the inverse temperature transition (coacervation starting from a concentration of 40 mg/ml) for 4%-Glu-PPP in PBS at pH 3.5 is an endothermic 0.3 cal/g (C.-H. Luan and D.W.U., unpublished data). Since dissolution is limited by cross-linking in X^{20}-4%-Glu-PPP, the heat of transition would be somewhat less for the elastomer. This estimate of the heat of the transition is within a factor of 2 of the calculated chemical work that brought about the length changes observed in Fig. 3*A*, that is,

$$A = [\mu(\text{pH } 3.3) - \mu(\text{pH } 4.3)]\,[n_{O^-}(\text{pH } 3.3) - n_{O^-}(\text{pH } 4.3)] \simeq 0.2 \text{ cal/g}, \qquad [6]$$

where n_{O^-} is the number of moles of carboxylates at the indicated pH values. The mechanical work, $f\Delta L$, as approximated from Fig. 3*A*, is a much smaller quantity, about 0.002 cal/g. Apparently, the amount of mechanical work achieved on going from pH 4.3 to 3.3 is of the order of 1% of the chemical work, with the chemical work (chemical free energy) primarily providing for the endothermic transition.

Interestingly, the elastomer can repeatedly pick up and set down a weight 1000 times its dry weight.

Related Mechanochemical Systems. The 4%-Glu-[Ile^1]polypentapeptide, (L-Ile-L-Pro-Gly-ϕ-Gly)$_n$, where ϕ is Val or Glu at a ratio of 4:1, has been synthesized and partially characterized. This Ile^1 analog exhibits transitions that are shifted to lower temperature, which provides the opportunity of adding additional polar residues. For example, in place of the Glu residues could be introduced a Ser or Thr residue in proper relationship to an Arg or Lys residue in an effort to have a protein kinase site for phosphorylation. In such a case, phosphorylation could cause a relaxation, whereas dephosphorylation could cause a contraction. The proposal that the hydrophobicity be modulated by phosphorylation and dephosphorylation in order to turn off and on elastomeric force and therefore to cause relaxation and contraction or other structural transitions has been made recently (29, 30). This brings the work reported here to the issues of free energy transduction in biological systems so elegantly treated by Hill and colleagues (31–34), whether the molecular system be a soluble enzyme, a contractile filament, a cytosolic component of a membrane pump or channel, or a coupling component of oxidative phosphorylation.

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Review

Entropic Elastic Processes in Protein Mechanisms. II. Simple (Passive) and Coupled (Active) Development of Elastic Forces

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The first part of this review on entropic elastic processes in protein mechanisms (Urry, 1988) demonstrated with the polypentapeptide of elastin (Val[1]-Pro[2]-Gly[3]-Val[4]-Gly[5])$_n$ that elastic structure develops as the result of an inverse temperature transition and that entropic elasticity is due to internal chain dynamics in a regular nonrandom structure. This demonstration is contrary to the pervasive perspective of entropic protein elasticity of the past three decades wherein a network of random chains has been considered the necessary structural consequence of the occurrence of dominantly entropic elastomeric force. That this is not the case provides a new opportunity for understanding the occurrence and role of entropic elastic processes in protein mechanisms. Entropic elastic processes are considered in two classes: passive and active. The development of elastomeric force on deformation is class I (passive) and the development of elastomeric force as the result of a chemical process shifting the temperature of a transition is class II (active). Examples of class I are elastin, the elastic filament of muscle, elastic force changes in enzyme catalysis resulting from binding processes and resulting in the straining of a scissile bond, and in the turning on and off of channels due to changes in transmembrane potential. Demonstration of the consequences of elastomeric force developing as the result of an inverse temperature transition are seen in elastin, where elastic recoil is lost on oxidation, i.e., on decreasing the hydrophobicity of the chain and shifting the temperature for the development of elastomeric force to temperatures greater than physiological. This is relevant in general to loss of elasticity on aging and more specifically to the development of pulmonary emphysema. Since random chain networks are not the products of inverse temperature transitions and the temperature at which an inverse temperature transition occurs depends on the hydrophobicity of the polypeptide chain, it now becomes possible to consider chemical processes for turning elastomeric force on and off by reversibly changing the hydrophobicity of the polypeptide chain. This is herein called mechanochemical coupling of the first kind; this is the chemical modulation of the temperature for the transition from a less-ordered less elastic state to a more-ordered more elastic state. In the usual considerations to date, development of elastomeric force is the result of a standard transition from a more-ordered less elastic state to a less-ordered more elastic state. When this is chemically modulated, it is herein called mechanochemical coupling of the second kind. For elastin and the polypentapeptide of elastin, since entropic elastomeric force results on formation of a regular nonrandom structure and thermal randomization of chains results in loss of elastic modulus to levels of limited use in protein mechanisms, consideration of regular spiral-like structures rather than ramdom chain networks or random coils are proposed for mechanochemical coupling of the second kind. Chemical processes to effect mechanochemical coupling in biological systems are most obviously phosphorylation-dephosphorylation and changes in calcium ion activity but also changes in pH. These issues are considered in the events attending parturition in muscle contraction and in cell motility.

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KEY WORDS: elastin; elastogenesis; muscle contraction; cell motility; mechanochemical coupling; entropic elasticity.

At every crossway on the road that leads to the future,
tradition, has placed against each
of us, 10,000 men to guard the pass.

Maurice Maeterlinck, 1907

I. INTRODUCTION

Elastic processes in proteins may be considered as one of two classes: class I, passive and simple elastic proceses, those demonstrated when the protein is acted on by an external force with the result of substantial deformation, and class II, active and coupled elastic processes, those resulting for example from a change in chemical potential which effects transition between two structural states, one of which can exert an elastomeric force. Class II includes interesting cases of mechanochemical coupling. Examples of class I involve the obvious deformations that occur when a joint flexes stretching ligaments and overlying skin, when lung is expanded and when a pressure pulse passes through a blood vessel. The resting tension of muscle, particularly when the muscle is pulled beyond the overlap of thick and thin filaments, may be considered an example of the first class. Elastic deformation can also be considered to result from binding processes as for example in the binding of a substrate to an enzyme by the induced fit mechanism of enzyme catalysis and in the binding of allosteric effectors; both could manipulate elastic forces in a manner that would reduce the activation energy for the catalytic event. Deformations of membrane proteins due to the presence of an electric field across the membrane could occur; an example would involve the mechanism for opening and closing of ion-selective transmembrane channels.

Most dramatic examples of active elastic processes (class II) would be the onset of elastic forces in muscle contraction and in cell motility in general. The essential element of the concept outlined here is the transition to an entropic elastic state at a fixed temperature by means of a change in chemical potential where the change in chemical potential changes the temperature of a transition between two states with, for example, the higher temperature state being the entropic elastic state. The transition can be an inverse temperature transition (from higher to lower chain entropy on increasing temperature), in which case a change in the hydrophobicity of the chain segment effects the shift in midpoint of the transition temperature; this can be considered mechanochemical coupling of the first kind. Alternatively, the transition can be from a low entropy state to a higher entropy state in which case chemical destabilization of the lower entropy state or stabilization of the higher entropy state can shift the temperature of the transition. This is what has generally been considered for mechanochemical coupling; it is referred to as mechanochemical coupling of the second kind. Mechanochemical coupling therefore is taken to be a chemical process converting one structural state to second state capable of exerting an entropic elastomeric force. In order to provide an entropic motive force most effectively, the second elastic state should be energetically preferred when in the extended state such that while extended, an entropic elastomeric force would result in an elastic recoil to a less extended state. Under these circumstances, entropic

elastomeric force would be particularly favorable rather than an internal energy component of elastomeric force.

Elastic processes of the first class will be considered in regard to elastin, to the third (elastic) filament of muscle, to enzyme catalysis and to conductance state changes in channels. With respect to elastin, oxidative processes that alter structure and elastic function with relevance to wound repair and to environmentally induced lung disease are discussed. Finally, elastic processes of the second class which involve the reversible turning on and off of elastic forces at a fixed temperature will be considered in relation to synthetic elastomers, to events attending parturition and their reversal, to muscle contraction and to cell migration in a concentration gradient. The approach to the additional biological elastic processes will follow the inquiry as to what properties of, and concepts derived from, the elastin system might be relevant to the observed properties of the additional systems in which elastic forces are involved.

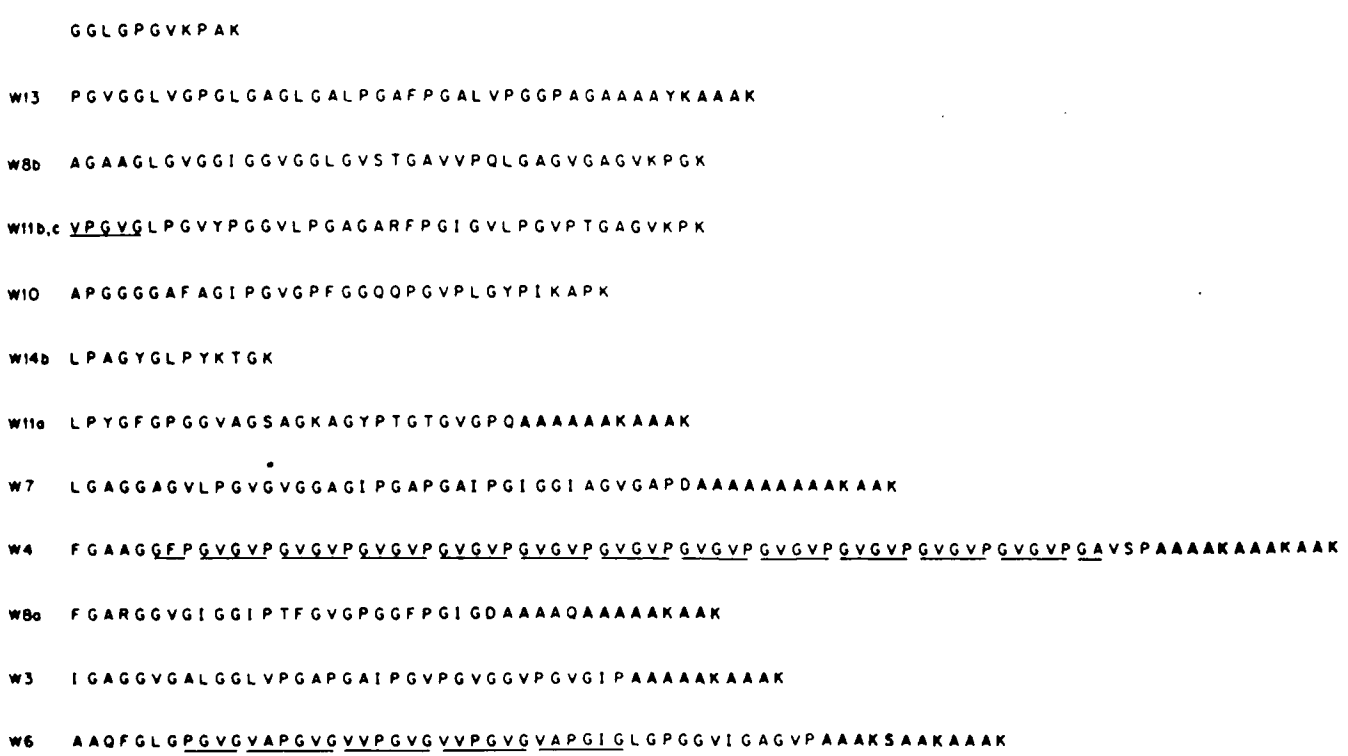
2. ELASTIC PROCESSES IN PROTEIN SYSTEMS: CLASS I

2.1. Elastin

For the past three decades, the dominant perspective of the nature of protein elasticity as represented by elastin has been that of the classical theory of rubber elasticity (Hoeve and Flory, 1958). This perspective was reaffirmed in 1974 with the summarizing statement that "A network of random chains within the elastin fibers, like that in a typical rubber, is clearly indicated" (Hoeve and Flory, 1974). In what follows, the random chain network perspective will be shown to be incorrect and with a new perspective come new insights into elastic processes in protein function and dysfunction. The most striking primary structural feature of elastin is the repeating pentapeptide sequence characterized in Urry, (1988); its prominence in the primary structure of elastin is shown in Fig. 1 (Sandberg *et al.*, 1981, 1985; Yeh *et al.*, 1987), where the primary structure is listed in terms of the sequences between crosslinking lysine residues. It was shown (Urry, 1988) that crosslinked high polymers of the repeating pentamer sequence develop elastomeric force in concert with the occurrence of an inverse temperature transition and that the entropically elastic crosslinked polymers lose elastomeric force by means of thermal denaturation. Networks of random chains are not the products of inverse temperature transitions and networks of random chains do not exhibit thermal denaturation. This section demonstrates that elastin also develops elastomeric force by means of an inverse temperature transition and loses elastomeric force by means of thermal denaturation.

2.1.1. Elastogenesis

The precursor protein of elastin, tropoelastin (Smith *et al.*, 1968; Sandberg *et al.*, 1969; Smith *et al.*, 1972), and a 70,000-m_r chemical fragmentation product of elastin, α-elastin (Partridge *et al.*, 1955; Partridge and Davis, 1955), are each soluble in water below 20°C. When the temperature is raised, tropoelastin and α-elastin aggregate, as shown for the polypentapeptide of elastin (Urry, 1988). When drops of these cloudy solutions are placed on a carbon-coated grid and similarly stained, these molecules randomly dispersed in solution at lower temperature are seen to


GGLGPGVKPAK
W13 PGVGGLVGPGLGAGLGALPGAFPGALVPGGPAGAAAAAYKAAAK
W8b AGAAGLGVGGIGGVGGLGVSTGAVVPQLGAGVGAGVKPGK
W11b,c VPGVGLPGVYPGGVLPGAGARFPGIGVLPGVPTGAGVKPK
W10 APGGGGAFAGIPGVGPFGGQQPGVPLGYPIKAPK
W14b LPAGYGLPYKTGK
W11a LPYGFGPGGVAGSAGKAGYPTGTGVGPQAAAAAAAKAAAK
W7 LGAGGAGVLPGVGVGGAGIPGAPGAIPGIGGIAGVGAPDAAAAAAAAAAKAAK
W4 FGAAGGFPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGAVSPAAAAKAAAKAAK
W8a FGARGGVGIGGIPTFGVGPGGFPGIGDAAAAQAAAAAKAAK
W3 IGAGGVGALGGLVPGAPGAIPGVPGVGGVPGVGIPAAAAAKAAAK
W6 AAQFGLGPGVGVAPGVGVVPGVGVVPGVGVAPGIGLGPGGVIGAGVPAAAKSAAKAAAK

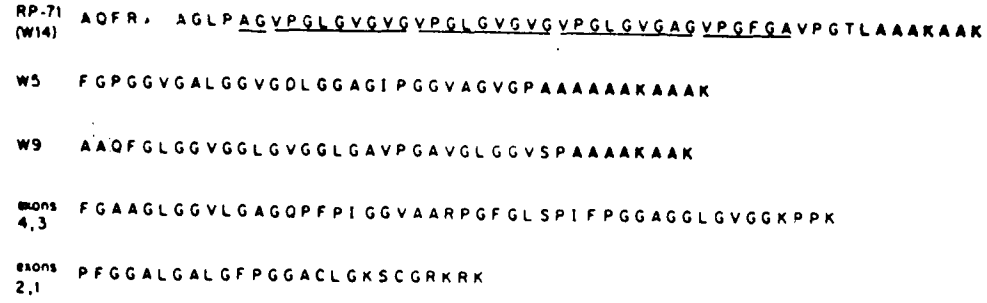


Fig. 1. Primary structure of bovine ligamentum nuchae elastin. These sequence data, reported by Yeh *et al.* (1987), are arranged according to sequences between crosslink-forming lysines and labeled according to Sandberg *et al.* (1985) in analogy with porcine aortic elastin. The recognized desmosine and isodesmosine forming sequences are boldfaced and are KAAK and KAAAK generally associated with string of alanines, A. At the heart of the primary structure is the longest sequence, W4; it contains the polypentapeptide of elastin, which was extensively characterized in Urry, (1988). The next longest sequence, W6, contains a repeating hexapeptide followed in length by RP-71 (W14) which contains a repeating nonapeptide. Also notable are serines, S, threonines, T, and tyrosines, Y, in sequential proximity to charged lysine, K, or argenine, R, residues. These are potential phosphorylation sites.

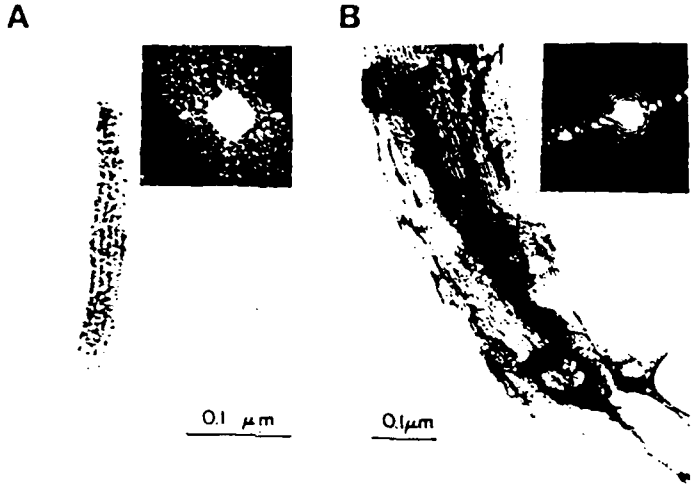


Fig. 2. Transmission electron micrographs of negatively stained incipent coacervates of tropoelastin (*A*), and of α-elastin (*B*), with optical diffraction patterns included. The primary diffraction spots are near 5 nm. It seems apparent on raising the temperature of aqueous solutions of tropoelastin and of α-elastin that these molecules molecularly dispersed in solution below 20°C can self-assemble into filamentous arrays on raising the temperature to 37°C. This demonstrates the intermolecular component of an inverse temperature transition. (Micrographs reproduced from Cox *et al.*, 1973, 1974, and diffraction patterns from Volpin *et al.*, 1976.)

have self-assembled in the formation of parallel-aligned filaments as shown in Fig. 2 (Cox *et al.*, 1973 and 1974). Optical diffraction of the micrographs demonstrate 5-nm periodicities (Volpin *et al.*, 1976). Thus, elastogenesis is the result of an inverse temperature transition, just as polypentapeptide fibrillogenesis is the result of an inverse temperature transition. Circular dichroism data of solution and coacervates of α-elastin similarly show an increase in intramolecular order with increase in temperature (Starcher *et al.*, 1973).

2.2.2. Characterization of the Elasticity of Elastin

When a sample of ligamentum nuchae elastin is stretched to 60% extension at 40°C and then the temperature dependence of elastomeric force is determined, there is a steep development of elastomeric force in the 20–40°C temperature range (Fig. 3). This is analogous to that of the polypentapeptide of elastin though the transition is not as sharp. In the 50–60°C temperature range when the data are plotted as ln [force/temperature (°K)] versus temperature, the slope is near zero. If the study is carried out in 30% ethylene glycol and 70% water as done by Hoeve and Flory (1958), the rise in elastomeric force is shifted to lower temperature and the temperature range, in which the near-zero slope is obtained, is wider (unpublished data). This is the solvent system on which Hoeve and Flory (1958, 1974) concluded

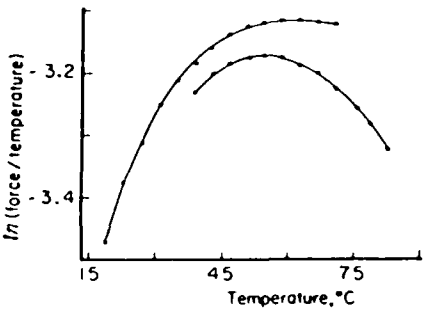


Fig. 3. Thermoelasticity study of bovine ligamentum nuchae elastin at 60% extension measured at 40°C. In the upper curve, the temperature was raised from 20°C at a rate of 4 h per data point to 40°C then at a rate of 30 min per data point. The temperature was then reduced to 40°C and the temperature raised at a rate of 4 h per data point. When the temperature is raised at the faster rate above 40°C, a near-zero slope is obtained. The curve is not reproduced on lowering the temperature, and a slower rise in temperature shows a marked loss of elastomeric force above 55°C. As confirmed in Fig. 4, this irreversible change is due to thermal denaturation.

that elastin was dominantly an entropic elastomer. The result is obtained when the temperature is raised relatively fast, taking only 20–30 min per 4°C from 40 to 75°C. If, however, the data are obtained at a rate of four hours per four degree rise in temperature as subsequently done in Fig. 3, there is observed an irreversible loss of elastomeric force above 55°C. This is thermal denaturation as can also be shown by determining stress–strain curves with interceding 24-h periods of heating at 80°C and 0% extension, as shown in Fig. 4. When the ln(elastic modulus) is plotted against the time of heating at 80°C, a half-life for the thermal denaturation is obtained. The half-life at 80°C is long, about 10 days, as may be obtained from the insert of Fig. 4. Thus, elastin can also be observed to develop elastomeric force as the result of an inverse temperature transition and to lose elastomeric force due to thermal denaturation. These are not the properties of random chain networks. In fact, the randomized networks resulting from thermal denaturation exhibit significantly reduced elastic moduli. If thermal randomization of polypeptide chains in ligamentum nuchae elastin is representative, then with the number of crosslinks no more than in elastin, the elastic moduli of random polypeptide chains would seem to be of limited value to many elastic processes in proteins.

To characterize further the nature of the elasticity of elastin the temperature dependence of length under zero load has been determined (Urry *et al.*, 1986), and elastin is found to exhibit lengthening on lowering the temperature as observed for the polypentapeptide but unlike the results for Latex rubber (see Urry, 1988, Fig. 13 therein). Furthermore, a dielectric relaxation study was carried out on the coacervate of α-elastin (Urry *et al.*, 1985). As shown in Fig. 5, α-elastin exhibits a dielectric relaxation spectrum in the 1-MHz to 1-GHz frequency range similar to that of the polypentapeptide of elastin shown in Urry (1988, Fig. 12, therein). The inert of Fig. 5 shows the temperature dependence of correlation time from which the energy barrier to mobility is approximated to be 1.5 kcal/mole (Urry *et al.*, 1985). This is the same value obtained for the backbone mobility of the polypentapeptide of elastin and, interestingly, it is essentially the same value obtained by Aaron and Gosline (1980, 1981) for single elastic fibers from optical anisotropy studies. Accordingly, even in this brief summary, the data demonstrate elastin to exhibit dominantly entropic elastomeric force but to do so by means of a nonrandom chain

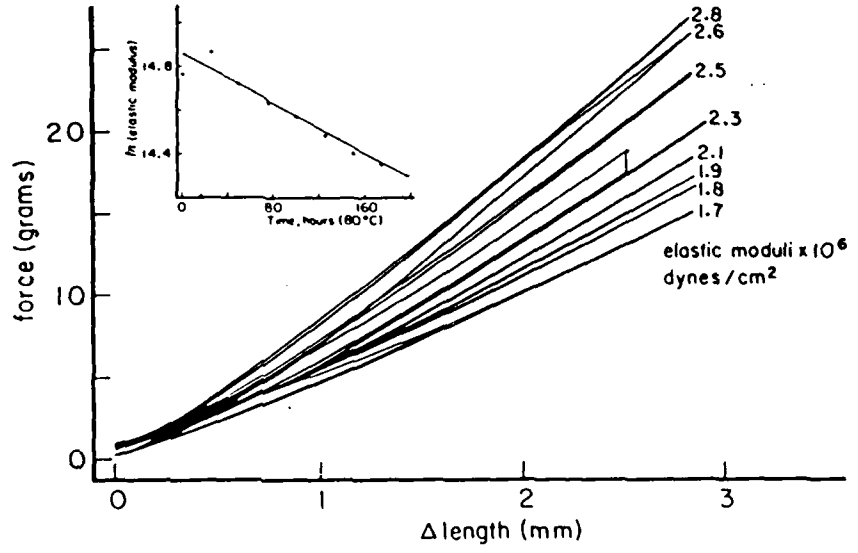


Fig. 4. Series of stress-strain curves for ligamentum nuchae elastin determined at 40°C to an extension of 60%. *Between subsequent curves, the sample* was returned to zero extension and heated at 80°C for approximately 24 h. Heating at 80°C results in a progressive loss of elastic modulus. A plot of ln(elastic modulus) versus time at 80°C is given *in the insert, from* which a half-life of about 10 days is obtained. This data and that of Figure 3 demonstrate a slow thermal denaturation of ligamentum nuchea elastin. Thermal randomization of chains causes loss of elastic *modulus* (D. W. Urry, B. Haynes, and R. D. Harris, unpublished results). This is difficult to reconcile with the perspective that the source of elastomeric force is due to random chain networks and argues, where significant elastic forces are observed in protein mechanisms, that nonrandom structures require consideration.

network. These results become central to understanding elastin function and pathology (see Section 3: Oxidative Processes Altering Structure and Elastic Function).

2.2. The Elastic (Third) Filament of Muscle

The third most abundant protein in skeletal muscle (Wang, 1985) after myosin of the thick filament and actin of the thin filament is a megadalton elastic protein called connectin by Maruyama (Maruyama *et al.*, 1976, 1977) and titin by Wang (Wang and Ramirez-Mitchell, 1979 and Wang *et al.*, 1979). In the electron microscope, connectin(titin) can be observed as long, slender filaments (Fig. 6). This protein is now considered the source of the gap filaments so apparent in insect flight muscle (Trombitas and Tigyi-Sebes, 1974). In 1974, A. F. Huxley noted:

> Additional filaments, which are seen when the myosin is dissolved away or when the muscle is stretched so far that there is a gap between the thick and thin filaments, are not shown because it is not yet known where they attach to the other structures in the filament array.

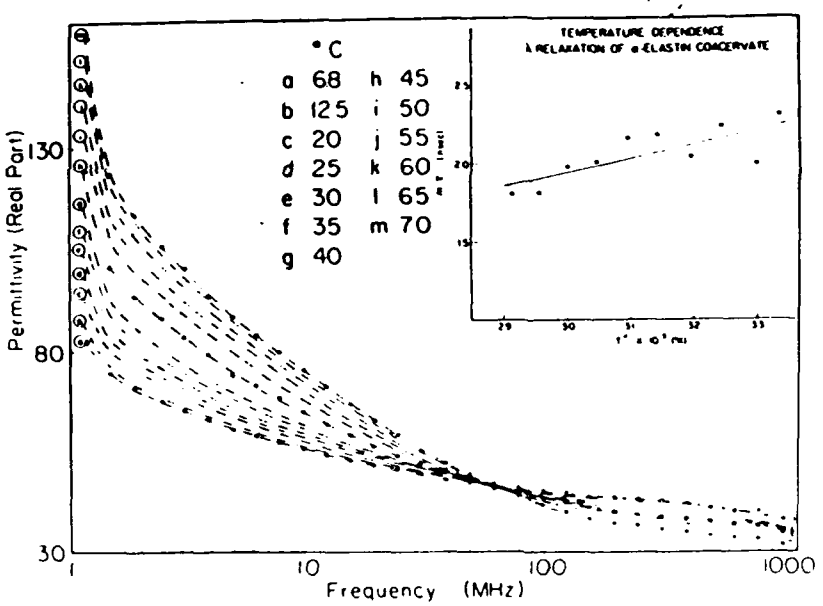


Fig. 5. Temperature dependence of the dielectric permittivity (real part) in the 1-1000 MHz frequency range of the α-elastin coacervate. On raising the temperature, a localized relaxation develops near 20 MHz. The temperature dependence of the correlation time in the insert indicates an activation energy for polypeptide backbone motion of approximately 1.5 kcal/mole, a value also found from NMR studies of the polypentapeptide of elastin. This backbone librational process is labeled the λ-relaxation. From Urry *et al.*, 1985.)

The titin protein has now been identified by Wang and co-workers (K. Wang, personal communication) to form a filament that spans from the Z line to the M line with evidence that a single polypeptide chain of 1 to 2×10^4 residues spans the total distance of about 1.2 μm (1.2×10^4 Å). It is thought to be responsible for the resting tension of muscle and to contribute to the tension in stretched muscle fibers when the thick and thin filaments have slid past each other to beyond overlap (Magid *et al.*, 1984). Titin has now been observed in vertebrate and invertebrate skeletal and cardiac muscle (Wang, 1985). A representation of the three-filament model of the sarcomere is shown in Fig. 7 (Magid *et al.*, 1984).

Information on the nature of the elasticity of this protein was provided by Maruyama *et al.* (1977), who demonstrated that the temperature coefficient of tension is positive and that this is the case at all extensions examined. This is a statement of the second term, $T(\delta S/\delta L)$, [see Urry, 1988, (2)]; i.e., this protein exhibits entropic elastomeric force. A megadalton protein that can be observed as long slender filaments and that is thought to span a 1.2-μm length cannot readily be characterized as having a random distribution of end-to-end lengths as required by the classic theory of rubber elasticity. It seems therefore that it is more readily described in terms of spiral structures with analogy to the β-spiral of the polypentapeptide of elastin. The molecular weight and the distance spanned by a single

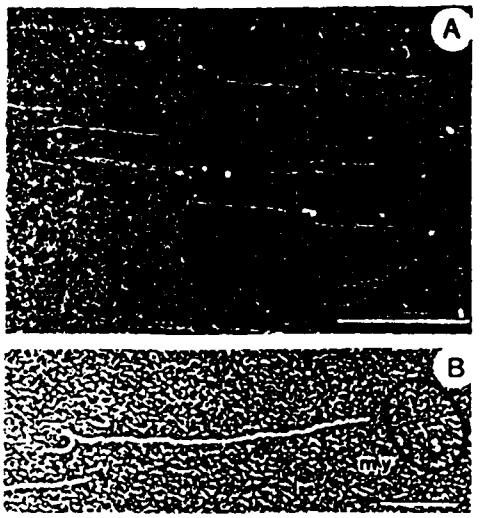


Fig. 6. Electron micrographs showing titin to form long filamentous structures. Calibration bars: (A) 1 μm, and (B) 0.2 μm. The lengths of the titin molecules are of the order of 1 μm with diameters of 4-5 nm. Circled below is a myosin dimer showing the two heads (cross-bridges). (From Wang *et al.*, 1984.)

chain mean that there are $1\text{-}2 \times 10^4$ residues spanning a distance of as many angstroms with from 1.0 to 0.5 Å/residue. This is the range of the number of angstroms per residue along the spiral axis of the β-spiral of the polypentapeptide of elastin. Interestingly, "titin contains an inordinate amount of proline (8-9%)" (K. Wang, private communication), and there is a similar amount of glycine (Wang, 1985). Although not a requirement for a librational entropy mechanism of elasticity, this allows the possibility of the occurrence of Pro^2-Gly^3 type II β-turns. The circular dichroism data so far reported are consistent with this possibility (Trinick *et al.*, 1984; Maruyama *et al.*, 1986). Interestingly, a dielectric relaxation spectrum of muscle (see Fig. 8) (Grant *et al.*, 1978; Schwan, 1974) demonstrates relaxations in the same frequency range as observed for elastin in Fig. 5 and for the polypentapeptide in Urry (1988, Fig. 12). Additional features in the amino acid composition are of relevance to mechanochemical coupling of the first kind to be discussed below (see Section 4: Chemical Modulation of Elastic Forces: Class II). Some 12-15% of the residues are serines and threonines; this, with an equivalent amount of lysines and arginines, raises the possibility for kinase sites, i.e., sites for phosphorylation. Indeed, purified rabbit titin is reported to contain 2-3 moles phosphate bound per mole of protein (Somerville and Wang, 1983). The effects of phosphorylation-dephosphorylation provide means of turning elastomeric force off and on in mechanochemical coupling of the first kind by increasing or decreasing the hydrophilicity with a resultant increase or decrease in length of the chain (see Section 4).

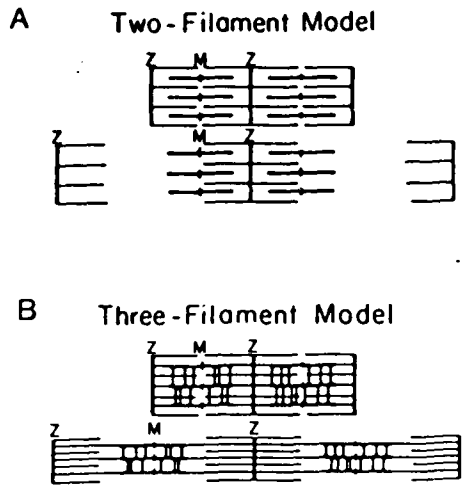


Fig. 7. (A) Two-filament model comprised of thick filaments of myosin centrally aligned in the sarcomere and thin filaments originating at the Z-line and interdigitating with the thick filaments. (B) Three-filament model where the third elastic filament runs from the Z-line into the thick filament. The third filament is shown to be continuous when the muscle has been stretched beyond overlap of thick and thin filaments. (From Magid *et al.*, 1984.) From the work of Wang and colleagues, the third filament (titin) is reported to span from the Z-line to the M-line at the midpoint of the thick filament.

2.3. Entropic Elastomeric Force in Enzyme Catalysis

In a discussion of the separation of an observed elastomeric force into its internal energy and entropy components (Urry, 1988), it was apparent that an entropic component of force would be just as effective in inducing bond strain as an internal energy component of force due to bond strain. This becomes of interest in the concepts of elastomeric force inducing strain in enzyme catalysis (Lumry and Eyring, 1954; Lumry and Gregory, 1986), particularly when considered in the induced fit mechanism of Koshland (1963). Furthermore, as noted in Urry, (1988), a peptide segment of but a few residues by the librational entropy mechanism could exhibit significant entropic elastomeric force. Because of data and analysis already available, carboxypeptidase A provides an interesting example to consider in these regards. Using the following form of the Kramer's (1940) expression

$$k_{cat} = 1/\tau_s \exp(-\Delta U/RT) \tag{1}$$

where k_{cat} is the initial rate of enzyme catalysis; τ_s is the structural relaxation time relevant to the catalytic event, and ΔU is the potential energy barrier for the reaction, Gavish (1986) reported a plot of $\ln k_{cat}$ versus ln (viscosity of the medium at constant

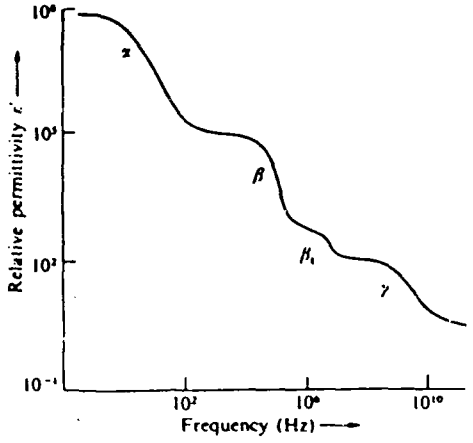


Fig. 8. Relative permittivity (real part) of muscle tissue. The β_1-relaxation occurs in the frequency range identified in the polypentapeptide of elastin (see Urry, 1988, Fig. 12) and in elastin (see Fig. 5) as due to peptide backbone librational motions where it was labeled the λ-relaxation. (From Grant *et al.*, 1980, using data from Schwan, 1974.)

temperature) and determined $1/\tau_s$ for carboxypeptidase A hydrolysis of a tripeptide to be 7.5×10^7/sec. Interestingly, this is a τ_s of 13 nsec, which is similar to the relaxation that develops as elastin and the polypentapeptide of elastin develop their regular, highly elastic states (see Fig. 5; see also Urry, 1988, Fig. 12). It is also in the range of the ultrasonic absorption maximum of proteins (Barnes *et al.*, 1985; Pethig, 1979; Zana and Tondre, 1972; Schneider *et al.*, 1969; Cho *et al.*, 1985). Interestingly, an increase in ultrasonic absorption has been reported on conversion from zymogen to active enzyme (Cerf, 1985). Figure 9 (Gavish, 1986) demonstrates that "the protein is capable of straining the substrate in a highly oriented way . . . ," i.e., the elastic forces used need to be anisotropic whereas random chain networks or random coils are isotropic.

Continuing with consideration of carboxypeptidase A and using the crystal structures of the enzyme free and when complexed with Gly-Tyr (Lipscomb *et al.*, 1968; Lipscomb, 1980; Rees *et al.*, 1980, 1981), analysis of the atom locations by Leibman *et al.* (1985) shows the major difference in a linear distance plot to involve residues 272 and 273. These residues are contiguous with the catalytically important residue Glu-270, in which, in one possible mechanism, the carboxylate forms an intermediate mixed anhydride with the carbonyl carbon of the scissile bond. The results appear consistent with a perturbation of the peptide segment that is contiguous with a functional group having critical electrostatic interactions with one side of the scissile bond. Figure 10 represents an attempt to depict this process from the standpoint of the librational entropy mechanism of elasticity in combination with the induced fit mechanism of Koshland (1963). On binding of the substrate to

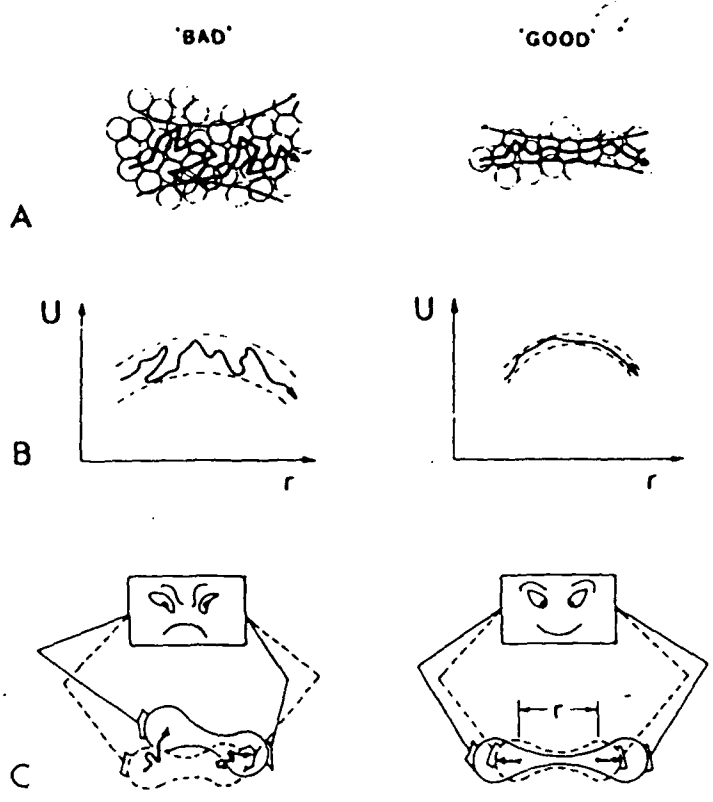


Fig. 9. Representations demonstrating the capability of an enzyme to produce oriented stresses (elastic forces) to be considered in achieving strain in a scissile bond of a substrate. In the BAD example, the mobility is more random in nature, and well-oriented catalytically useful stresses do not result. In the GOOD example, the motions occur in such a manner as to produce oriented elastic forces that can produce strain in a substrate bond. (From Gavish, 1986.) The BAD result would be exemplified by elastic forces in a random coil or random network of chains, whereas the GOOD result can occur when elastic forces derive from changes in internal chain dynamics in a nonrandom structure as in the librational entropy mechanism of elasticity.

the enzyme, a conformational change is induced in the enzyme that arises from the formation of favorable electrostatic and possibly hydrophobic interactions. In the process of the induced fit, a peptide segment, schematically represented as a spiral, becomes stretched. The essential element is that there occur on binding a damping of librations with but a small extension in a peptide segment contiguous with an electrostatic interaction at one side of the scissile bond. The damping, which could involve only a few residues and the side chain at the active site and need not involve the schematic spiral structure of Fig. 10, results in an entropic elastomeric force. Through the electrostatic attachment of the active site side chain, the entropic

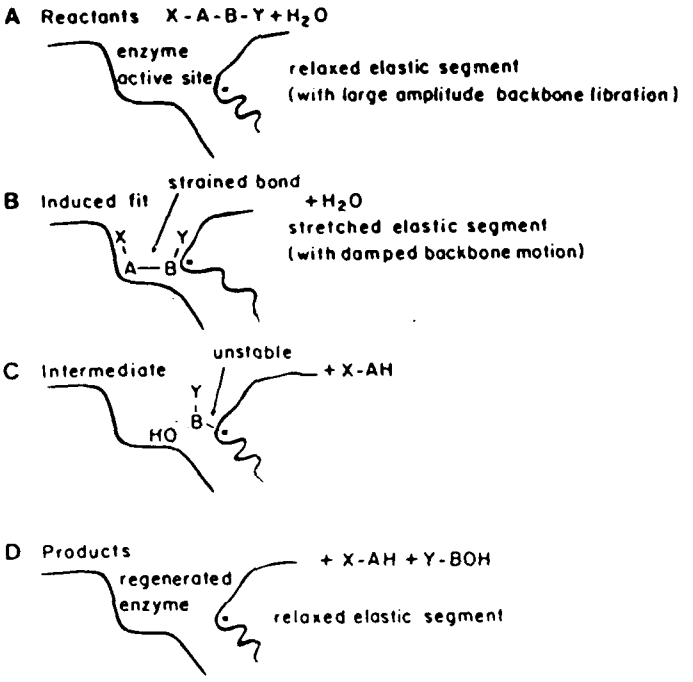


Fig. 10. Schematic representation of an elastic chain segment resulting in bond strain during enzyme catalysis. (A) The enzyme active site before interaction with substrate with a relaxed elastic chain segment shown as a spiral structure. (B) On binding of the substrate in an induced fit process, the elastic segment becomes stretched, which effects a damping of motion in the polypeptide backbone and in the side chain that interacts electrostatically with atom B. This results in an oriented stress, producing a strain in the A-B scissile bond. (C) An enzyme intermediate with B covalently attached to the side chain that is contiguous with the polypeptide chain segment where binding of substrate caused damping of librational motions. (D) The regenerated enzyme with relaxed elastic chain segment and with products of the catalysis. Although the elastic polypeptide chain segment is represented as a spiral, it could involve as few as two or three residues. Substrate binding causes an effective extension of a chain segment and damping of librational motions within it. This results in the development of an entropic elastomeric force that can produce a strain in the scissile bond, thereby facilitating bond cleavage.

elastomeric force due to damping of motion in the polypeptide backbone and the active site side chain exerts a strain on the scissile bond. A random chain network is not required; the entropic elastic force can be derived from a short peptide segment and can arise in an oriented anisotropic manner. To have an entropic elastomeric force induce a strain would be an effective means of reducing the internal energy or potential energy barrier for bond cleavage.

2.4. Entropic Elastomeric Force in Channel Conductance State Changes

Robinson (1986) recently carried out an analysis of the closed-channel-open-channel equilibrium for the sodium channel of nerve that is relevant to elastic processes in protein mechanisms. He considered the probability of the open state as a function of transmembrane potential. The system was modeled as charge moving in an electric field while tethered to a peptide chain segment. The force constant for the charge confined by an harmonic potential was determined to be approximately 20 dyne/cm and was used to estimate an elastic modulus. The value for the elastic modulus is obtained on assuming a value for the ratio of cross-sectional area to length for the elastomeric segment. Using 400 Å for this ratio, the elastic modulus would be 5×10^6 dyne/cm^2, essentially the value for elastin. The results suggest the importance of an equilibrium between two states in an electric field in which interconversion involves the stretching of an elastic peptide segment. The effect of phosphorylation/dephosphorylation in modulating the elastic forces is considered in section 4; this could be important in modulation of channel state.

3. OXIDATIVE PROCESSES ALTERING STRUCTURE AND ELASTIC FUNCTION

It has been shown that the development of both elastin fibers and of elastomeric force is the result of an inverse temperature transition. As shown in Urry (1988, Fig. 14), when the hydrophobicity of the polypeptide is changed, the temperature for association to form fibers changes, as does the temperature at which intramolecular order develops to produce the highly elastic state. These changes occur in an enitrely predictable way. When the hydrophobicity is increased, the transitions occur at lower temperature; when the hydrophobicity is decreased, which is equivalent to an increase in hydrophilicity (i.e., to making the polypeptide chain more polar), the transitions occur at higher temperature. This realization is fundamental to understanding function and pathology of elastin.

3.1. Effect of Prolyl Hydroxylation on Fiber Formation

One means of increasing the hydrophilicity of tropoelastin, the precursor protein of elastin, occurs naturally. The means is prolyl hydroxylation by the enzyme prolyl hydroxylase. This enzyme is essential to the formation of functional collagen; it represents a post-translational modification of collagen that is important for collagen release from the cell (Prockop *et al.*, 1976), that stabilizes the triple stranded collagen helix (Uitto *et al.*, 1976; Berg and Prockop, 1973; Rosenbloom *et al.*, 1973; Ramachandran *et al.*, 1973; Ramachandran *et al.*, 1975) and that makes collagen resistant to nonspecific proteolytic degradation (Berg and Prockop, 1973). The same enzyme can hydroxylate prolyl residues of tropoelastin (Uitto *et al.*, 1976; Sandberg, 1976; Rucker and Tinker, 1977) with very different consequences. Using the PPP of elastin as the model system, it has been shown by means of chemical synthesis that the temperature for aggregation of $(Val^1\text{-}Hyp^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ is above 60°C (Urry *et al.*, 1979); with just 10% of the prolyl residues replaced by hydroxyproline (Hyp), the temperature for aggregation is shifted 7°C to higher temperature (Fig.

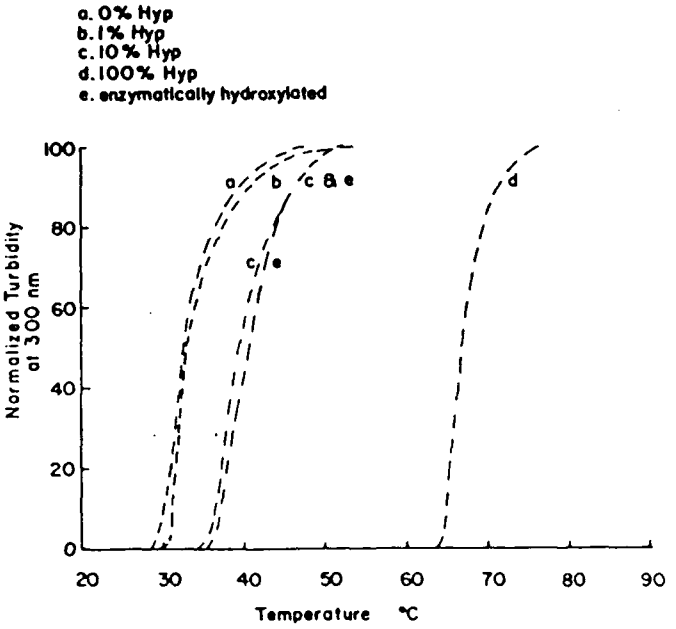


Fig. 11. Temperature profiles for the coacervation (aggregation) of the polypentapeptide of elastin in which the Pro residue has been partially or totally replaced by hydroxyproline (Hyp). Curve a is 0% Hyp, i.e. $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; curve c is when on the average one in 10 pentamers contains a Hyp, that is, one in 50 residues has been altered by addition of a hydroxyl moiety; aggregation is shown to be delayed on raising the temperature by 7°C. Curve d is 100% Hyp, i.e. $(Val^1\text{-}Hyp^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; and onset of aggregation requires an increase in temperature by about 35°C. Increase in the hydrophilicity, i.e., a decrease in hydrophobicity, markedly raises the temperature for the onset of the inverse temperature transition.

11). The action of prolyl hydroxylase on the polypentapeptide of elastin results in only about 1% hydroxlyation (Bhatnagar *et al.*, 1978) and yet increase the temperature equivalent to 10% hydroxylation achieved chemically. This enhanced effect of the enzyme in shifting the temperature of the transition may be due to the distribution of Hyp along the polypeptide or possibly because the enzymatic process results in a single enantiomer, whereas in the unresolved chemically prepared Hyp, the two enantiomers could be incorporated more nearly equivalently in the peptide synthesis. Thus, hydroxylation of proline, increasing hydrophilicity of the polypeptide, impairs fiber formation. This was proposed to be the reason for impaired elastic fiber under conditions of a repair response as in wound repair and pulmonary fibrosis (Urry *et al.*, 1980). In the case of wound repair, this information provides for an understanding of why elastin fibers are so sparse in scar tissue. Quoting from the volume entitled "Wound Repair" by Peacock (1984):

In a scar, where there are few elastic fibers and where collagen fibers become oriented primarily along lies of tension, there is little "give," and stretching and relaxation are not possible. This accounts for rigidity of scar tissue and inability to undergo repeated deformation and recovery as needed in skin covering a joint or other moving part. Failure to include new elastic fibers in repair tissue until long after collagen fibers are formed is another example of the inferiority of scar tissue to normal tissue and has obvious implications in the repair of skin defects, ligamentous structures, and large arteries.

The repair process appears to have evolved for quick wound closure at the expense of quality, possibly due to the need to limit the opportunity for infection. With the antibiotics now available, quality can become of primary concern. As an understanding of elastogenesis unfolds, such as the identification of chemotactic peptides that recruit fibroblasts that have already differentiated for elastin synthesis (Senior *et al.*, 1984), it is hoped that the quality of wound repair can be improved.

That interference with fiber formation results from high activities of prolylhydroxylase was recently demonstrated in cell culture by Franzblau and coworkers (Barone *et al.*, 1985). Prolylhydroxylase requires ascorbate as a cofactor. This group has made the important finding that ascorbate treatment of neonatal rat aortic smooth muscle cells in culture results in overhydroxylation of elastin, results in increase in the solubility of the precursor protein at 37°C, and results in a decrease in the formation of insoluble elastin, i.e., a decrease in fiber formation. Therefore, simple extrapolation from the demonstration that elastin forms by means of an inverse temperature transition has significant biological consequences. This is even more dramatically demonstrated in the following discussion.

3.2. Oxidative Loss of Elastic Recoil

It is a simple prediction of our understanding of the nature of the entropic elasticity of elastin that increasing the hydrophilicity (decreasing hydrophobicity) would raise the temperature of the inverse temperature transition. The result at 37°C would be for the β-spiral-like structures to unwind with the resultant loss of elastomeric force, particularly when the degree of extension is limited. This prediction derives from the data in Urry (1988, Fig. 14). Any oxidative process (increase in hydrophilicity) should result in a loss of elastic recoil exhibited by elastin. This perspective can be tested by means of a superoxide generating system. When a series of stress-strain curves is determined on ligamentum nuchae elastin over a period of hours, during which superoxide is enzymatically released to the bathing solution, the elastic modulus is seen to decrease progressively, and the percentage extension required before a resistance is encountered increases with time. The stress-strain curves are shown in Fig. 12. A plot of the ln(elastic modulus) versus time (see insert in Fig. 12B) indicates a half-life for the loss of elastic modulus of about one-half day. This can be compared with the rate of thermal denaturation at 80°C in Fig. 4, above which a half-life of about 10 days is observed. Oxidative processes are a potent means of destructuring elastin. Observation along the x axis of the stress-strain curves as a function of time in Fig. 12 shows the time dependence of lengthening (of unwinding). With increased time of exposure to superoxide, progressively greater extensions are required before elastin resists extension. Therefore, when elastin becomes oxidized, i.e., more hydrophilic, the structures developed by means of an

inverse temperature transition, i.e., normally largely complete by 37°C, simply unwind because the increase in hydrophilicity shifts the inverse temperature transition to higher temperatures. This simple understanding of pathology provides an explanation for the loss of elastic recoil with age, e.g., in the sagging and wrinkling of skin. Slowing oxidative processes becomes an obvious intervention. The insights also provide a new molecular basis for the understanding of environmentally induced lung disease.

3.3. Relevance to Environmentally Induced Lung Disease

In the progressive chronic disorder pulmonary emphysema, the elastic fibers become fragmented and dysfunctional with a primary symptom being loss of elastic recoil (Clark *et al.*, 1983). In an effort to understand this elastic fiber dysfunction, an animal model for pulmonary emphysema has been developed in which the initial insult is instillation of elastase directly into the lung (Kuhn *et al.*, 1976; Osman *et al.*, 1985*b*). This has supported the concept that a lack of proteinase inhibitors, which would otherwise block proteolytic degradation, might be resonsible (Snider, 1984; Janoff, 1985; Stone, 1983). In examining the causes of decreased antiprotease activity, studies have been carried out that demonstrate that α1-proteinase inhibitor is damaged by superoxide; increased proteolytic activity would be a consequence. With the above demonstration of the loss of elastic recoil of elastin resulting directly from oxidative processes, this becomes a reasonable primary event, with a possible scenario that the action of proteolytic enzyme activity could even be part of a repair response that is not necessarily harmful. Elastolytic removal of the oxidized chains and replacement by new elastin, while the elastic fiber is still present as a site of aggregation and nucleus for fiber growth would seem to be a potential remedial process. With the demonstrated oxidative loss of elastic recoil in Fig. 12, it would appear that attention should be directed toward the sources of oxidants, for example in tobacco smoke (Osman *et al.*, 1985*a*), and the natural and prophylactic defensive mechanisms that may be relevant.

4. CHEMICAL MODULATION OF ELASTIC FORCES: CLASS II

In the above consideration of elastic processes in protein mechanisms, passive elastic processes (class I) were considered. In this section are considered the interesting active elastic processes, examples of class II. In the active elastic process, the protein is of a design whereby elastic forces may be turned on or off by a reversible change in a chemical element related to the system. This is mechanochemical coupling. For example, there can be a change in the activity of a chemical species such as a change in the pH or a change in the concentration of calcium ion, a ΔpCa, or there could be a change in the hydrophilicity of the system by phosphorylation or dephosphorylation. Figure 13 represents structural changes for the two kinds of mechanochemical coupling. The first kind involves an inverse temperature transition. As has been demonstrated in the foregoing cases, a change

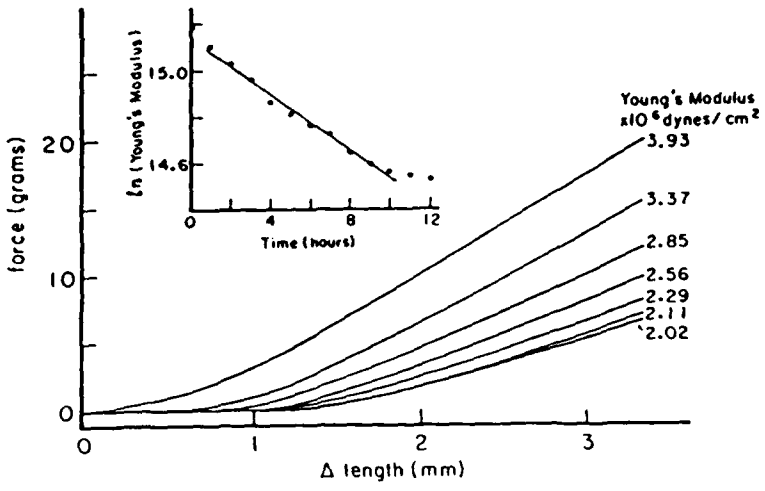


Fig. 12. Effect of a xanthine oxidase superoxide generating system on the elastic properties of ligamentum nuchae elastin. The experiment was followed for 12 h at 37°C, with the superoxide generating system being replenished every 2 h. Oxidation of ligamentum nuchae elastin results in a systematic loss of elastic modulus and an increase in the percent age of elogation required before the elastomer begins to resist deformation. A plot of the ln (elastic modulus) versus time shown as the insert gives a half-life of about 12 h whereas, as shown in Fig. 4, when heating at 80°C in the absence of the superoxide generating system, the half-life was as many days (D. W. Urry, B. Haynes, M. M. Long, and B. Freeman, unpublished data). The prediction from the previous studies that an increase in hydrophilicity at 37°C would cause an unwinding of the elastin structure with loss of elastomeric force for a given extension appears to be borne out by the experimental result. Such oxidative processes in the lung would lead to loss of elastic recoil, as found in pulmonary emphysema.

in the hydrophobicity or hydrophilicity can change the temperature of the inverse temperature transition. This was demonstrated with synthetic polypeptides of different hydrophobicity (Urry, 1988, Fig. 14), and oxidative processes demonstrated the resulting increase in temperature for the inverse temperature transition in Fig. 11 and 12. The challenge for mechanochemical coupling of the first kind then becomes one of changing the hydrophilicity reversibly. In Fig. 13A, a largely extended series of β-turns coil up into a regular β-spiral structure as the structural change of an inverse temperature transition where intramolecular hydrophobic side-chain interactions are optimized on forming a spiral with interturn hydrophobic contacts. If the hydrophobicity can be decreased while setting at 37°C, for example, the transition temperature would be raised and the spiral structure would unwind, turning off elastomeric force.

In mechanochemical coupling of the first kind, polypeptide elastomeric force develops on going from a higher entropy state to a lower entropy state, i.e., due to an inverse temperature transition. In mechanochemical coupling of the second kind, a change in chemical potential shifts the temperature for a regular transition, going

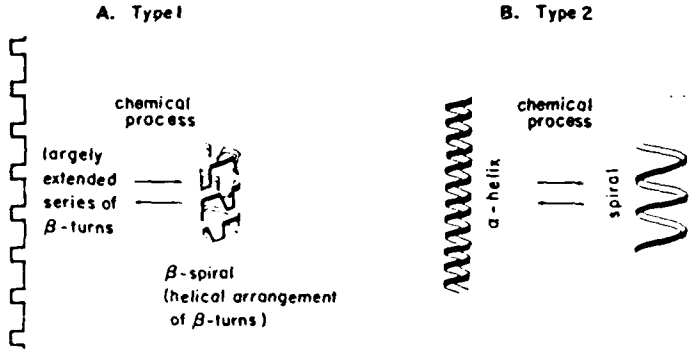


Fig. 13. Structural transitions for mechanochemical coupling. (A) Mechanochemical coupling of the first kind where a relatively less-ordered and less elastic state undergoes an inverse temperature transition to a more-ordered more elastic state, and a reversible chemical process that changes the hydrophobicity of the polypeptide chain shifts the temperature of the inverse temperature transition. In the specific example, a relatively disordered, largely extended series of β-turns winds up into an elastic β-spiral structure, defined as a helical arrangement of β-turns, in which the intramolecular hydrophobic interactions are optimized. For a given polypeptide chain hydrophobicity, e.g., for the polypentapeptide of elastin, this inverse temperature transition occurs on raising the temperature from 20 to 40°C. If at 37°C the polypeptide chain were made less hydrophobic (more hydrophilic), e.g., by a phosphorylation, the transition temperature would be raised and the structure would unwind with loss of capacity to exert an elastomeric force. Phosphorylation would then have turned off elastomeric force. (B) Mechanochemical coupling of the second kind, where a relatively more-ordered less elastic state undergoes a standard transition on raising the temperature to a less ordered more elastic state and where a chemical process can change the temperature over which a transition occurs. In the specific example, a relatively inelastic α-helix converts to a more elastic spiral structure, which derives its elastic force from the increase in internal chain dynamics within the regular structure. Any chemical process that increased the free energy of the α-helix or decreased the free energy of the spiral state would lower the temperature of the transition and could turn on an elastomeric force.

from a less elastic lower entropy state to an elastic higher entropy state. The chemical process would lower the temperature of the transition to turn on the elastomeric force. In the example in Figure 13B, an inelastic α-helix, which is an extended state, is converted to a highly elastic spiral state, which then shortens to the extent permitted by the load. The result is an elastic contraction. Any chemical process that raises the free energy of the α-helix and/or lowers the free energy of the spiral state would bring about a contraction.

What is here called mechanochemical coupling of the second kind is what has generally been meant by mechanochemical coupling as for example by free energy transduction in muscle contraction (Eisenberg and Hill, 1985). Mechanochemical coupling of the first kind becomes possible on having demonstrated that elastomeric force can develop as the result of an inverse temperature transition. This latter insight is uniquely derived from the elastin studies.

4.1. Elastic Molecular Machines and a Motive Force in Protein Mechanisms

4.1.1. Using Temperature Change to Turn Elastomeric Force On and Off

The lifting of a weight against gravitational force is the performance of work and a device capable of performing work may be called a machine. The crosslinked polypentapeptide of elastin is an elastic molecular machine. When a strip of crosslinked polypentapeptide of elastin is attached to a weight at 20°C and the temperature

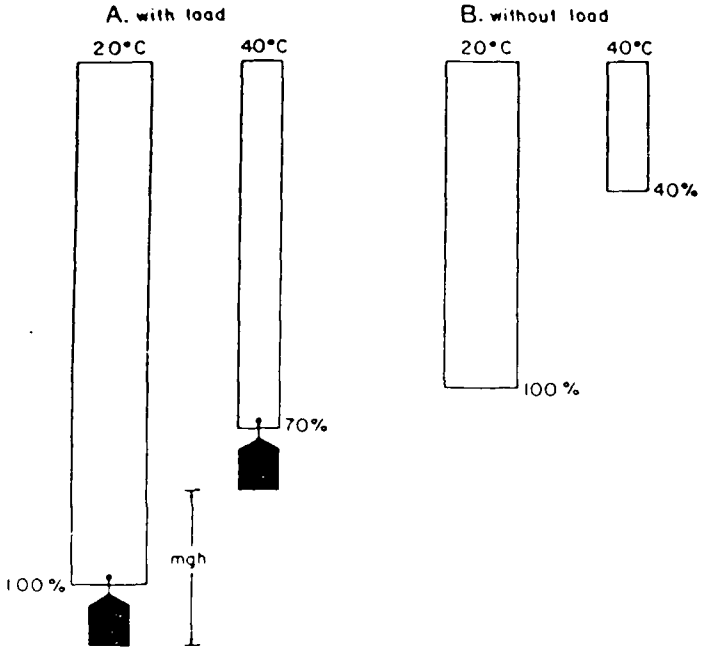


Fig. 14. Sketch showing the capacity of the γ-irradiation crosslinked polypentapeptide of elastin to function as an elastic molecular machine. (A) To a strip of crosslinked polypentapeptide at 20°C is loaded a weight. On raising the temperature from 20 to 40°C, the weight is lifted as the elastomer shortens to 70% of its original length (Urry *et al.*, 1986). Latex rubber also shortens on raising the temperature over this interval, but the change is only about 5%. The reason for the greater capacity of the crosslinked polypentapeptide to lift a weight is due to the occurrence of an inverse temperature transition wherein the polypentapeptide chain wraps up into a β-spiral structure, as shown in Fig. 13A. (B) The contraction, decrease in dimensions, of the crosslinked polypentapeptide of elastin without load on raising the temperature from 20 to 40°C. Whereas Latex rubber expands on raising the temperature in the absence of a load, the crosslinked polypentapeptide of elastin contracts to about 40% of its original length (see Urry, 1988, Fig. 13). This simple experiment clearly shows the remarkable difference between the elasticity of polypeptide in water and that of a classic rubber. The chemical modulation of this inverse temperature transition provides for a new motive force in protein mechanisms.

is raised to 40°C, the weight is lifted (Urry *et al.*, 1986) (Fig. 14A). This is a much greater shortening than occurs, for example, for Latex rubber, where the change is only 5% for the same temperature interval and weight. The reason for the performance of more work by the polypentapeptide is the inverse temperature transition that occurs between 20 and 40°C. The magnitude of the change that occurs without a load is shown in Fig. 14B. The length change is the result of the wrapping up of the largely extended series of β-turns into a β-spiral structure as shown in Fig. 13A. By analogy, this could be called thermomechanical transduction of the first kind.

Temperature change may also be used to turn on elastic force in the case of the structural changes considered in situations relevant to mechanochemical coupling of the second kind. A weight may be attached to the end of an α-helix (Fig. 15A). If there were a graded instability of the helix with respect to an increase in temperature with the lower portion of the helix being less stable, then on raising the temperature, the lower part of the helix undergoes transition first, as shown in Fig. 15B. A portion of α-helix is converted to an extended elastic spiral structure that shortens lifting the weight. A further rise in temperature converts additional α-helix to spiral and further lifts the weight. It is helpful to have the graded instability in order to facilitate reversibility in a condensed system. This could be called thermomechanical transduction of the second kind. If, instead of a temperature change, the temperature of the transition could be lowered by a chemical process, mechanochemical coupling of the second kind would be achieved.

4.1.2. Using a Change in pH to Turn Elastomeric Force Off and On

The principle of using change in pH as the chemical process for mechanochemical coupling of the first kind can be shown by the effect of introducing

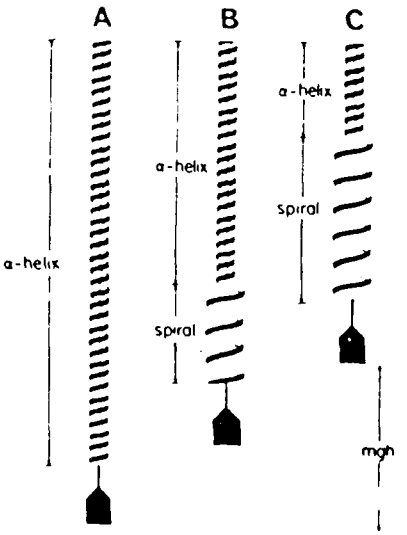


Fig. 15. Elastic contraction due to a temperature elicited structural conversion from an α-helix to a spiral structure. This is the result of a standard transition from a more-ordered to a less-ordered state on raising the temperature. An elastic spiral structure is shown rather than a random coil structure because thermal randomization of the polypentapeptide of elastin (see Urry, 1988, Fig. 16) and of elastin (see Fig. 4) causes a loss of elastic force rather than an increase in elastic force. As drawn, the lower end of the α-helix is more thermally labile and the thermal instability decreases as one progresses up the helix such that the structural transition occurs in a regular manner as the temperature is raised. This graded thermal instability would facilitate reversibility. Chemical modulation of the temperature of the transition would be mechanochemical coupling of the second kind.

an occasional Glu residue in position 4 of the polypentapeptide When the ratio of Glu to Val in position 4 is 1:4, the temperature for the inverse temperature transition for the onset of the intermolecular component of the inverse temperature transition is raised from 25°C for the pure polypentapeptide to 37°C at pH 2 for the Glu-containing polypentapeptide. The change of one residue in 25 from Val to Glu (COOH) increases hydrophicility (polarity) sufficient to shift the transition by 12°C. On raising the pH to 6, the temperature for the onset of aggregation of the 20% Glu^4-polypentapeptide is raised to 49°C. The change of one side chain in 25 residues from COOH to COO^- again increases the temperature of the transition by 12°C. (Fig. 16). A crosslinked matrix for demonstrating shifts in elastomeric force development has yet to be formed but it is expected that it will provide the first demonstration of mechanochemical coupling of the first kind. In order to lower the temperatures for the transitions, the Ile^1-PPP can be used. The principle is shown in Fig. 17 in terms of thermoelasticity curves. Elastomeric force develops between 20 and 40°C. If the polypeptide elastomer is made more polar, for example, by ionization of the carboxyl side chains of the of the included glutamic acid residues, then the transition is shifted some 20°C higher in temperature and comparable elastomeric force does not develop until 60°C. A change in the pH from 2 to 7 at 37°C would change from

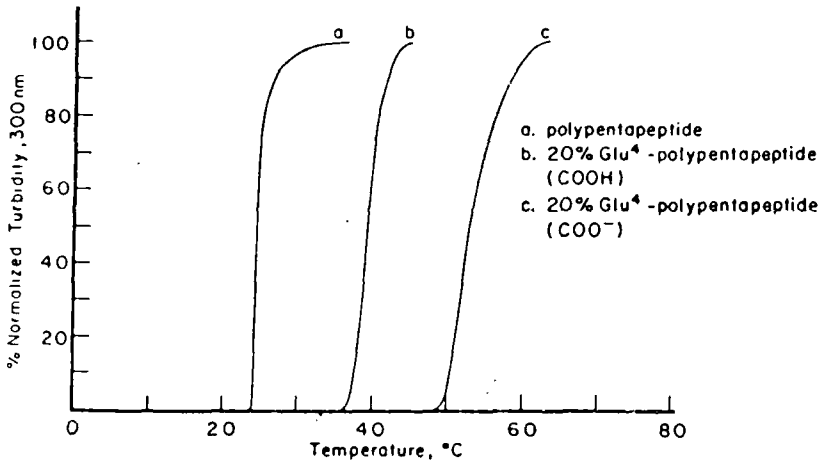


Fig. 16. Temperature profiles of coacervation, i.e., of the intermolecular component of the inverse temperature transition; for the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, in curve a; for 20% Glu^4-polypentapeptide at pH 2, where the carboxyl group is un-ionized (COOH) in curve b; and for 20% Glu^4-polypentapeptide at pH 6 where the carboxyl group is ionized (COO^-). The effect of decreasing the hydrophobicity (increasing the hydrophilicity) by introducing one glutamic acid residue in 25 residues raises the temperature for the onset of the inverse temperature by 12°C. Conversion of one residue in 25 from a COOH side chain to a COO^- side chain further raises the temperature for the onset of the inverse temperature transition by another 12°C. As this inverse temperature transition has been shown to turn the elastomeric force on and off in the crosslinked polypentapeptide, this result raises the possibility of turning on and off elastomeric force at 50°C by changing the pH. This would be mechanochemical coupling of the first kind. This data was obtained in distilled water. (D. W. Urry, M. Iqbal, and K. U. Prasad, unpublished data)

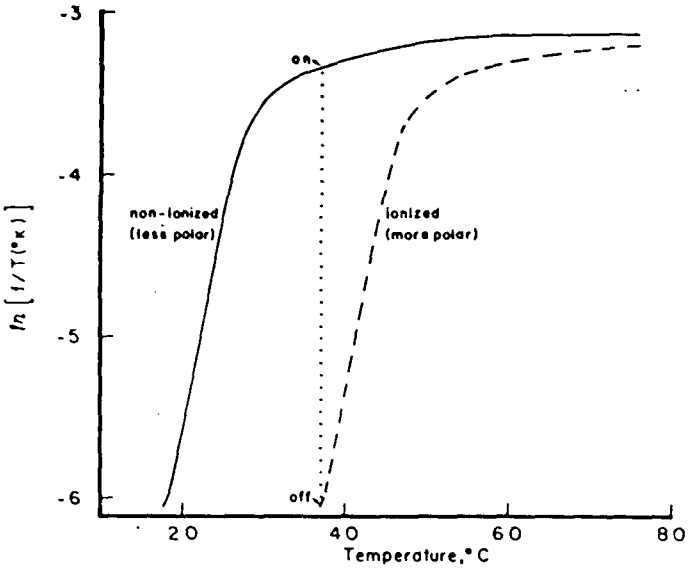


Fig. 17. Curves demonstrating mechanochemical coupling of the first kind. Solid curve: Thermoelasticity curve of 20 Mrad γ-irradiation crosslinked polypentapeptide of elastin, where on raising the temperature from 20° to 40°C there is a dramatic development of elastomeric force f, plotted as $\ln[f/T(°K)]$ versus temperature (°C). This development of elastomeric force is due to an inverse temperature transition. On making the polypeptide less hydrophobic which can also be stated as more hydrophilic or more polar, the temperature of the inverse temperature transition for the development of elastomeric force is increased to give the dashed curve. This effect of changing chain polarity of shifting the inverse temperature transition is shown in Figs. 11 and 16. When the change in chain polarity can be achieved reversibly, elastomeric force can be turned on and off by a chemical process. Examples of chemical means could be ionization-deionization (as in Fig. 16) or phosphorylation-dephosphorylation. This effect of phosphorylation could be operational in a fibrous structural protein or within a globular protein to modulate function by turning on and off elastic forces.

one curve to the other; and elastomeric force would be turned off by this change in chemical potential. Thus, just as changing the temperature from 20 to 40°C raises the weight in Fig. 14, lowering the pH from 7 to 2 at constant temperature is expected to turn on an elastomeric force and perform work, e.g., lift a weight. As this occurs due to the development of an entropic elastomeric force, it could appropriately be called entropic motive force (EMF).

It should be noted that almost four decades ago, Katchalsky (1951) demonstrated mechanochemical coupling with polyelectrolytes and considered the structural interconversions from rigid sphere at low ionization to statistical (random) coils at about 10% ionization to extended rigid rods at greater than 50% ionization. This would be relevant to mechanochemical coupling of the second kind and the mechanism would be charge-charge repulsion.

4.1.3. Possible Chemical Processes at Constant pH and Temperature

In general, for mechanochemical coupling to be of use to warm-blooded animals, it would best occur at a constant temperature and pH. Transient local pH changes would seem to be of limited use; since proton diffusion is so much faster than the structural change, the pH change will have dissipated before the structural response can have occurred. In those special cases in which a change of pH was maintained for sufficiently long times, pH change would be a useful on-off switch.

4.1.3.a. Deamidation-Amidation as an On-Off Switch. If deamidation of glutamine and/or asparagine in a polypeptide chain could be achieved, the result at pH 7 would be the production of an anionic carboxylate side chain. This is equivalent to the deprotonation of the carboxylic acid chain shown in Fig. 16 and would on deamidation, raise the temperature of the inverse transition of a 20% Gln^4-PPP with the result of turning off elastomeric force (Fig. 17). Conversely, the amidation of a carboxylate side chain could be used to turn on elastomeric force. While glutamine transaminase is a well-known enzyme system, it acts on the free amino acid. A search of the literature for amidases turned up no enzymes that could function as an amidase for glutamine or asparagine while in a polypeptide chain. An amidaselike activity of calpain (Ca^{2+}-dependent cysteine proteinase) I and II, which hydrolyzes the carboxyl-terminal amide of substance P but leaves the glutamine residue of substance P intact (Hatanaka *et al.*, 1985), is reported. There is also a so-called ω-amidase pathway for the degradation of glutamine which converts a carboxamide to a carboxylate but the action is on α-ketoglutaramate to produce α-ketoglutarate and ammonia (Calderón *et al.*, 1985).

4.1.3.b. Dephosphorylation-Phosphorylation as an On-Off Switch. The use of phosphorylation to activate a group (e.g., COOH) for synthesis in a metabolic process, such as in the glutamine transaminase-catalyzed reaction, is reasonably well understood. Just how the energy of adenosine triphosphate hydrolysis is used in muscle contraction, in cell motility, in modulation of enzyme processes and channels, in membrane transport, and so forth, is less well understood. The timely release of kinases for phosphorylation and phosphatases for dephosphorylation is central to mechanochemical coupling in cell motility. The mechanism that emerges from the study of the elastic polypentapeptide and its analogues is the turning on and off of elastomeric force by changing the temperature of a transition between two states, one of which can exert an elastomeric force. Just as ionization changes the hydrophobicity of the elastomeric sequential polypeptide and thereby changes the temperature of an inverse temperature transition, phosphorylation of a polypeptide by Fig. 17 could similarly be expected to change the temperature of an inverse temperature transition and to turn off an elastomeric force while dephosphorylation could turn it back on. This would be a case for mechanochemical coupling of the first kind. As has been more commonly appreciated, it would also be possible for a change in phosphorylation to shift the equilibrium for a standard transition from a lower to a higher entropy state as the on-off switch in mechanochemical coupling of the second kind.

4.1.3.c. Changes in Calcium Ion Activity as an On-Off Switch. Calcium ion has the capacity to bind polypeptide and protein with binding constants as large as $\geq 10^6$/M. This means that changing calcium ion activity could also be used as an on-off switch for shifting between two states, one elastomeric and the other relatively inelastic. The binding of calcium ion to troponin C, calmodulin, parvalbumin, and related proteins is well appreciated and in the former two cases are an important element in determining the state of muscle contraction in striated and smooth muscle. The introduction of a ΔpCa as a mechanism for turning elastomeric force on and off in the present context of the possible implications from understandings developed on the sequential polypeptides of elastin and their analogs, could relate to consideration of the repeating sequences in myosin and possible modifications of the Harrington (1971, 1979) and the Huxley and Simmons (1971) models for the occurrence of elastic forces in muscle contraction.

4.2. Events Attending Parturition and their Reversal

The remarkable changes that facilitate birth and their reversal center on two anatomical sites: the pubic symphysis and the uterine cervix. In the days immediately preceding delivery, the cartilaginous pubic symphysis converts to an interpubic ligament that relaxes the size constraints on the pelvic girdle (birth canal); the uterine cervix softens and can open wide to allow natural birth to occur. Both development of interpubic ligament and cervical softening occur in response to the relaxation factor first recognized by Hisaw (1926) in the guinea pig. The relaxation factor has been identified as an insulin-related hormone called relaxin (for reviews, see Kemp and Niall, 1984; Weiss, 1984). Both in the pubic symphysis (Braddon, 1978) and in the uterus (Judson *et al.*, 1980), a response to the administration of relaxin is the increase in concentration of cyclic adenosine monophosphate (cAMP), which activates a cAMP-dependent kinase (Kemp and Niall, 1984). This enzyme phosphorylates serine and threonine residues in sequential proximity to arginine and lysine residues (Sparks and Brautigan, 1986). The phosphorylation of protein associated with a relaxation of tension immediately suggests mechanochemical coupling of the first kind discussed above, which perhaps even more interestingly also provides the basis for reversal, i.e., for the active pulling of the structures back to their near-predelivery state by dephosphorylation.

4.2.1. Interpubic Ligament Formation and Reversal

In virgin mice, the pubic bones are separated by less than one millimeter; in the half-dozen days before parturition, the gap increases, becoming bridged by a 5- to 6-mm-long ligament at term; after delivery, "the gap rapidly closes" to <2 mm (Hall, 1947) (Fig. 18). The dominant protein components of ligament are elastin and collagen, there being 80-90% elastin in ligamentum nuchae. As collagen is nonextensible, partial removal or detachment would seem necessary in order for elongation to occur. Elastin present could simply unwind on phosphrylation, if an appropriate extracellular kinase activity were present (Kübler *et al.*, 1983). It has been seen with the polypentapeptide of elastin that the relaxing of force that occurs on the low temperature side of the inverse temperature transition leads to an extension by a factor approaching 2.5 in the absence of an extending force (a load)

(see Fig. 14, see also Urry, 1988, Fig. 13). At a constant temperature, the relaxation would occur as phosphorylation (increased polarity) raised the temperature of the transition as shown in Fig. 17. If there were no extracellular kinase, tropoelastin could be phosphorylated intracellularly, for example, by the cAMP-dependent kinase, and then elastin fibers would be formed extracellularly in the relaxed, extended state. Interestingly, as shown in Fig. 1 for bovine ligamentum nuchae elastin, there are serines and threonines in sequential proximity to arginines and lysines. It might be inquired as to why all ligaments, and elastin containing tissues, would not be equally relaxed in late pregnancy. The answer could be the *de novo* synthesis of elastin by cells at the targeted anatomical sites and/or it could involve the interesting splicing out of sequences between crosslinks that has been observed by Yeh *et al.*, (1987) and/or it could involve the localized release of extracellular kinases (Kübler *et al.*, 1983). Perhaps more significant than the relaxing effect would be the capacity to turn on the elastomeric force, to shorten the ligament and draw the pubic bones back toward their prerelaxed dimensions as depicted in Fig. 18. Extracellular dephosphorylation following delivery would provide the mechanism for decreasing chain polarity and for turning on elastomeric force by the winding up of the β-spiral structure, as shown in Fig. 13A, thereby drawing the pubic bones back to their near-predelivery state.

4.2.2. Cervical Ripening

In the uterine cervix, the elastin fibers could also undergo relaxation by phosphorylation as proposed for the interpubic ligament. If there are titinlike proteins in

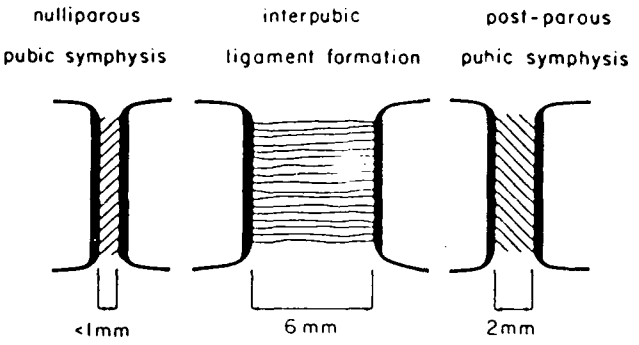


Fig. 18. Schematic representation of changes in the pubic symphysis that attend parturition following the descriptions of Hall (1947) based on studies in mice. In virgin mice, the connective tissue connecting the pubic bones, the pubic symphysis, is a dense connective tissue filling a gap of less than 1 mm. In the few days preceding delivery, the gap between the pubic bones has become an interpubic ligament 5-6 mm in length. After delivery, the gap closes to about 2 mm with the regeneration of the pubic symphysis. Based on the perspectives gained from the elastin studies, it is proposed that the elastic forces required to draw the pubic bones back together result from dephosphorylation which would result in the development of elastic spiral structures and the turning on of elastic forces. This would be an application of mechanochemical coupling of the first kind.

smooth muscle, however, as in striated and cardiac muscle (Wang, 1985), phosphorylation of this or a similar elastic protein would be another means of relaxing the cervical closure, and dephosphorylation could result in reformation of the near-nulliparous uterine cervix. This would be relevant to the uterus in general. Titin is isolated with 2-3 moles phosphate per mole of protein (Wang, 1985). Again, it is not so much the relaxation and softening that is remarkable as is the reversal of the effects and the development of the forces necessary to return to the near-normal cervical opening. Should such a capacity exist in smooth muscle cells to modulate tension exhibited by a supramicron length elastic protein, the role of such a protein in some forms of essential hypertension would become of interest. In smooth muscle, it might be noted in consideration of possible relevance of the elastin studies in relationship to the effects of phosphorylation-dephosphorylation as an on-off switch for structural change that phosphorylation occurs by myosin light-chain kinase (Dabrowska *et al.*, 1977) and reversal by myosin light-chain phosphatase (Aksoy *et al.*, 1976), which accompanies smooth muscle contraction and that proteolytically modified myosin light-chain kinase can activate actin-dependent myosin ATPase resulting in contraction without calcium ion or cal· ·dulin (Walsh *et al.*, 1983).

4.3. Muscle Contraction

Muscle contraction can be characterized as the turning on and off of elastic forces (Hibberd and Trentham, 1986). Accordingly, it is hoped that the insights provided by the studies on elastin peptides and related analogues, the new perspective on the so··ce of entropic elastomeric force that result, and the concepts that emerge for turning elastic forces on and off justify a few considerations from one not expert in the area of muscle contraction. In keeping with phosphorylation and dephosphorylation as a means of altering chain polarity and of reversibly shifting the temperature for a structural transition between highly elastic and less elastic states of different end-to-end chain lengths, in relationship to the interactions with ATP, one means whereby the elastin results could be relevant would be to the proposed structural changes involving the head or S-1 fragment of myosin. In this case, length changes could be considered which might relax the requirement for large angle changes in cross-bridge rotation. One scenario could be ATP-dependent relaxation, permitting the cross-bridge to extend and attach to the actin filament followed by release of phosphates and elastic shortening for the powerstroke.

The foregoing understandings of elasticity were developed on polypeptides composed of repeating sequences and the new class of conformations (spirals) that result are intermediate in entropy between α-helices, β-sheets, for example on the one hand and random chain networks or random coils on the other. The obvious repeating sequences, heptamers grouped as 28mes (McLachlan, 1984), in the myosin rod segment suggest consideration of elastic spiral structures in muscle contraction. Studies on the β-spirals of the polypentapeptide, polytetrapeptide, polyhexapeptides, and polynonapeptides of elastin indicate spiral structures of the order of 15 (Venkatachalam and Urry, 1981; Urry *et al.*, 1981), 16 (Khaled *et al.*, 1985), 12 (Urry and Long, 1976), and 18 (Chang *et al.*, unpublished data) residues per turn, respectively, with values in the range of 0.5 Å/residue for translation along the

spiral axis in the relaxed state. A spiral structure with some 14 residues per turn would seem possible for the repeat sequences of the myosin rod. With the concentration of positive charge in every fourth heptamer (McLachlan, 1984), a 14-residue-per-turn spiral would have the positive charge in alternate turns. Such a spiral structure could provide for an elastic collapse of length on going from α-helix to spiral as indicated in Figs. 13 and 15. A structural transition of this type could be relevant to the proposed mechanisms of Huxley and Simmons (1971) and Harrington (Harrington, 1971, 1979; Tsong *et al.*, 1979, 1983). Using a chemical process to lower the temperature of the transition in an α-helix to spiral transition would seem to overcome the Skolnick (1987) criticism of the Harrington model of an α-helix to random coil transition in the S-2 segment of the myosin rod. What is considered here would be modification of the Harrington model where a nonrandom spiral structure of the order of 0.5 Å/residue replaced the random coil and where a chemical process such as a change in the activity of calcium ion or change of polarity by phosphates would lower the temperature of the transition and trigger contraction. In the above-noted case of smooth muscle, myosin light-chain phosphorylation-dephosphorylation (Dabrowski *et al.*, 1977; Aksoy *et al.*, 1976; Walsh *et al.*, 1983) could provide a switching mechanism. The added negative charge of the phosphate could, by electrostatic repulsion, destabilize the α-helix much as the α-helix poly-L-glutamate is destabilized at a greater than 0.3 degree of ionization (Urry, 1968, see Fig. 35). With the demonstrated irreversibility of thermal denaturation in the condensed phases of the polypentapeptide of elastin (Urry, 1988) and of elastin itself (see Fig. 4), where random coils presumably do develop, the conversion from α-helix to spiral could be expected to occur with great reversibility. Also with the low elastic modulus implied on complete thermal randomization of chains in elastin (see Fig. 4), random chains do not appear to be the source of sufficient elastic moduli whereas spiral-like structures that derived their entropic elastomeric force from internal chain dynamics could provide the elastic modulus required in muscle contraction.

Consideration of the charge distribution in the rod segment shows that the greatest excess of negative side chains over positive side chains occurs in the range of the S-2 segment, residues 300'-520' in the McLachlan (1984) numbering, and the excess negative charge persists but decreases as one proceeds in both directions toward the amino and carboxyl ends. The charge distribution suggests that this segment should be a focal point for the collapse of α-helix to spiral and that as the change in chemical potential progressed, the collapse could spread in an orderly fashion in both directions from a focal point. Such a double-zipper mechanism (a term made perhaps even more appropriate by the dimeric α-helical structure of the myosin rod) could facilitate reversibility and could possibly provide explanation for the long stroke (sliding) distance of >600 Å for a single ATP cycle reported by Yanagida *et al.* (1985). The interesting stretch activation exhibited by insect flight muscle and possibly mammalian heart muscle (Pringle, 1978) could be a mechanical stretching that converted spiral back to α-helix, even under chemical conditions that in the absence of the mechanical stretch would favor elastic spiral over α-helix. Stretch activation could also be relevant to the possible length changes considered above in the S-1 fragment. In terms of the two kinds of mechanochemical coupling, that of the putative involvement of the S-1 segment could possibly be

mechanochemical coupling of the first kind, whereas that of the S-2 segment and the rod in general could be mechanochemical coupling of the second kind.

4.4. Cell Motility

As with the other protein systems and processes considered, in this section on cell motility, molecular mechanisms are put forward that interject the concepts of elasticity and of turning on and off elastomeric force, which were derived from studies on the elastin system; the purpose remains to stimulate inclusion of these perspectives in consideration of, in this case, cell migration by those who are expert in this area. The points drawn from the elastin system are the analogy between coacervation and a sol→gel transformation and the effects of changing polypeptide chain polarity in a way that relaxes elastic constraints. Another, but not obligatory, point that can be introduced is the demonstration in the fibronectin-fibroblast system that cell attachment and chemotaxis can use the same peptide sequence (Long *et al.*, 1987).

Perhaps an interesting starting point would be one in which the only directionality present would be due to a concentration gradient of chemoattractant (CA) (Fig. 19). Consider a cell of essentially circular shape attached to its appropriate substrate. Within the cell is a cytoskeletal network in which microfilaments span from cell membrane to cell membrane, criss-crossing the cell as an essentially isotropic network. Importantly, these microfilaments exert an elastic constraint on the cell membrane. The chemoattractants on the high concentration side of the cell bind to

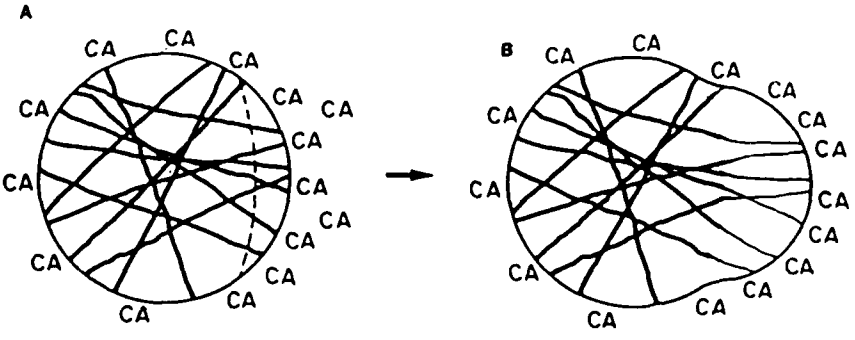


Fig. 19. Schematic representation of possible cell migration by pseudopod formation in response to concentration gradient of chemoattractant, CA. (A) The cell membrane is elastically constrained by elastic filaments that criss-cross the cell. They could equally well span, for example, from the nuclear membrane to the plasma membrane. On the side of the cell where the concentration of chemoattractant is higher, binding to cell receptors would cause local intracellular release of cyclic adenosine monophosphate (cAMP), which locally activitates cAMP-dependent kinases. (B) The kinases have phosphorylated locally the elastic filaments increasing the hydrophilicity of the polypeptide chains and causing the elastic filaments to unwind and to release the elastic constraint on the membrane. This relaxation of elastic constraint and the natural water uptake that attends the reversal of an inverse temperature transition results in pseudopod formation initiating cell migration in the direction of the increasing concentration of chemoattractant.

their receptors with the following sequelae. With the possibility of a commonality of cell attachment peptide sequence and chemotactic peptide sequence, the chemoattractant competitively releases cell attachment to the substrate and binds to the cell receptor with the result of localized cAMP release within the cell. The released cAMP activates cAMP-dependent kinases locally that phosphorylate nearby elastic microfilaments, e.g., one end of a titinlike molecule. Phosphorylation *raises* the temperature of the inverse temperature transition and causes the elastic microfilament to unwind and to turn off the local elastomeric constraints on the membrane. The unwinding of the microfilament has elements equivalent to the reversal of coacervation, i.e., a gel→sol transition that causes an osmotic flow of water to the site much as the crosslinked polypentapeptide of elastin swells with a factor of 10 increase in volume on lowering the temperature below the onset of the inverse temperature transition (see Fig. 14B). Raising the temperature of the inverse temperature transition by phosphorylation at a fixed temperature is equivalent to lowering the temperature to below the onset of the inverse temperature transition. Phosphorylation would be the equivalent in Fig. 17 of going at 37°C from the solid curve, where the elastomeric force is turned on, to the dashed curve, where elastomeric force is turned off.

With the simultaneous local relaxation of elastic constraint on the membrane and the local swelling of the gel→sol transition, the cell membrane protrudes by pseudopod formation in the direction of increasing concentration. By these processes, an isotropic cell becomes anisotropic and achieves movement in the direction of the concentration gradient. Reattachment to the substrate, dephosphorylation of the local microfilaments, and possibly engaging the actomyosin motive system with organization of stress fibers to draw up the rear of the cell could all presumably follow appropriately. Cell migration in a concentration gradient was recently shown to occur apparently without functional myosin (Solomon, 1987; Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). This finding directs attention toward elastic proteins and forces outside of the actomyosin system. The foregoing is but one of a number of possible scenarios that could use what has been learned from studies of the nature of polypeptide elasticity.

NOTE ADDED IN PROOF

Preparation of a γ-irradiation cross-linked 4% Glu polypentapeptide matrix has been achieved which demonstrates pH dependent contraction and relaxation. The schematic curves of Fig. 17 are almost exactly what is found for the matrix contracted at 37 °C and pH 3.3 (solid curve) and relaxed at 37 °C and pH 4.3 (dashed curve) for phosphate buffered saline. This represents the first demonstration of mechanochemical coupling of the first kind which, of course, is a mechanism where the chemical process shifts the temperature of an inverse temperature transition. This mechanism should be considered as a possibility whenever free energy transduction occurs in protein mechanisms.

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Review

Entropic Elastic Processes in Protein Mechanisms. I. Elastic Structure Due to an Inverse Temperature Transition and Elasticity Due to Internal Chain Dynamics

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Numerous physical characterizations clearly demonstrate that the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ in water undergoes an inverse temperature transition. Increase in order occurs both intermolecularly and intramolecularly on raising the temperature from 20 to 40°C. The physical characterizations used to demonstrate the inverse temperature transition include microscopy, light scattering, circular dichroism, the nuclear Overhauser effect, temperature dependence of composition, nuclear magnetic resonance (NMR) relaxation, dielectric relaxation, and temperature dependence of elastomer length. At fixed extension of the crosslinked polypentapeptide elastomer, the development of elastomeric force is seen to correlate with increase in intramolecular order, that is, with the inverse temperature transition. Reversible thermal denaturation of the ordered polypentapeptide is observed with composition and circular dichroism studies, and thermal denaturation of the crosslinked elastomer is also observed with loss of elastomeric force and elastic modulus. Thus, elastomeric force is lost when the polypeptide chains are randomized due to heating at high temperature. Clearly, elastomeric force is due to nonrandom polypeptide structure. In spite of this, elastomeric force is demonstrated to be dominantly entropic in origin. The source of the entropic elastomeric force is demonstrated to be the result of internal chain dynamics, and the mechanism is called the librational entropy mechanism of elasticity. There is significant application to the finding that elastomeric force develops due to an inverse temperature transition. By changing the hydrophobicity of the polypeptide, the temperature range for the inverse temperature transition can be changed in a predictable way, and the temperature range for the development of elastomeric force follows. Thus, elastomers have been prepared where the development of elastomeric force is shifted over a 40°C temperature range from a midpoint temperature of 30°C for the polypentapeptide to 10°C by increasing hydrophobicity with addition of a single CH_2 moiety per pentamer and to 50°C by decreasing hydrophobicity. The implications of these findings to elastic processes in protein mechanisms are (1) When elastic processes are observed in proteins, it is unnecessary, and possibly incorrect, to attempt description in terms of random chain networks and random coils; (2) rather than requiring a random chain network characterized by a random distribution of end-to-end chain lengths, entropic elastomeric force can be exhibited by a single, short peptide segment; (3) perhaps of greatest significance, whether occurring in a short peptide segment or in a fibrillar protein, it should be possible reversibly to turn elastomeric force on and off by reversibly changing the hydrophobicity of the polypeptide. Phosphorylation and dephosphorylation would be the most obvious means of changing the hydrophobicity of a polypeptide. These considerations are treated in Part 2: Simple (Passive) and Coupled (Active) Development of Elastic Forces (see Urry, 1988).

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KEY WORDS: inverse temperature transition; entropic elasticity; thermomechanical transduction; mechanochemical coupling; polypentapeptide of elastin; librational entropy mechanism of elasticity; internal chain dynamics.

> At every crossway on the road that leads to the future, tradition, has placed against each of us, 10,000 men to guard the pass.
>
> (Maurice Maeterlinck, 1907)

1. INTRODUCTION

It is the purpose of this, the first of two papers, to provide clarification of the basis of polypeptide entropic elastomeric force, its definition, the experimental basis for its identification, and, importantly, the source of entropy change on deformation (see also Urry, 1988). The nature of entropic elastomeric force is demonstrated with the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ with the emergence of a new mechanism for the elasticity.[2] Entropic elasticity of this and related sequential polypeptides derives from regular spiral structures in which internal chain dynamics (librational motion) provides the entropic elastomeric force. The central point derived from studies on the polypentapeptide and its analogues is that entropic protein and polypeptide elasticity can be exhibited by regular, nonrandom conformations that can be denatured at temperatures greater than 60°C with loss of elastic force and of elastic modulus. Polypeptide elasticity does not require random coils or networks of random chains. In point of fact, thermally denatured polypeptide elastomers, i.e., the randomized polypeptide structures with no change in the number of crosslinks, exhibit greatly reduced elastic moduli.

The specific case demonstrated is one in which entropic elastic states of polypeptides are obtained on the more ordered side of an inverse temperature transition. Since the temperature of the inverse temperature transition depends on the hydrophobicity of the polypeptide chain, it becomes apparent at a given temperature that elastomeric force can be turned on and off by changing the hydrophobicity of the polypeptide chain. Examples of chemical processes whereby the hydrophobicity of the polypeptide chain could be reversibly changed, i.e., whereby the temperature of the transition could be reversibly shifted, are ionization-deionization as in the titration of a weak acid or base, enzymatic amidation-deamidation of carboxylates, and phosphorylation-dephosphorylation. The second paper (Part II) considers the application of these concepts to entropic elastic processes of additional polypeptide and protein systems.

2. ENTROPIC ELASTOMERIC FORCE

2.1. Definition of Entropic Elastomeric Force

A material displays elastomeric force f when it resists and recovers from deformation. The elastomeric force is defined as the change in maximum work function (Helmholtz free energy) ∂A with change in length ∂L at constant volume

[2] Presumably because of the firmly held view that the elasticity of elastin derived from random chain networks, the only published physical characterizations of the elastomeric sequential polypeptides of elastin are due to the work of this Laboratory. Because of this, the relevant work reviewed here, going back to the early 1970s will require that an uncommon proportion of the references be to our own publications.

V and constant temperature T; that is,

$$f \equiv (\partial A/\partial L)_{V,T} \tag{1}$$

But since

$$(\partial A/\partial L)_{V,T} = (\partial E/\partial L)_{V,T} - T(\partial S/\partial L)_{V,T} \tag{2}$$

where E is the internal energy and S is the entropy of the elastomeric system, the elastomeric force is described as having two components, an internal energy component f_e and an entropy component f_s, that is,

$$f = f_e + f_s \tag{3}$$

On deformation, f_e is attributable to the strain in bonds and f_s, the entropic elastomeric force, results from a decrease in the number of configurations in the lowest energy band of states. Since bond strain leads to bond breakage, a durable (i.e., ideal) elastomer would be one in which $f_e = 0$ and $f = f_s$. Elastin, the elastic protein of connective tissue, presents an extreme example of a durable elastomer. Single elastic fibers can last the lifetime of an individual (Partridge, 1962; Sandberg *et al.*, 1977). Within the normal lifetime of the individual, single elastic fibers will have undergone a billion stretch-relaxation cycles in the aortic arch and thoracic aorta, where there is twice as much elastin as collagen (Cleary and Moont, 1977). Clearly, one anticipates that this is a near-ideal elastomer, that elastin exhibits a dominantly entropic elastomeric force. Obviously, to be able to exert an elastomeric force without paying an internal energy price to do so would be a very useful mechanism in protein function. Just as apparent, an f_s in an elastomeric polypeptide segment should be usable to effect an f_e in an appropriately associated bond system. In particular, for it to be possible to use entropic elastomeric force to induce strain, that is to achieve an f_e, in a bond undergoing hydrolytic cleavage would be an effective means of reducing the internal energy barrier for a reaction and to increase the catalytic rate. But before such considerations have validity, it is necessary both to have means of identifying entropic elastomeric force and to understand the mechanism whereby entropic elastomeric force develops. For, in the above example of strain in enzyme catalysis, it was assumed that a single, short, anisotropic polypeptide chain segment, rather than an isotropic random network of chains, would be sufficient to exert an entropic elastomeric force.

2.2. Experimental Bases for Identifying Entropic Elastomeric Force

A means of evaluating the magnitude of the elastomeric force f_s is in experimentally determining the f_e/f ratio. Following Flory *et al.* (1960), Andrady and Mark (1980), and Queslel and Mark (1986),

$$f_e/f = -T(\partial \ln[f/T]/\partial T)_{V,L,n} \tag{4}$$

Accordingly, the experiment is to stretch the sample to a fixed length L and for conditions of constant volume V and constant composition n, to determine the temperature dependence of force. The f_e/f ratio is obtained from a plot of $\ln(f/T)$ versus temperature, where the slope times the mean of the absolute temperature for

the temperature interval of interest is the f_e/f ratio. For a dominantly entropic elastomer, f_e/f is less than 0.5. Two complications with the approach are that it is not common to be able to hold V and n constant with varying temperature and that T is large, usually greater than 300K, requiring accurate determination of the slope. To overcome the former complication, an approximate correction term has been developed for conditions of constant pressure P constant length and for an elastomer in equilibrium eq with a solvent. The expression becomes (Dorrington and McCrum, 1977)

$$f_e/f = -T(\partial \ln[f/T]/\partial T)_{P,L,eq} - \frac{\beta_{eq} T}{\alpha^3(V_i/V) - 1} \tag{5}$$

where $\beta_{eq} = (\partial \ln V/\partial T)_{P,L,eq}$ is the thermal expansion coefficient; α is the fractional increase in length, L/L_i where L_i is the initial length and L the length at fixed extension, and V_i and V are the volumes of the elastomer before and after elongation. This correction term is an approximation for an isotropic network of chains with a gaussian distribution of end-to-end lengths. As shown below, the polypentapeptide of elastin is not isotropic and would not have a broad gaussian distribution of end-to-end chain lengths. Fortunately, as will also be shown, in the temperature range from 40 to 60°C, the volume of the nonextended state and the composition are nearly constant, such that Eq. (4) can be considered.

Because of the limitations of Eqs. (4) and (5), it is useful to have additional means of evaluating the entropic nature of a polypeptide. In order for a polypeptide to exhibit entropic elastomeric force, the backbone must be able to undergo substantial motion and the states represented by the motion must be obtainable with only low-energy barriers for going from one state to another. Considering this, the temperature dependence of the backbone motion, e.g., of the correlation time for the motion, can be determined. If the energy of activation for the motion is several kcal/mole or higher, the states could not be sufficiently interconverting at body temperature to give a large entropy to the backbone. By contrast, if the activation energy is low, e.g., less than 2 kcal/mole, the states would be reasonably obtainable at body temperature, and the polypeptide backbone would have high entropy. The temperature dependence of backbone correlation time can be obtained from NMR and dielectric relaxation studies.

2.3. Proposed Mechanisms of Entropic Protein Elasticity

Having defined and arrived at a means of identifying entropic elastomeric force (EEF), it is necessary to develop an understanding of the mechanism, as this determines the potential for EEF to be a factor in protein mechanisms.

2.3.1. Classic Theory of Rubber Elasticity: Decrease in Entropy on Deformation Due to Displacement from Random Distribution of End-to-End Chain Lengths

In the classic theory of rubber elasticity, the description is one of a random chain network in which there is a random distribution of end-to-end chain lengths

(Flory, 1953; Mandelkern, 1983). This is shown in Fig. 1, where the probability of a given end-to-end chain length, $W(r)$, is plotted against the value of the end-to-end chain length for a freely jointed chain composed of 10,000 units with a repeat length of 0.25 nm (Queslel and Mark, 1986; Flory, 1953). The end-to-end chain lengths vary from 0 nm to fully extended, peaking near 20 nm. A random chain network is an isotropic system. One random-chain configuration with the end-to-end chain length, indicated as r, is given in Fig. 2 (Flory, 1953). A collection of such chains with a random distribution of end-to-end chain lengths is represented by the solid curve in Fig. 1. As developed by Flory *et al.* (1960), calculation of the f_e/f ratio is achieved by the expression

$$f_e/f = T(d \ln\langle r^2\rangle_0/dT) \tag{6}$$

where $\langle r^2\rangle_0$ is the mean square end-to-end chain length calculated, beginning with the equation of Eyring (1932). That the elasticity of elastin is due to random chain networks is a staunchly held view (Hoeve and Flory, 1958, 1974; Aaron and Gosline, 1980, 1981; Torchia *et al.*, 1983; Torchia and Piez, 1973; Lyerla and Torchia, 1975; Gosline and Rosenbloom, 1984; Andrady and Mark, 1980; Fleming *et al.*, 1980).

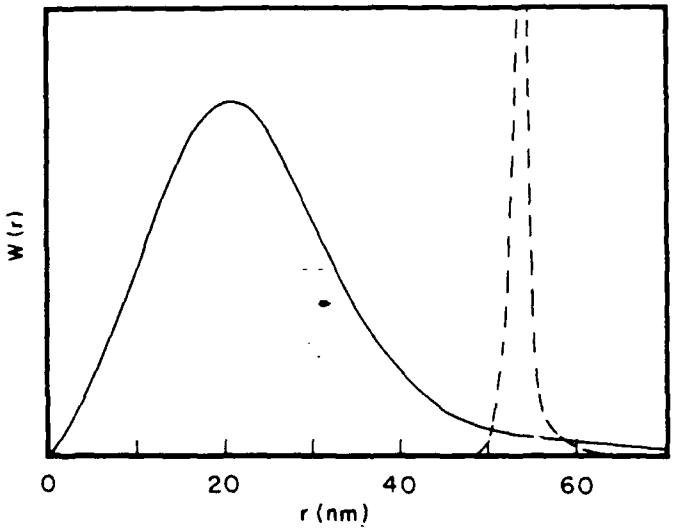

Fig. 1. Distribution, $W(r)$, of end-to-end chain lengths for a freely jointed chain of 10,000 segments, each 0.25 nm in length. The most probable end-to-end length is near 20 nm with occurrences ranging from overlapping of ends to the fully extended chain. (Redrawn from Flory, 1953; Queslel and Mark, 1986.) (- - -) Schematic representation of the distribution of end-to-end chain lengths that could occur for well-oriented 800-residue polypentapeptide of elastin in the β-spiral conformation when in an unstretched crosslinked elastomeric matrix. It is the unwinding of a β-spiral structure in the crosslinked matrix on reversing the inverse temperature transition, i.e., on lowering the temperature from 40° to 20°C, that results in an increase in length of the elastomer by more than 200% (see Fig. 13).

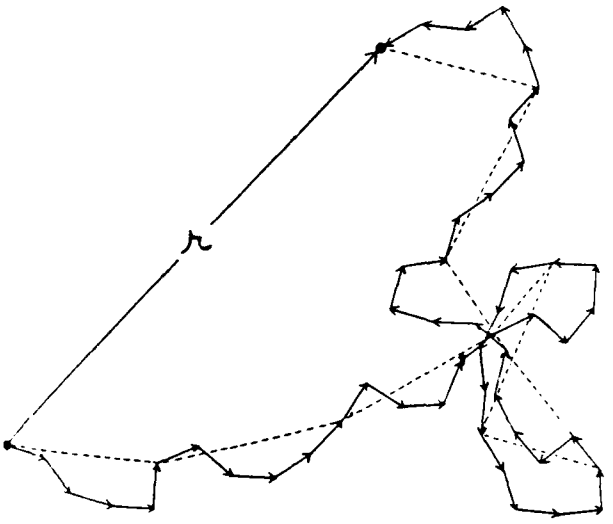

Fig. 2. A 50-segment random chain in two dimensions with allowed angles ranging from +90° to −90° and with the end-to-end distance vector indicated as *r*. (From Flory, 1953.)

2.3.2. *Solvent Entropy: Decrease in Entropy on Deformation Due to Exposure of Hydrophobic Side Chains That Become Surrounded by Clathrate-like Water of Lower Entropy Than Bulk Water*

The amino acid composition of mammalian elastin, for example, is one of 60% hydrophobic residues, one-third glycine residues, and the remainder largely due to lysine residues that become crosslinks with loss of charge (Petruska and Sandberg, 1968; Franzblau and Lent, 1969). Thus, in terms of amino acid composition, elastin is a hydrophobic protein. On stretching of elastin, it is to be expected that hydrophobic association of side chains will become disrupted and that less-ordered bulk water will surround the exposed hydrophobic side chains and become more-ordered clathrate-like water. Thus, a decrease in solvent entropy is expected on extension of elastin; this has been proposed to contribute to entropic elastomeric force (Weis-Fogh and Andersen, 1970; Gosline, 1978, 1980; Gray *et al.*, 1973).

2.3.3. *Librational Entropy Mechanism of Elasticity: Decrease in Entropy on Deformation Due to Damping of Internal Chain Dynamics*

The librational entropy mechanism (LEM) of elasticity was derived from studies on the conformation and function of the polypentapeptide of elastin (Urry, 1982; Urry *et al.*, 1982, 1985*f*; Urry and Venkatachalam, 1983).[3] The mechanism, however, is entirely general. In the nonstretched state, a polypeptide chain segment is able to exhibit internal chain dynamics involving large-amplitude low-frequency rocking

[3] The polypentapeptide of elastin $(VPGVG)_n$ recurs 11plus times in porcine and bovine elastin (Sandberg *et al.*, 1981; J. Rosenbloom, personal communication), it forms the longest sequence between crosslinks and is centrally located in the sequence (J. Rosenbloom, personal communication; see also Urry, 1988, Fig. 1 therein).

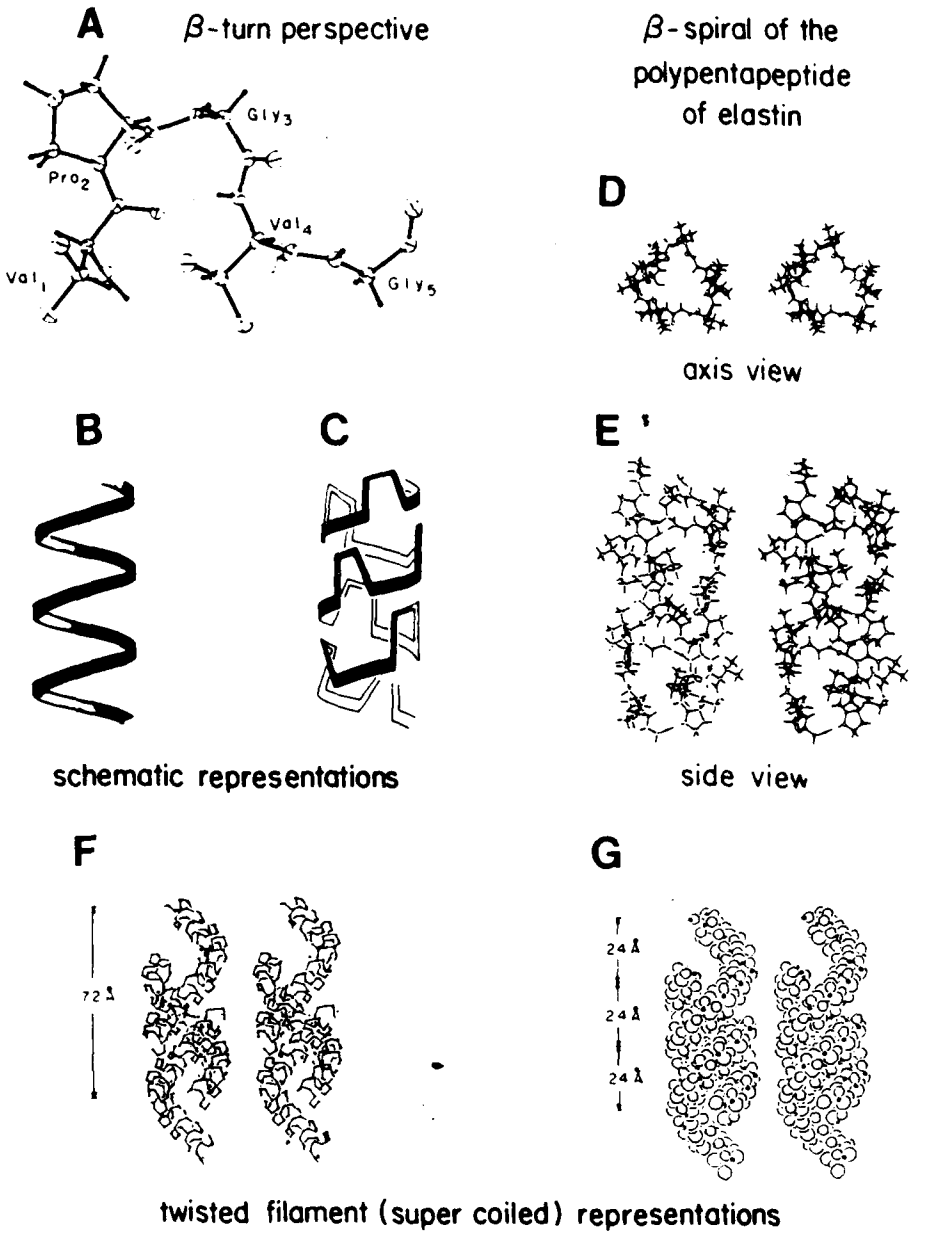

Fig. 3. Molecular structure of the polypentapeptide of elastin. (A) β-turn of the pentamer, Val^1-Pro^2-Gly^3-Val^4-Gly^5, with the $Val^1C{=}O \ldots HNVal^4$ 10 atom hydrogen-bonded ring, with the Pro^5-Gly^3 sequence at the corners of the turn and with the intervening peptide moiety with its C—O, the Pro C—O, directed on the side opposite to that of the Pro α—CH, called a type II Pro^2-Gly^3 β-turn. (Adapted from Cook *et al.*, 1980.) (B) Schematic representation of a helical structure with the helical parameters those of the β-spiral of the PPP. (C) Schematic representation of the β-spiral showing the β-turns functioning as spacers between the turns of the helix (spiral). The β-spiral is the result of optimizing intramolecular hydrophobic interactions. (D) Spiral axis view in stereo pair of a detailed plot showing one of a class of closely related β-spiral conformations. There is room for several columns of water within the β-spiral and shown suspended between β-turns is the suspended peptide segment, within which the peptide moieties can undergo large amplitude rocking motions. (B–D from Urry, 1983.) (E) Stereo pair side view showing one of a class of closely related β-spiral conformations (the same as in D). β-turns are seen to function as spacers between turns of the spiral, with the interturn contacts using the hydrophobic Val and Pro side chains. There are spaces in the surface of the β-spiral, where intraspiral water can exchange with extraspiral water. The suspended segments are seen to be essentially surrounded by space to be filled with water and are therefore free to exhibit large librational motions (see Fig. 4). (From Venkatachalam and Urry, 1981.) (F) Stereo pair of supercoiling of three β-spirals in αC—αC virtual bond representation. (G) Stereo pair representing the structure resulting from supercoiling of three β-spirals. The residues are represented as spheres of different radii centered on the α—C of different residues, this approximating the twisted filaments shown in Fig. 8A (F) and (G). (From Urry *et al.*, 1982.)

motions of composite moieties, the simplest of which would be the peptide moiety. On stretching, the amplitude of the rocking motions (of the librations) is damped and may be shifted to higher frequency. This constitutes a decrease in entropy of the chain segment.

In its range of expressions of elastic force, the LEM may involve only a small chain segment composed of but a few residues. Alternatively, the LEM may involve a regular dynamic structure. The regular elastic structure, for example, composed of repeating peptide sequences, is intermediate in its entropic state between the more rigid α-helix, β-sheets, and triple-stranded collagen structures at the one extreme and the classically considered random chain network (random coil structure)

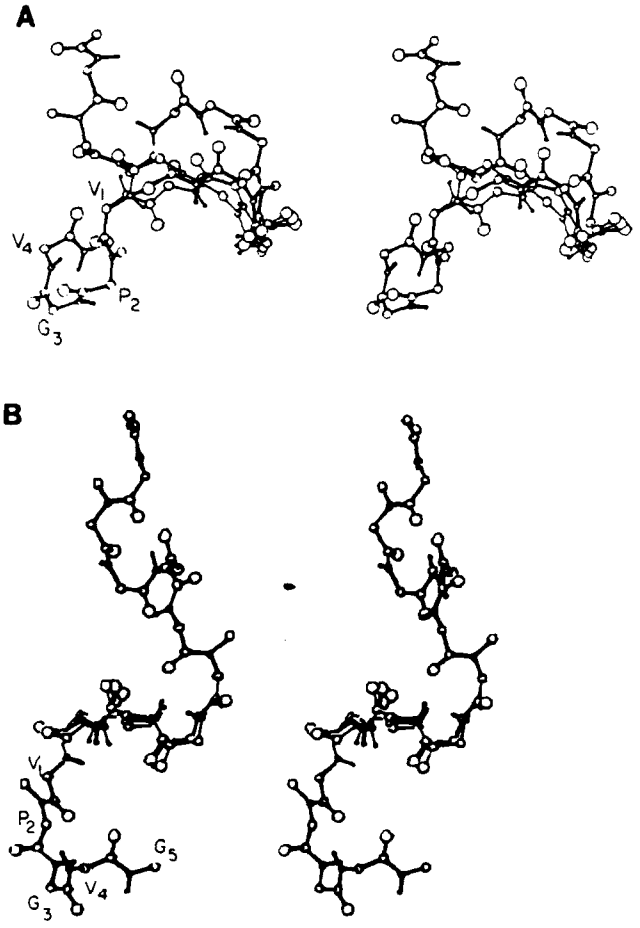

Fig. 4. Stereo pair representation of a pentadecapeptide fragment in (A) relaxed and (B) extended (130%) states. A central pentamer from the Val[1] α-carbon of one repeat to the Val[1] α-carbon of the next repeat is shown with the maximal torsional oscillations allowed for a 2 kcal/mole-residue cutoff energy. Note the large-amplitude rocking possible in (A) and the greatly damped rocking on stretching (B). This shows the decrease in backbone entropy tht occurs on extension. (From Urry and Venkatachalam, 1983.)

at the other. An example of such a regular structure is the polypentapeptide of elastin $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$. Stages in the development of the conformation of the polypentapeptide of elastin have been reviewed elsewhere (Urry and Long, 1976; Urry, 1982, 1984). Here, one member of the class of conformations is demonstrated in the relaxed and extended states. The molecular conformation of a relaxed β-spiral of the polypentapeptide is shown in Fig. 3 (Venkatachalam and Urry, 1981; Urry, 1983; Urry *et al.*, 1982; Cook *et al.*, 1980). A spiral is defined as the helical recurrence of a repeating conformational feature (Urry, 1972, 1974). The prefix β- signifies that the β-turn (Fig. 3A) is the dominant secondary structural feature in the spiral. The β-spiral of the polypentapeptide of elastin is a helical structure (Fig. 3B, C) resulting from an inverse temperature transition in which intramolecular hydrophobic contacts are optimized. The β-turns function as spacers between turns of the spiral, and between the β-turns are suspended segments within which large-amplitude low-frequency librations can occur. Figure 4A shows a pentadecapeptide in which a central pentamer has been allowed to undergo rocking motions (librations), the amplitudes of which are within an energy cutoff of 2 kcal/mole residue (Urry and Venkatachalam, 1983). On extension along the β-spiral axis to a length of 130%, the amplitudes of the librations within the central pentamer for the same cutoff energy are seen to have become markedly damped (Fig. 4B). This decrease in amplitude of the librations on extension constitutes a large decrease in the entropy of the pentapeptide segment. The decrease in entropy provides resistance to extension and the increase in entropy on return to the relaxed (nonstretched) state constitutes the driving force for recovery from deformation. This is called the librational entropy mechanism of elasticity and the resulting entropic elastomeric force can occur within a single short peptide segment or within a regular spiral structure.

3. DEVELOPMENT OF STRUCTURE AND ENTROPIC ELASTOMERIC FORCE DUE TO AN INVERSE TEMPERATURE TRANSITION

3.1. Definition of an Inverse Temperature Transition

One statement of the second law of thermodynamics is that entropy of a total system of constant composition must increase with increased temperature. When the system is multicomponent and one component within the total system undergoes a decrease in entropy with increase in temperature, that component is said to have undergone an inverse temperature transition. When the total system is the two-component polypeptide-plus-water system and the polypeptide exhibits the inverse temperature transition, the water component must have undergone an even greater increase in entropy through the temperature range of the inverse temperature transition; thus the total system will have experienced an increase in entropy on passing through the temperature range of note. This is understood by realizing that, below the transition, water surrounding hydrophobic side chains is more ordered than bulk water (Frank and Evans, 1945; Kauzmann, 1959; Tanford, 1973). This more-ordered water is called clathrate-like water, which is thought to be characterized by a hydrogen-bonding network referred to as a pentagonal dodecahedron (Swaminathan *et al.*, 1978). On raising the temperature through the transition range, the clathrate-like water becomes less-ordered bulk water as the hydrophobic side

chains associate increasing the order in the polypeptide. With polymers of the repeat peptides of elastin, the increase in order of the polypeptide part of the system with the increase in temperature has been demonstrated in numerous ways: by light and electron microscopy, by circular dichroism, by dielectric relaxation, by NMR, and by the nuclear Overhauser effect, which provided direct observation of hydrophobic side-chain associations attending the inverse temperature transition. There is also evidence in the same system using dielectric relaxation that the amount of clathrate-like water decreases rapidly with increase in temperature through the same temperature range as the order in the polypeptide increases (Buchet *et al.*, 1988).

3.2. Coacervation of the Polypentapeptide (Fibrillogenesis)

3.2.1. Definition of Coacervation

The polypentapeptide of elastin, PPP, is soluble in water below 25°C. On raising the temperature above 25°C, aggregation occurs. Shown in Fig. 5 are curves indicating the development of turbidity at 300 nm for different concentrations. The development is seen to be concentration dependent in an interesting way. On raising the concentration, the temperature profile for turbidity formation shifts to lower temperature and becomes steeper until a high concentration limit is reached, a limit that depends on molecular weight (Urry *et al.*, 1985*e*). When the turbid solution is allowed to stand, the more dense aggregates settle. When the process is reversible and the more

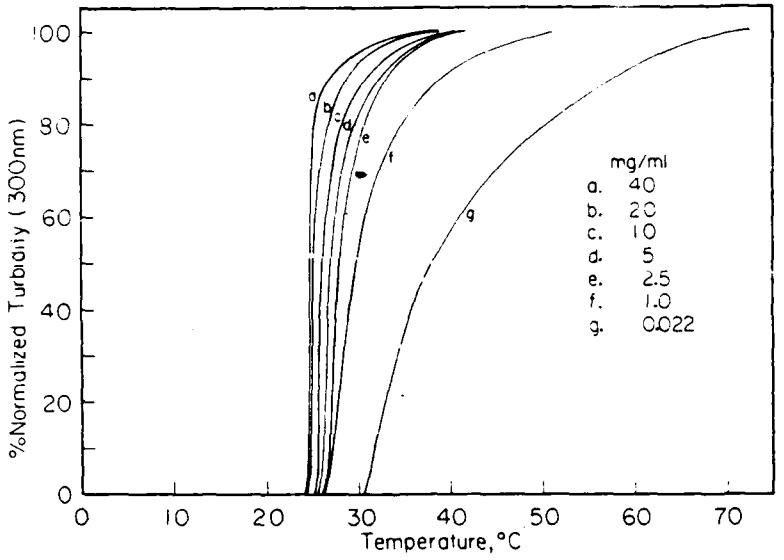

Fig. 5. Temperature profiles for aggregation followed as the development of turbidity at 300 nm on raising the temperature of a range of concentrations of polypentapeptide. As the concentration is raised, aggregation begins at an increasingly lower temperature and exhibits a steeper rise in turbidity, until a high concentration limit is reached, above which increases in concentration cause no further lowering of temperature. The temperature of the high concentration limit can can be used in a plot versus ln molecular weight to estimate molecular weight. (From Urry *et al.*, 1985*e*.)

dense phase is viscoelastic and of fixed composition, as it is for the polypentapeptide in water, the process is called coacervation (Bungenberg de Jong and Kruyt, 1929, 1930; Bungenberg de Jong, 1949), and the more dense phase is called the coacervate. The overlying solution is called an equilibrium solution, and the concentration of PPP in the equilibrium solution decreases as the molecular weight of the PPP is increased. The curves in Fig. 5 can then be called temperature profiles for coacervation (Urry *et al.*, 1985*e*).

3.2.2. Development of the Phase Diagram

The temperature dependence of the composition of the PPP-plus-water system is shown in Fig. 6 (Urry *et al.*, 1985*e*; Urry, 1988). At 20°C, the polypentapeptide and water are miscible in all proportions. On raising the temperature to 30°C when there is more than 63% water by weight, there is a phase separation (coacervation occurs) with the development of an overlying equilibrium solution. At this temperature, the equilibrium solution is removed and the tube is resealed. The 30°C composition of the coacervate is 37% peptide and 63% water by weight. Continuing to raise the temperature to 60°C shows only a small expression of an overlying equilibrium solution such that at 60°C the composition is 38% peptide and 62% water by weight. In the temperature interval of 40–60°C, the coefficient of thermal expansion, $\beta_{eq} = (\partial \ln V/\partial T)$, is 2.4×10^{-4}/deg. Very interestingly above 60°C there

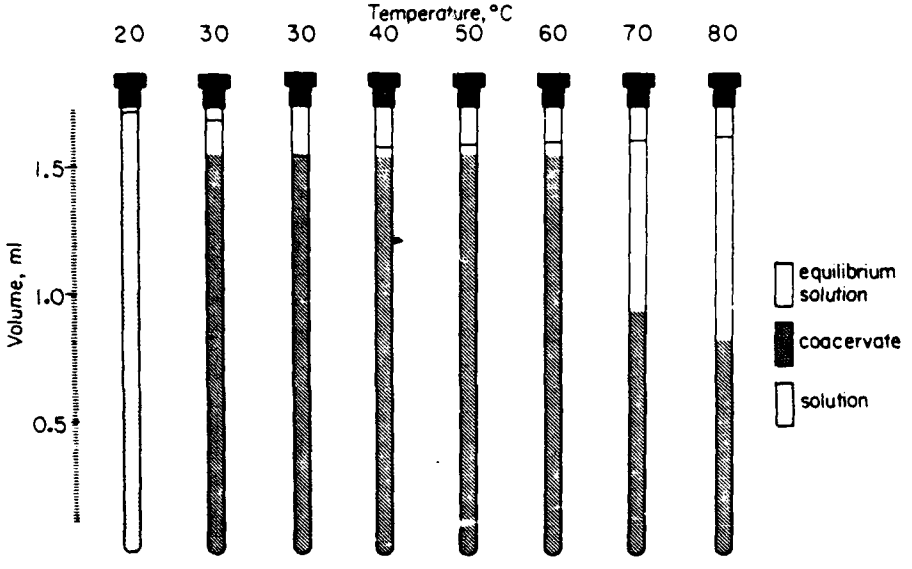

Fig. 6. Composition study of the polypentapeptide-water system as a function of temperature. At 20°C, the polypentapeptide and water are miscible in all proportions. At 30°C, a phase separation has occurred in which the more dense phase, called the coacervate, is 37% peptide, 63% water by weight. The overlying equilibrium solution, which contains more peptide the lower the molecular weight of the polypentapeptide, can be removed and the tube resealed. From 40° to 60°C there is the expression from the coacervate of very little equilibrium solution. Above 60°C, however, there is a dramatic release of water from the coacervate phase, indicating a second transition. Circular dichroism demonstrates that this corresponds to a decrease in intramolecular order. (From Urry, 1987.)

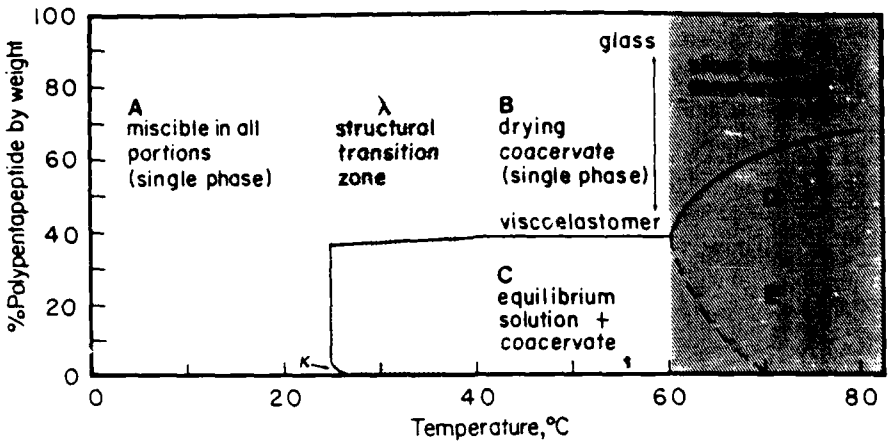

Fig. 7. Phase-structure diagram of the polypentapeptide-water system developed primarily from the data of Fig. 6 and supplemented by other physical characterizations. (Adapted from Urry, 1985.)

is a dramatic expulsion of water reaching at 80°C a composition of 68% peptide and 32% water by weight. The entire process is reversible. With the data in Fig. 6, a phase diagram can be constructed as shown in Fig. 7, which also includes information on associated polypeptide structural transitions (Urry *et al.*, 1985*e*; Urry, 1985).

3.2.3. Fibrillogenesis

The process of coacervation is also a process of fibrillogenesis (Fig. 8). The polypentapeptide of elastin self-assembles to form fibers. As shown in the scanning electron micrograph depicted in Fig. 8B, a single fiber is seen to splay out into many parallel aligned fibrils and to recoalesce to form the same-sized fiber (Urry *et al.*, 1976). In Fig. 8A, a transmission electron micrograph with negative staining shows a fibril to be composed of parallel aligned filaments with a filament width of about 5 nm (Volpin *et al.*, 1976). As follows from Fig. 3, intramolecular ordering during coacervation provides the β-spirals, which associate to form the filaments for the intermolecular ordering of Fig. 8; the expulsion of water above 60°C (see Fig. 6), is the denaturation of the ordered polypentapeptide, as would occur with the loss of the intraspiral water within the structure shown in Fig. 3D, E.

3.3. Correlation of Structure Development with Elastomeric Force Development

3.3.1. Formation of Polypentapeptide Elastomers

When a tube containing coacervate at a composition of about 38% peptide and 62% water by weight is γ-irradiation crosslinked with a 20×10^6 radiation absorbed dose (20 mrad) (Fig. 6), a cylindrical elastomeric matrix is produced (Urry *et al.*, 1986*d*) (Fig. 9A). By NMR analysis, there is little or no detectable change in the [^{13}C]-NMR and [^{15}N]-NMR spectra (even with 90% plus enrichment) resulting from the γ-irradiation, and a longitudinal relaxation rate study as a function of temperature demonstrates a transition to decreased mobility on going from 25° to

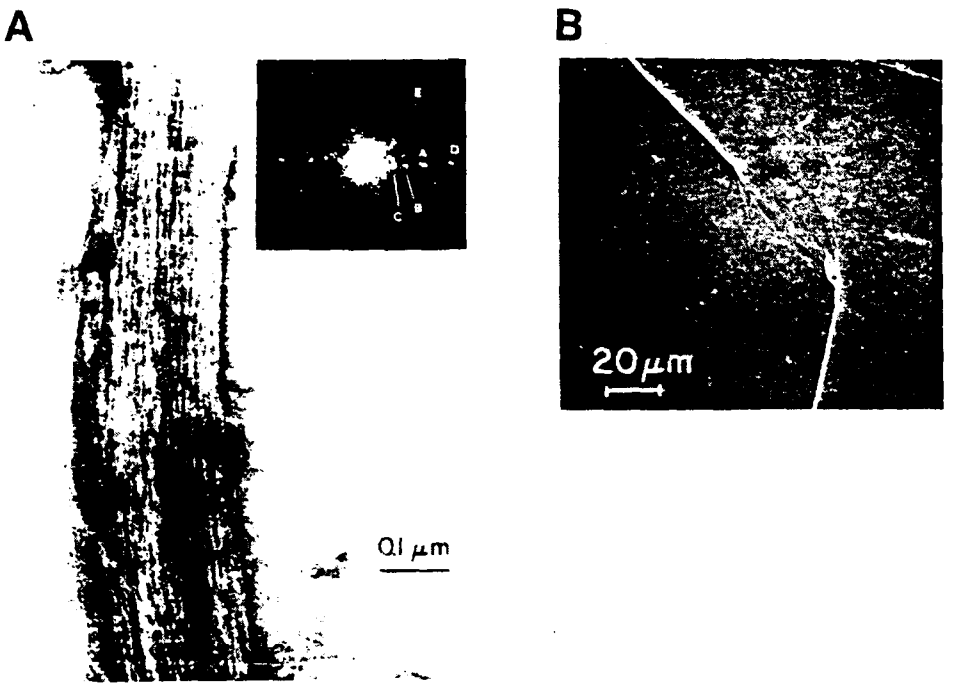

Fig. 8. Self-assembly into filaments, fibrils, and fibers. (A) Aggregates from the cloudy solution of Fig. 5 prepared for examination by transition electron microscopy using the negative staining technique. The molecules dissolved in water below 25°C aggregate when the temperature is raised, to form filaments. Optical diffraction of the micrograph shows a major equatorial reflection near 5 nm, which corresponds to the interfilament distance. At about 40° off the meridian is another spot suggesting a twist to the filament, as may be observed directly on close examination of the micrograph. The twisted filaments are modelled using polypentapeptide β-spirals in Fig. 3E, F. (From Volpin *et al.*, 1976.) (B) Polypentapeptide chemically crosslinked during aggregation of the inverse temperature transition is seen by scanning electron microscopy to have self-assembled into fibers composed of parallel aligned fibrils (see text for further detail). (From Urry *et al.*, 1976.)

40°C that is essentially the same for coacervate as for elastomer, i.e., 20 mrad crosslinked coacervate (Fig. 9C) (Urry *et al.*, 1985*d*, 1986*d*). With the cylindrical shape, the cross-sectional area can be accurately determined and the initial elastic modulus is found to be 1×10^6 dynes/cm^2. Also, the change in volume on extension is easily determined with the cylindrical elastomer, such that the V_i/V ratio of Eq. (5) is known. This NMR study demonstrates, in the coacervate and in the elastomer, that a new characterization of an inverse temperature transition is a decrease in backbone mobility with increasing temperature at constant composition; it also demonstrates the equivalence of the coacervate and γ-irradiation crosslinked elastomeric states.

For a more practical shape for mechanical characterizations in the studies to be discussed, the coacervate is formed in the bottom of a cryotube, and a pestle containing a channel is inserted into the tube (Urry and Prasad, 1985). The coacervate flows into the circular channel. On γ-irradiation, circular elastomeric bands are formed of whatever desired dimensions. Commonly the channel is cut about 0.7 mm

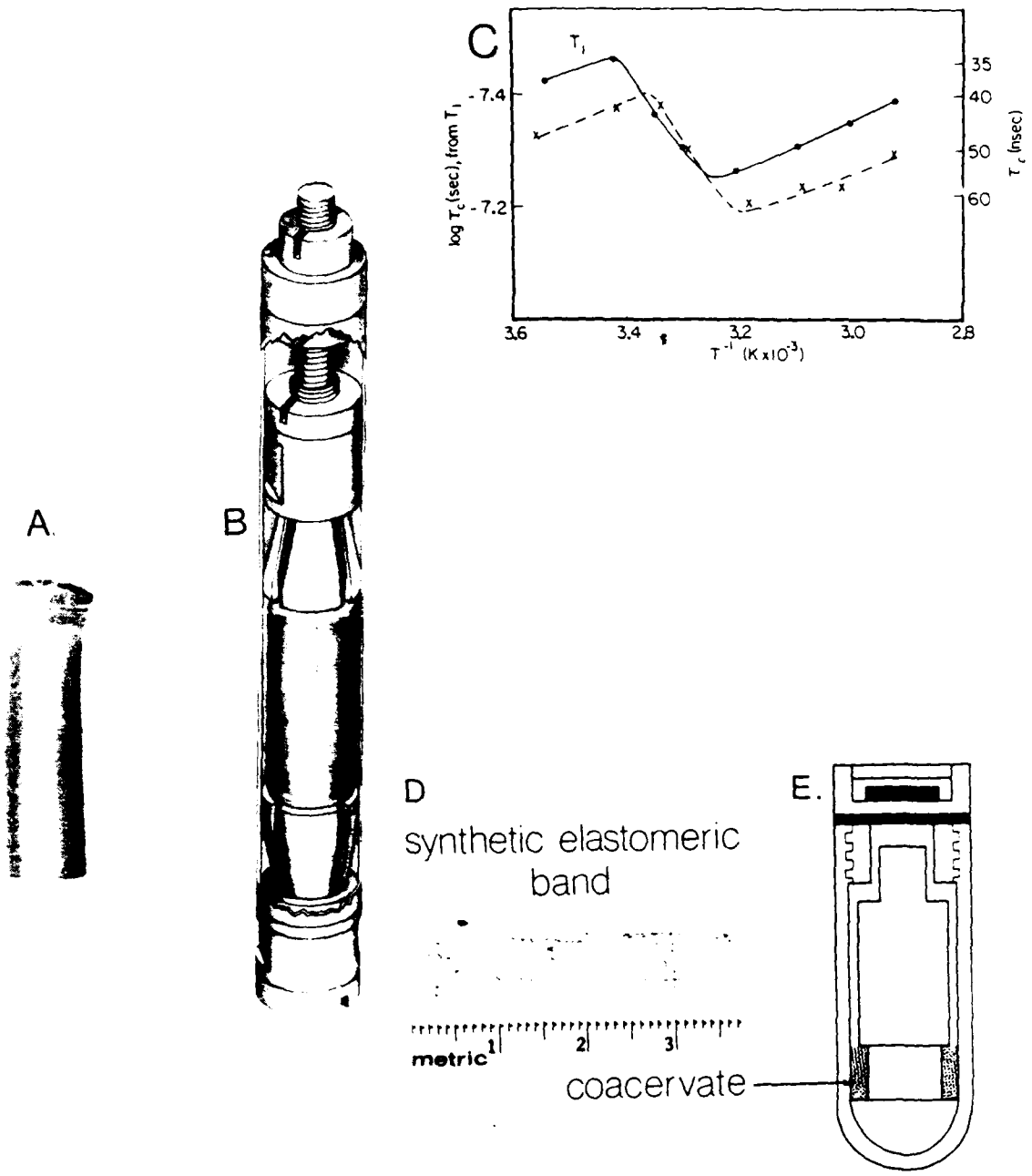

Fig. 9. Preparation and characterization of the crosslinked polypentapeptide. (A) When polypentapeptide coacervate is formed in a tube as in Fig. 6 and the 40°C composition is γ-irradiation crosslinked using a 20×10^6 radiation-absorbed dose (20 mrad), an elastomeric cylinder is prepared. (B) The elastomeric cylinder can be racked up for NMR studies. (C) The temperature dependence of peptide ^{15}NH correlation time of ^{15}N Gly^5-PPP is plotted for both coacervate (——) and (- - -) elastomeric cylinder. The inverse temperature transition is seen as a decrease in backbone mobility on going from 20° to 40°C. At temperatures above the transition, the temperature dependence of correlation time plotted versus T^{-1} (°K) shows an energy barrier to backbone motion of about 1.5 kcal/mole for both the coacervate and the elastomer. (From Urry *et al.*, 1986*d*.) (D) Synthetic elastomeric band prepared on 20-mrad crosslinking of coacervate band formed within the channel of a pestle when in a cryotube as in E.

deep and 7 mm wide, and a tube size is commonly chosen that gives a band of about 30 mm in circumference before cutting. A resulting elastomeric band is shown in Fig. 9D along with the tube and pestle (Fig. 9E).

3.3.2. *Demonstration of Entropic Elastomeric Force*

Following Eqs. (4) and (5), a small strip of the synthetic elastomeric band of Fig. 9D is placed in a pair of grips and stretched to a fixed extension while immersed in water. The force is then monitored as a function of temperature, and the resulting data are plotted in Fig. 10 as $f/T(°K)$ versus temperature; when plotted as $\ln[f/T(°K)]$ versus temperature following Eqs. (4) and (5) above 40°C there is a near-zero slope (-4.5×10^{-4}/deg). Because of the equivalence of the coacervate and the synthetic elastomeric band of PPP, the value of β_{eq} in Eq. (5) can be determined from the data in Fig. 6 to be 2.4×10^{-4}/deg and using a value for the volume ratio, V_i/V, determined from stretching the cylinder (Fig. 9B) to the 63% extension, the calculated f_e/f ratio for the data in Fig. 10 in the temperature interval of 40–65°C is 0.12. The result is consistent in this temperature range with the PPP being dominantly an entropic elastomer. There are problems, however, with using the second term on the right-hand side of Eq. (5); an isotropic network of chains with a gaussian distribution of end-to-end lengths has been assumed to make this correction, whereas the data in Fig. 8 demonstrate an anisotropic fibrillar system at the molecular level. This situation is aided somewhat by the near-constant volume and composition demonstrated in Fig. 6 for the 40–60°C temperature range, such that Eq. (4) is approximately correct; from Eq. (4), the f_e/f ratio would be 0.15. There is, however, the added complication that the near-zero slope of Fig. 10 may be due to the development of elastomeric force resulting from the inverse temperature transition in the 20–40°C temperature range and a decrease in elastomeric force above 60°C due to thermal denaturation (see Fig. 16, below).

Another measure of the entropic nature of the elastomeric force can be the determination of the temperature dependence of the backbone motions. The requisite data are shown in Fig. 9C. Above 40°C, there is a linear increase in mobility, with increase in temperature plotted as the inverse of the absolute temperature T^{-1} (°K). From the plot for both the coacervate and the cylindrical elastomer, the energy barrier for backbone mobility is approximately 1.5 kcal/mole (Urry *et al.*, 1986*d*). This indicates that backbone motion occurs with a sufficiently low energy of activation that a large number of states can be achieved at physiological temperatures as required of an entropic elastomer. This is further demonstrated by the large-amplitude low-frequency backbone librations observed in the dielectric relaxation data (see Fig. 12, below). Thus, it is concluded that the polypentapeptide of elastin exhibits a dominantly entropic elastomeric force.

3.3.3. *Correlations of Inverse Temperature Transition (Structure Development) with Development of Force at Fixed Extension*

Figure 10 shows a dramatic increase of elastomeric force at 20–40°C for an elastomer that had been stretched 63% at 40°C. This development of elastomeric force correlates with a structural transition, many different facets of which have been observed by a range of different physical characterizations (Urry, 1984, 1985;

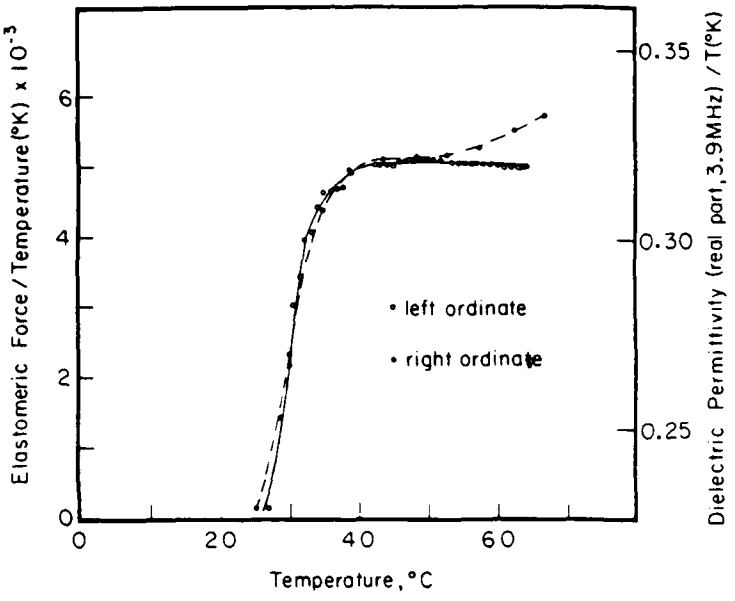

Fig. 10. Thermoelasticity data (○) and dielectric permittivity, real part at 3.9 MHz (●) on the polypentapeptide of elastin. Both sets of data have been divided by the absolute temperature (°K). For the thermoelasticity study, the 40°C coacervate concentration was 20 mrad crosslinked; the resulting elastomeric band was stretched to 63% at 40°C and the elastomeric force measured as a function of temperature. The dramatic rise in force at 25–40°C is due to inverse temperature transition (i.e., elastomeric force increases on increasing order), and the near zero slope above 40°C indicates a dominantly entropic elastomer. For the dielectric relaxation study, a 40°C coacervate was injected from below into a coaxial line cell, and the dielectric permittivity (real part) was determined as a function of temperature over the frequency range 1 MHz to 1 GHz (see Fig. 12A). The real part of the dielectric permittivity at 3.9 MHz divided by the absolute temperature (°K) is plotted as a function of temperature. The development of the local intense relaxation centered above 10 MHz (see Fig. 12) correlates with the development of elastomeric force. Thus, the development of a regular structure in which the peptide moieties are librating near a 10-MHz frequency is responsible for the force development.

Urry *et al.*, 1985*b*, 1988). The physical characterizations range from the macroscopic, where the unaided eye is the analytical tool, to the microscopic, to the molecular, and to the atomic levels of characterization. The sum of the results leaves little question but that the molecular basis for the development of entropic elastomeric force is internal dynamics within a regular nonrandom structure. It is because the elasticity being examined is attributable to a sequential polypeptide, namely, the polypentapeptide of elastin that the demonstration and conclusion of a new molecular basis for entropic elastomeric force is so uncompromised. A brief listing of some of the telling characterizations follows.

3.3.3.1. Temperature Profiles for Coacervation. Figure 5 shows that clear solutions of polypentapeptide (PPP) in water become cloudy as the temperature is

raised above 25°C. The onset of aggregation (turbidity) correlates closely with the rise in elastomeric force seen in Fig. 10.

3.3.3.2. Microscopy. When a droplet of cloudy solution (Fig. 5) is placed on a carbon-coated grid, negatively stained with uranyl acetate and oxalic acid at *p*H 6.2, and examined in the transmission electron microscope, the filamentous aggregates of Fig. 8A are observed. Optical diffraction of the micrographs demonstrate periodicities, most prominently a 5-nm lateral spacing of the filaments as well as an off-meridional reflection indicating an underlying helicity to the filaments (Volpin *et al.*, 1976). When two syntheses of the PPP are carried out, one having an occasional Glu residue in position 4 and the second having an occasional Lys residue in position 4, and when solutions of the two syntheses are combined and a water-soluble carbodiimide coupling reagent is added during aggregation, crosslinking occurs and the molecular system is seen to have self-assembled into fibers. This can be observed with a light microscope with no fixative (Urry, 1983) and in the scanning electron microscope with the aluminum coating, as shown in Fig. 8B (Urry *et al.*, 1976). Accordingly, during the development of elastomeric force in the 20–40°C temperature range, at the molecular level there is a self-assembly to form fibrillar structures.

3.3.3.3. Circular Dichroism Studies. When a series of circular dichroism (CD) curves are obtained at different temperatures on a 2.3-mg/ml solution of PPP in water and the ellipticity at 197 nm is plotted as a function of temperature on the left-hand ordinate and the elastomeric force of 20 mrad crosslinked PPP coacervate at 60% extension is plotted on the right-hand ordinate as in Fig. 11, the two curves are found to coincide (Urry *et al.*, 1985*c*). The complete CD spectrum below 25°C is more nearly that of a disordered polypeptide, whereas the CD spectrum at elevated temperatures is characteristic of a polypeptide containing recurring type II β-turns

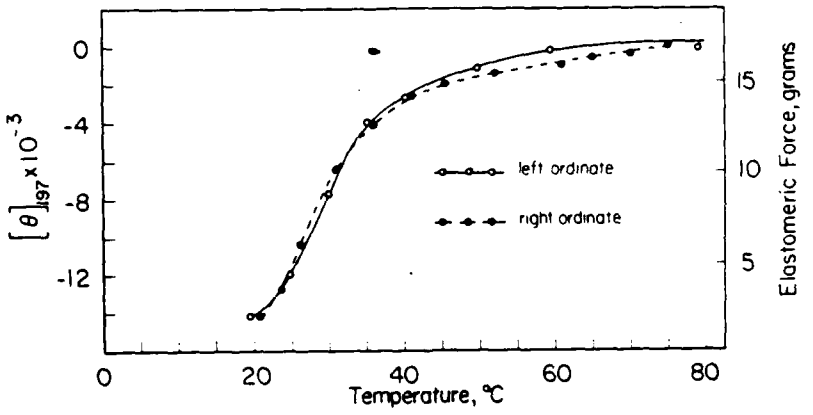

Fig. 11. Ellipticity data at 197 nm of the polypentapeptide of elastin in water (2.3 mg/ml) as a function of temperature (○). The ellipticity changes from that more indicative of a less ordered state at 20°C to that indicative of a recurring β-turn conformation at ≥40°C. Force plotted as a function of temperature for a crosslinked band of polypentapeptide having been stretched to 60% at 40°C (●). Elastomeric force development correlates with development of intramolecular order, as monitored by circular dichroism. (From Urry *et al.*, 1985*c*.)

(see Fig. 3A for the β-turn and Fig. 15 below for the complete CD spectra). Thus intramolecular order increases as elastomeric force develops.

3.3.3.4. Nuclear Overhauser Effect Studies. Specific intramolecular hydrophobic side-chain associations attending the inverse temperature transition of the elastomeric des Val^4 analogue of the PPP of elastin (Urry *et al.*, 1977) as well as for the PPP itself (M. A. Khaled and D. W. Urry, unpublished data) have been determined by means of nuclear Overhauser enhancement studies. On raising the temperature into the range of the inverse temperature transition, there develops a close proximity of the Val $^1\gamma C\underline{H}_3$ moieties with the Pro^2 $\delta C\underline{H}_2$ moieties. This identifies an intrapentamer part of the intramolecular hydrophobic association responsible for the inverse temperature transition. Evidence has most recently been obtained for Val $\gamma C\underline{H}_3$-Pro $\beta C\underline{H}_2$ interturn, intramolecular hydrophobic interactions (Urry, Chang, Krishna, Huang, Trapane, and Prasad, unpublished data).

3.3.3.5. Composition Studies. Figure 6 shows a phase transition that occurs in the same temperature interval in which elastomeric force develops in Fig. 10. The composition of the coacervate as noted at 40°C is 38% peptide and 62% water by weight (Urry *et al.*, 1985*e*). This coacervate composition was used in the NMR and dielectric relaxation studies noted below.

3.3.3.6. Nuclear Magnetic Resonance Relaxation Studies. When NMR relaxation studies are carried out on the coacervate phase at the 40°C composition (Urry *et al.*, 1985*d*) and on the cylindrical elastomer (Urry *et al.*, 1986*d*) (Figs. 9A–C), the polypeptide backbone mobility increases when the temperature is raised to near 20°C. Above 20°C, a marked decrease in mobility occurs, without any change in water content, until the inverse temperature transition is over near 40°C. Above 40°C, the usual increase in mobility with increase in temperature resumes. As temperature is the measure of molecular motion, this inverse behavior of decrease in backbone motion with increase in temperature provides a new definition of an inverse temperature transition. Again, it is during this inverse behavior that elastomeric force develops.

3.3.3.7. Dielectric Relaxation Studies. In the polypentapeptide-plus-water system, the only dipole moments are those of the water molecules and those of the peptide moieties of the polypeptide backbone. Accordingly, a relaxation that develops with its frequency about 10 MHz with a low energy of activation is clearly due to the polypentapeptide backbone motion. When the real part of the dielectric permittivity at 3.9 MHz divided by the temperature (°K) is plotted for the 40°C coacervate composition as a function of temperature, as shown on the right-hand side of Fig. 10, the development of relaxation intensity corresponds with the development of elastomeric force (Urry *et al.*, 1984). The real part of the dielectric permittivity is plotted in Fig. 12A for the frequency range 1-MHz to 1-GHz for a series of temperatures through the transition (R. Henze and D. W. Urry, unpublished data). The relaxation is seen to occur with a localized frequency, as is most clearly shown in Fig. 12B, where the 40°C minus 20°C difference curve is plotted from the data of Henze and Urry (1985). The difference curve is seen to be a nearly ideal Debye-type

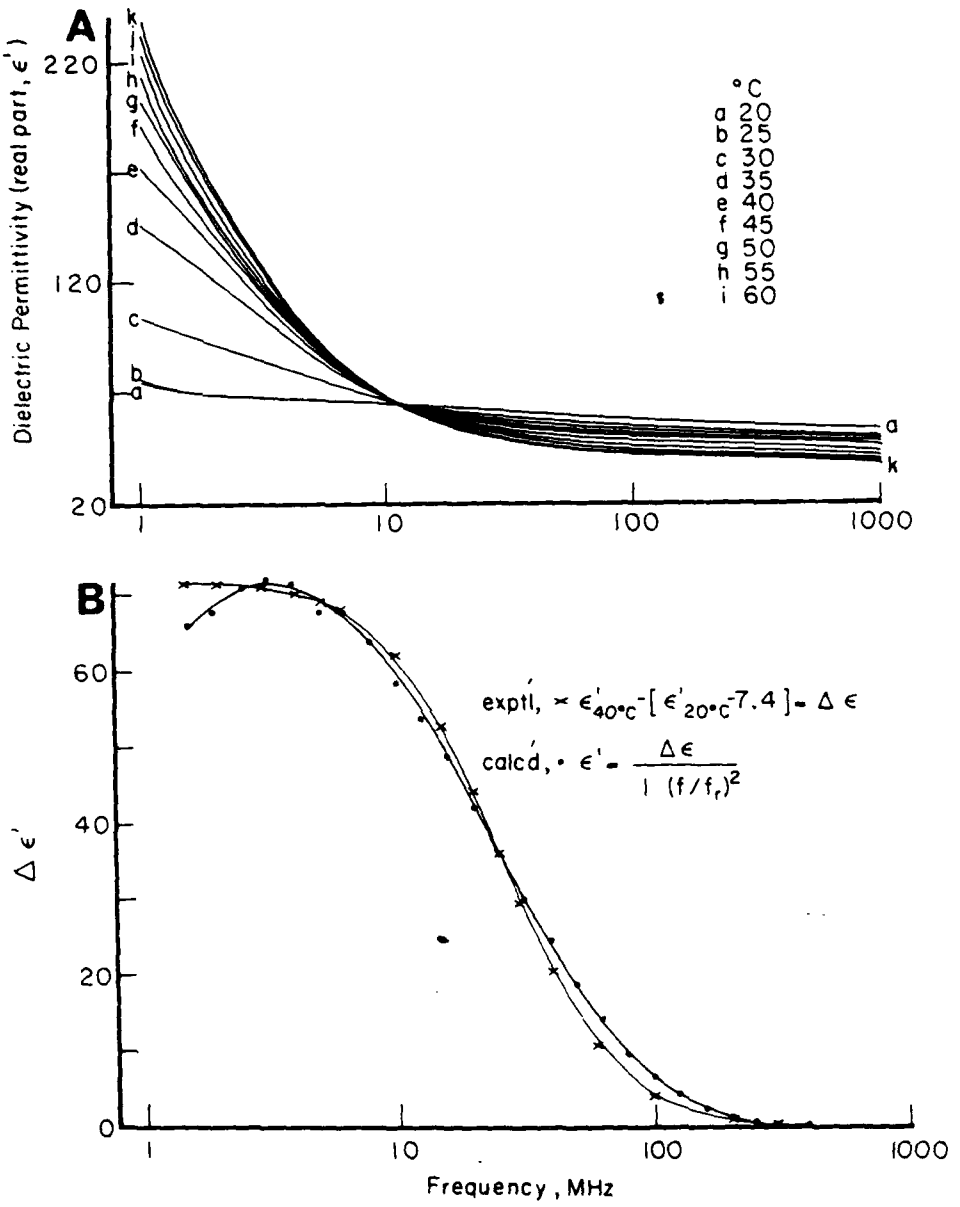

Fig. 12. *Dielectric relaxation* study of the 40°C concentration of polypentapeptide coacervate in the frequency range 1 MHz to 1 GHz. (A) As the temperature is raised above 20°C, an intense local relaxation develops above 10 MHz. (From R. Henze and D. W. Urry, unpublished data.) (B) The 40°C minus 20°C difference curve compared with a Debye curve. (From the data of R. Henze and D. W. Urry, 1985.) The close superposition indicates a remarkably localized frequency requiring a regular structure. The relaxation has been assigned to a peptide librational mode. As shown in Fig. 10 using the 3.9 MHz data point from part A, the intensity of this relaxation correlates with the development of elastomeric force.

relaxation. This requires that the peptide dipole moments responsible for the relaxation are all oscillating at the same frequency. Because the energetics for peptide rocking motions would be different for pentamers in different conformations, or in a random chain network (Lyerla and Torchia, 1975), the common frequency requires that each pentamer during the transition comes into the same conformation, i.e., a regular nonrandom conformation develops during the transition (Henze and Urry, 1985). Again, the development of elastomeric force is seen to correspond with the development of a regular structure, i.e., with an increase in intramolecular order.

3.3.3.8. Temperature Dependence of Elastomer Length. A strip of elastomeric PPP is equilibrated at 40°C and stretched to 60%; the force so developed is then maintained as the temperature is varied. Like all entropic elastomers, as the temperature is raised, the elastomer shortens. The interesting feature about the elastomeric PPP is that the length change is exaggerated in the 20-40°C temperature (Urry *et al.*, 1986*b*). Whereas Latex will shorten less than 10% from 100% at 20°C to 90% plus at 40°C, the elastomeric polypentapeptide shortens by 30%, from 100% at 20°C to near 70% at 40°C. This differential behavior is even more dramatic in the absence of a load. Latex will expand by about 5% on going from 20°C to 40°C

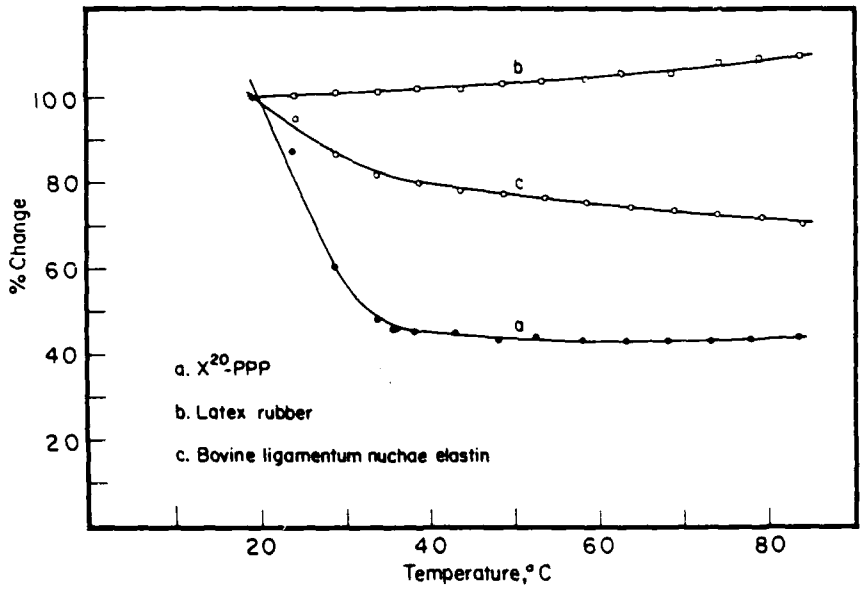

Fig. 13. Temperature dependence of length under zero load of the 20-mrad cross-linked 40°C coacervate concentration of the polypentapeptide of elastin (curve a). Note the dramatic shortening (contraction) that occurs on raising the temperature from 20° to 40°C. The magnitude of this shortening is equivalent to taking a near extended polypentapeptide chain with β-turns in place and wraping it up into the β-spiral shown in Fig. 3. This shortening is due to an inverse temperature transition in which intramolecular hydrophobic interactions are optimized. Note that a classic rubber, Latex, uniformly expands as the temperature is raised without load (curve c). Curve b shows the data for ligamentum nuchae elastin, as well as the inverse behavior, over a broader range, but again most steeply in the 20-40°C temperature range. Curves a and c are not those exhibited by random chain networks. (From Urry *et al.*, 1986*b*.)

in the absence of a load. The elastomeric polypentapeptide on going from 20°C to 40°C undergoes a remarkable shortening from 100% at 20°C to near 40% at 40°C as shown in Fig. 13. This dramatic shortening is the result of structure formation, i.e., of the winding up into a helical structure. Interestingly, the change in length is approximately what is to be expected if the β-spiral of Fig. 3 simply unwound with retention of β-turns (Thomas *et al.*, 1987). Thus, the development of elastomeric force in Figs. 10 and 11 is partly due to the shortening of end-to-end chain length when the β-spiral structure forms as the result of the inverse temperature transition.

3.4. Application of the Principle That Elastomeric Force Develops as the Result of an Inverse Temperature Transition

It has been established repeatedly that at fixed length, elastomeric force develops as the result of an inverse temperature transition. In general, the temperature range in which an inverse temperature transition occurs varies inversely with the hydrophobicity of the polypeptide chain. Accordingly, the correlation established it should be possible to change the temperature at which the development of elastomeric force occurs. This can be done by the replacement of a valyl residue with a more hydrophobic isoleucyl residue. This simple addition of a CH_2 moiety has no effect on the conformation of the polypentapeptide (Urry *et al.*, 1986*c*); yet, as shown in Fig. 14A, the temperature profiles of coacervation are shifted to lower temperatures. In Fig. 14B, the increase in intramolecular order with increase in temperature as followed by circular dichroism is shifted to lower temperatures. And very significantly as shown in Fig. 14C, the development of elastomeric force has shifted from a midpoint near 30°C for the PPP of elastin to a midpoint near 10°C for the Ile^1-PPP elastomer (Urry *et al.*, 1986*c*).

By decreasing the hydrophobicity (increasing the hydrophilicity) of the polypeptide, the temperature of the transition can be raised. This is achieved with the des Val^4-PPP, i.e., with the polytetrapeptide $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$. In Fig. 14A, the temperature profiles for coacervation are seen to be shifted to higher temperature, as is the development of intramolecular order (see Fig. 14B). As expected, the development of elastomeric force has also been shifted to higher temperatures to a midpoint for the transition of near 50°C, some 20°C higher than for the PPP (Urry *et al.*, 1986*a*). Thus, the fundamental point that the temperature range for the development of elastomeric force can be shifted by changing the hydrophobicity or hydrophilicity of the elastomeric polypeptide is established. This can be expected to be of significance in protein mechanisms (Urry, 1988).

3.5. Thermal Denaturation

It has been established that the elastomeric state of the PPP of elastin is one of a nonrandom regular structure. The proposed class of conformations for the relaxed elastomeric state is demonstrated by the β-spiral of Fig. 3. As the elastomeric state in the 40–60°C temperature range is a nonrandom (although dynamic) structured state, it follows that thermal denaturation should be observable. The dramatic (but very slow) expulsion of water from the coacervate on raising the temperature

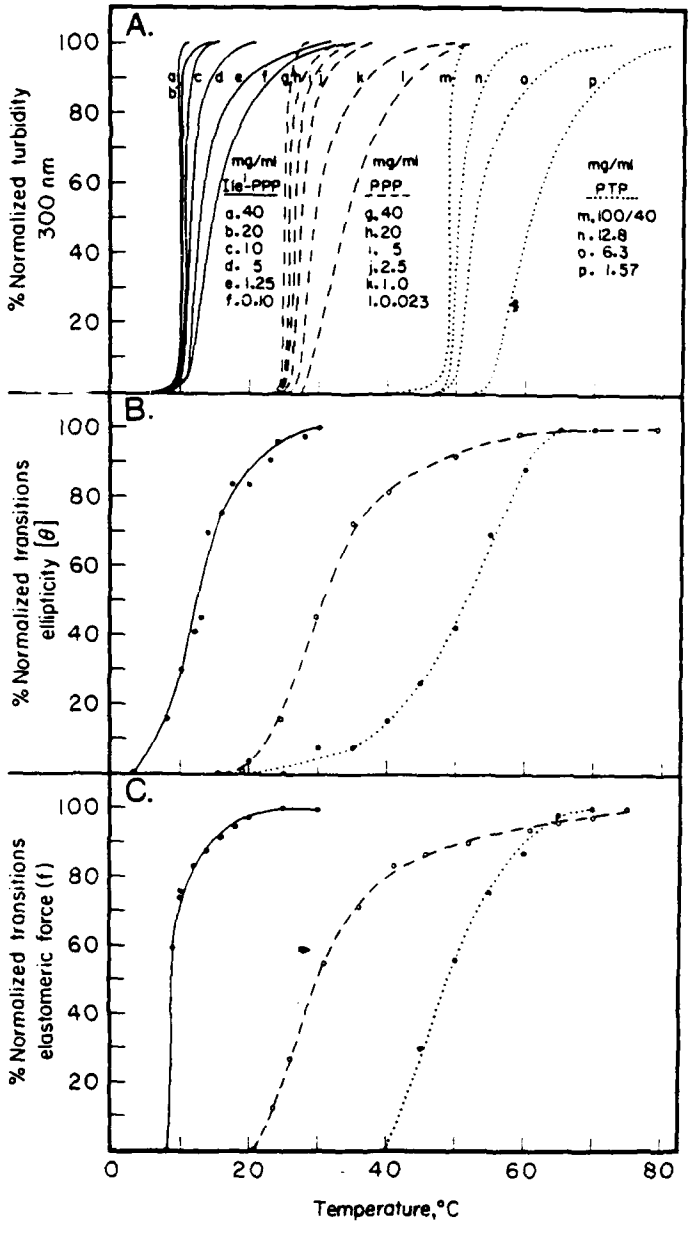

Fig. 14. Effect of polypeptide hydrophobicity on the temperature range of the inverse temperature transition and the temperature range for the development of elastomeric force for Ile^1-PPP $(Ile^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, for PPP $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ and for PTP $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$, where all polypeptides are greater than 50,000 M_r. (A) Temperature profiles for aggregation as a function of concentration for each of the three sequential polypeptides. (B) Ellipticity data showing the increase in order as a function of temperature for each of the three sequential polypeptides. (C) Development of elastomeric force as a function of temperature for each of the three sequential polypeptides after γ-irradiation in their coacervate states. The addition of a single CH_2 moiety within the pentamer increases the pentamer hydrophobicity and shifts the intermolecular hydrophobic interaction (A), shifts the intramolecular hydrophobic interaction (B), and shifts the development of elastomeric force to lower temperature. (Adapated from Urry *et al.*, 1986*a*.) Deletion of the hydrophobic Val^4 residue shifts the aggregation, the increase in intramolecular order and the development of elastomeric force to higher temperature. (Adapted from Urry *et al.*, 1986*a*, *c*.) The magnitudes in the shifts are proportional to the hydrophobicity change (Urry *et al.*, 1986*a*). Thus, the temperature range over which elastomeric force develops can be shifted by changing the hydrophobicity of the polypeptide.

from 60° to 80°C seen in Fig. 6 could be the result of denaturation of a water-containing structure. This can be checked by circular dichroism studies in which the change in CD pattern on standing at 80°C is determined (Fig. 15). The recurring β-turn CD pattern is observed when the spectrum of the sample is determined shortly after reaching 80°C. On standing at 80°C, the CD pattern slowly reverts toward that of the low-temperature (15°C) pattern (Urry *et al.*, 1985*e*). The 15°C CD pattern is obtained below the temperature for the onset of the inverse temperature transition and is indicative of a less-ordered state. As the CD pattern on prolonged heating at 80°C slowly shifts toward that of a less-ordered state, the process that occurs with expulsion of water in Fig. 6 is shown to be denaturation. The half-life for denaturation in the small coacervate droplets of the CD study is about 3 days. Under these circumstances, the thermal denaturation can be easily shown to be reversible by lowering the temperature to 15°C, where redissolution occurs.

It now becomes of particular interest to determine the effect of prolonged heating at 80°C on elastomeric force and on elastic modulus. Should denaturation result in significant losses of elastomeric force, it would seem apparent that random chain networks would have little to do with the entropic elastomeric force of the PPP elastomer. In Fig. 16, the stress-strain curve for the PPP elastomer is determined at 40°C to give an elastic modulus of 4.3×10^5 dynes/cm^2; the sample is then returned to zero extension, heated at 80°C for 24 hr while still in the stress-strain apparatus.

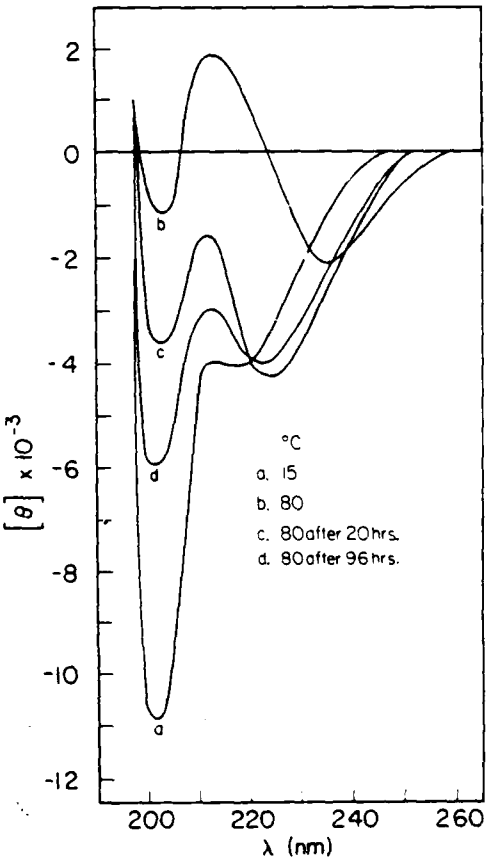

Fig. 15. Circular dichroism data on the polypentapeptide of elastin for 2.3 mg/ml in water. At 15°C, the CD pattern is characteristic of polypeptides with less order. A random polypeptide would have value of -4×10^4 at 195 nm and a slightly positive or no band near 220 nm. The presence of a negative band near 220 nm and the -1.1×10^4 band near 200 nm suggests the presence of some order, even at 15°C. On steadily raising the temperature to 80°C, the pattern changes to one characteristic of a repeating type II β-turn. However, on standing at 80°C, the pattern slowly changes back toward that of less order. This slow denaturation is entirely reversible by lowering the temperature below 25°C.

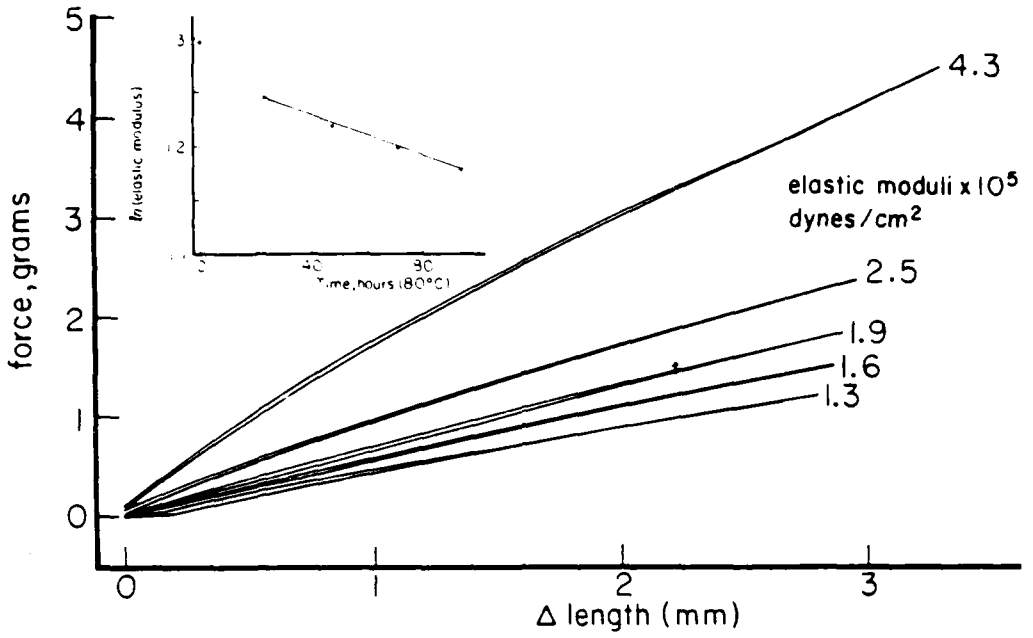

Fig. 16. Stress/strain data on the 20-mrad crosslinked 40°C coacervate concentration of the polypentapeptide as a function of time at 80°C and zero extension. The first stress/strain cycle to 60% elongation and back, determined at 40°C, gave an elastic modulus of 4.3×10^5 dynes/cm^2. The same at zero extension was then held at 80°C for 24 hr, after which the elastic modulus was again determined at 40°C to be 2.5×10^5 dynes/cm^2. This procedure was repeated three more times. After each 24 hr of heating at 80°C, the elastic modulus decreased. The insert shows a plot of ln (elastic modulus) versus time (in hours) at 80°C. From the plot, the half-life for thermal denaturation could be determined to be about 70 hr, similar to that shown in Fig. 15. Interestingly, in relationship to the data in Fig. 6, the elastomeric band also shortens on standing at 80°C. While the effect of prolonged heating at 80°C shown in Figs. 6 and 15 is reversible, in the condensed crosslinked matrix, where low-temperature dissolution is not possible, reversibility has not been demonstrated. Polypeptide chains once randomized in condensed phases apparently do not readily restructure. Importantly, the effects of thermal denaturation are directly observable as a loss of elastomeric force. (Urry, Haynes, and Harris, unpublished data)

The new length at zero force is determined at 40°C, and the stress-strain curve is run at 40°C to 60% extension. The elastic modulus has decreased to 2.5×10^4 dynes/cm^2. In Fig. 16, the 24-hr periods of heating at 80°C are repeated three more times and the elastic modulus determined after each 24-hr period. Heating at 80°C results in a loss of elastic modulus. The inset in Fig. 16 shows a plot of the ln(elastic modulus) versus hours at 80°C from which a half-life of about 70 hr is obtained. This half-life is similar to that found in the circular dichroism study (see Fig. 15). Interestingly there is also a shortening of the elastomer on heating at 80°C in analogy to the decrease in coacervate volume of Fig. 6 above 60°C. Reversibility of the denaturation in the crosslinked condensed phase has not been demonstrated, whereas for the CD study (Fig. 15) and the composition study (Fig. 6) reversibility was demonstrable by lowering the temperature to below 20°C, where redissolution occurs, after which the process can be repeated. Once the chains are denatured in the crosslinked sample, however, disentanglement has not been achieved even on

swelling in water at 20°C or less. It may also be noted that when the thermoelasticity curves as in Figs. 10 and 11 are run using 4 hr per data point above 40°C, an irreversible loss of force is observed above 60°C. Thus, thermal denaturation is demonstrable directly in terms of loss of elastomeric force. As thermal randomization of polypentapeptide chains results in loss of elastomeric force, clearly random chain networks are not responsible for the observed elastomeric force in the 40-60°C temperature range.

As an additional note, if the 40-60°C state is taken as the native state, heat denaturation is observed on raising the temperature above 60°C, and cold denaturation is observed on lowering the temperature below 40°C. Interestingly, however, cold denaturation results in a swelling of the elastomer, whereas heat denaturation causes a shrinking of the elastomer.

3.6. Conclusions on the Mechanism of Entropic Polypentapeptide Elasticity

The preceding data clearly demonstrate that the classical theory of rubber elasticity, requiring as it does a random chain network, is not relevant to the entropic elasticity of the polypentapeptide of elastin. With respect to the possible contribution of solvent entropy to the entropic elastomeric force, there are a few points to note. It is expected that clathrate-like water would form around the hydrophobic side chains exposed to solvent on stretching, but the slow denaturation, the slow loss of elastomeric force at 80°C, is not of a time scale relevant to the formation and loss of clathrate-like water that occurs with a frequency in the GHz range (Buchet *et al.*, 1988), and the half-life for denaturation is the same whether the elastomer is heated at 80°C while stretched or while at zero extension (Urry *et al.*, 1987*c*). Finally, as clathrate-like water has a weak temperature dependence (Buchet *et al.*, 1988), there is little clathrate-like (low entropy) water at temperatures near 80°C and greater. It seems clear that force is borne by polypeptide chains and since denatured structure with randomized chains does not contribute significantly to the entropic elastomeric force exhibited in the 40-60°C temperature range, the only alternative source of chain entropy would be the entropy attributable to internal chain dynamics. This gives the LEM of elasticity.

4. LIBRATIONAL ENTROPY MECHANISM OF ELASTICITY

4.1. Expressions of Entropy

4.1.1. Boltzmann's Relation

The state of a polypeptide or of a polypeptide segment can be completely specified by knowledge of the coordinates; q_i, and momenta p_i of each displaceable component. In this terminology, a peptide moiety or a methyl moiety may be considered a component. If n is the number of such components, a state is specified by a single point in $2n$-dimensional (q_i, p_i) space, referred to as phase space. The number of *a priori* equally probable points (states), W, accessible to the system is a measure of the entropy of the system (Eyring *et al.*, 1961). This bridge between statistical mechanics and thermodynamics is called Boltzmann's relation and can

be written

$$S = R \ln W \tag{7}$$

where R is the gas constant, 1.987 cal/mole deg. Taking the peptide moiety to be planar and bond angles and lengths to be fixed, the state of a polypeptide backbone can also be considered to be completely specified with knowledge of the ϕ and ψ torsion angles. One state can be represented as a point in conformation space. The sum of accessible states can be represented by the volume occupied by such points. If the peptide segment of interest involved three torsion angles, the range of torsion angle changes within a 1.5-kcal/mole cutoff energy (e.g., 0.5 kcal/mole for each degree of freedom) above the lowest energy state might be represented as $\psi_1 - \psi_1'$, $\phi_2 - \phi_2'$, and $\psi_2 - \psi_2'$. Plotting these three amplitudes of torsion-angle changes along each of three axes in a rectangular coordinate system describes a volume in three-dimensional space, and the volume would be proportional to the entropy of the peptide segment. Should there be n torsion angles, the space of interest would be n-dimensional, and the volume of this conformation space would again be proportional to the entropy of the system. For the pentapeptide (Val^1-Pro^2-Gly^3-Val^4-Gly^5), the ϕ(Pro) torsion angle would be fixed such that there would be nine backbone torsion angles. Considering the pentamer within the β-spiral conformation of Figs. 3 and 4A to be the span from the Val^1 α-carbon of one repeat to the Val^1 α-carbon of the next repeat, the question of interest becomes: How many ways can the pentamer segment span the gap from the position of one Val^1 α-carbon to the position of the next Val^1 α-carbon? Taking a 1-kcal/mole-residue cutoff energy and counting each 5° change in torsion angle as a new state, the number of states in the relaxed conformation (W^r), using the potential functions of Scheraga and co-workers (Momany *et al.*, 1974, 1975), is calculated to be 764 (Urry *et al.*, 1985*f*). Extending the β-spiral to 130%, as shown in Fig. 4b, and using the same procedure as for the relaxed state, calculated for the extended conformation to be 58 states, W^e. The change in entropy, ΔS, on deformation of the pentamer becomes

$$\begin{aligned} \Delta S = S^r - S^e &= R \ln W^r / W^e \\ &= 1.987 \ln 764/58 \\ &= 5.12 \text{ cal/mole-deg per pentamer} \end{aligned} \tag{8}$$

which is about one entropy unit per residue. To demonstrate that the entropy change is well behaved with changes in cutoff energy, values of 0.6 and 2.0 kcal/mole have been used, and the same values are obtained for the change in entropy. Alternatively, the Boltzmann summation overstated as $\sum_i e^{-\varepsilon_i/RT}$, can be used in the statistical mechanical definition of entropy, i.e.,

$$S = R \ln \sum_i e^{-\varepsilon_i/RT} + E/T$$

where ε_i is the energy per mole of each allowed state, and E is the total internal energy. The change in entropy becomes

$$\Delta S = R \ln \left(\sum_i^r e^{-\varepsilon_i^r/RT} \Big/ \sum_i^e e^{-\varepsilon_i^e/RT} \right) + (E^r - E^e)/T \tag{9}$$

It has been demonstrated that the polypentapeptide elastomer is almost entirely an entropic elastomer, i.e., that $f_e \simeq 0$, which means that there is little change in internal energy on stretching, such that $(E^t - E^s) \simeq 0$. When the energy values for each state with a 5° change in torsion angle are used in the summations, the entropy change is again one entropy unit per residue (Urry *et al.*, 1985*f*). This demonstration of an entropy change due to the damping of internal chain dynamics is relevant to any sized peptide segment in which deformation can effect a damping of torsional motions.

4.1.2. Entropy of an Harmonic Oscillator

Another useful expression for entropy derives from the consideration of the frequency of motions. While these large-amplitude low-frequency motions are expected to be highly anharmonic, useful qualitative insight is available from the statistical mechanical expression for entropy using the harmonic oscillator partition function (Dauber *et al.*, 1981). The expression for entropy becomes

$$S_i = R[\ln(1 - e^{-h\nu_i/kT})^{-1} + (h\nu_i/kT)(e^{h\nu_i/kT} - 1)^{-1}] \qquad (11)$$

where ν_i is the frequency, k is Boltzmann's constant, and h is Planck's constant (Eyring *et al.*, 1961). A plot of log ν_i versus S_i (left-hand ordinate) and TS_i (right-hand ordinate) is given in Fig. 17. What is apparent is that the lower the frequency, the larger the contribution to the entropy. A librational motion of 10-MHz frequency

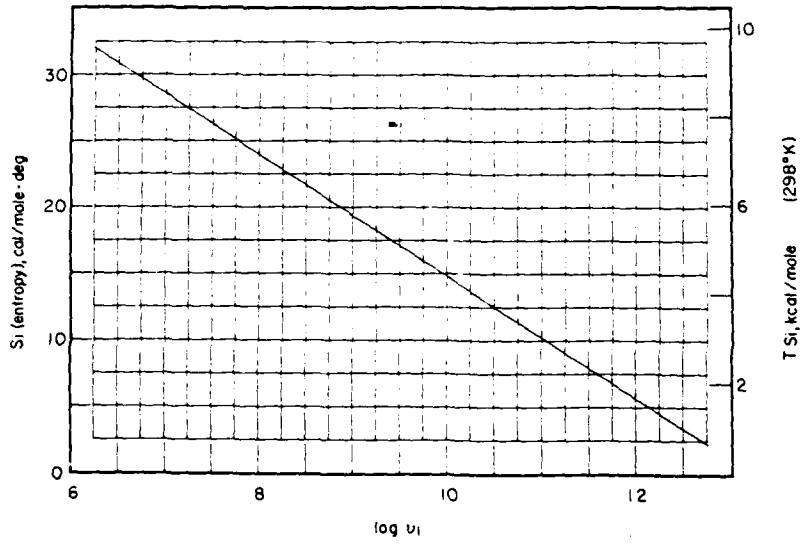

Fig. 17. Plot of Eq. (11) for the entropy S_i (in cal/mole-deg) on the left-hand ordinate and given as TS_i (in kcal/mole) on the right-hand ordinate. This demonstrates the increasing contribution of low-frequency motions to the entropy of a polypeptide chain.

would contribute some 9 kcal/mole to the free energy. The correlation time, $\tau_i = (1/2\pi\nu_i)$, corresponding to 10 MHz, is 16 nsec. This is more than two orders of magnitude longer than the currently longest trajectories calculable by means of molecular dynamics (Karplus and McCammon, 1981). With ultrasonic absorption of proteins showing a maximum in the 10-nsec range (Barnes *et al.*, 1985; Pethig, 1979; Zana and Tondre, 1972; Schneider *et al.*, 1969; Cho *et al.*, 1985; Cerf, 1985) and with the dielectric relaxation studies showing the importance of 10-nsec correlation times to entropic elasticity in polypeptides and proteins (Henze and Urry, 1985; Urry *et al.*, 1985), it is apparent that these low-frequency motions have important contributions to the free energy and to the structure and function of proteins.

4.2. λ Plots and Peptide Librational Motions

As shown in Fig. 4, it is the suspended Val^4 α-carbon to Val^1 α-carbon segment in which the larger-amplitude torsional motions are apparent. Accordingly, it becomes of interest to plot the allowed states on $\psi(Val^4)$ versus $\phi(Gly^5)$ and $\psi(Gly^5)$ versus $\phi(Val^1)$ maps. The four torsion angles of the suspended segment are paired as the torsion angles flanking each peptide moiety. The maps, called λ plots, are given in Fig. 18A for the relaxed conformation. Interestingly, each of the allowed states falls near a 45° diagonal; i.e., whenever there is a change in ψ_i, there is a *compensating* oppositely signed change in ϕ_{i+1} (Urry *et al.*, 1982; Urry and Venkatachalam, 1983). The λ plots indicate that the peptide moieties are undergoing large-amplitude rocking motions, i.e., peptide librations. This coupling of torsional angle changes is the basis for the identification of an LEM of elasticity. In the relaxed state, the amplitudes of the librations approach 180°, whereas on a 130% extension along the spiral axis (about a 30% increase in the Val_i^1 α-carbon-Val_{i+1}^1 α-carbon distance) the amplitudes of the librations are severely damped to about one-third the relaxed amplitude.

4.3. Dielectric Relaxation and the Amplitude of the Peptide Librational Process

As soon as the dynamic β-spiral in Fig. 3 was derived (Venkatachalam and Urry, 1981), the concept of librational entropy was immediately raised because of the apparent freedom of the suspended segment (Urry, 1982), and tests of the concept wre immediately initiated. These tests were the synthesis of the L · Ala^5-PPP and D · Ala^5-PPP analogues and the dielectric relaxation studies. The purpose of the former, replacing Gly^5 by Ala^5, was to introduce a side chain, a CH_2 moiety, in the middle of the suspended segment to see whether elasticity could be affected by the resulting limiting of peptide librations. The dielectric relaxation studies were conducted to see whether the librational process could be observed in PPP itself and, if observed, to see whether the librational amplitudes were damped in the Ala^5-PPP analogues. The L · Ala^5-PPP analogue is inelastic in spite of essentially identical conformations and temperature profiles of aggregation; the product of the temperature-induced aggregation is a granular precipitate rather than a viscoelastic

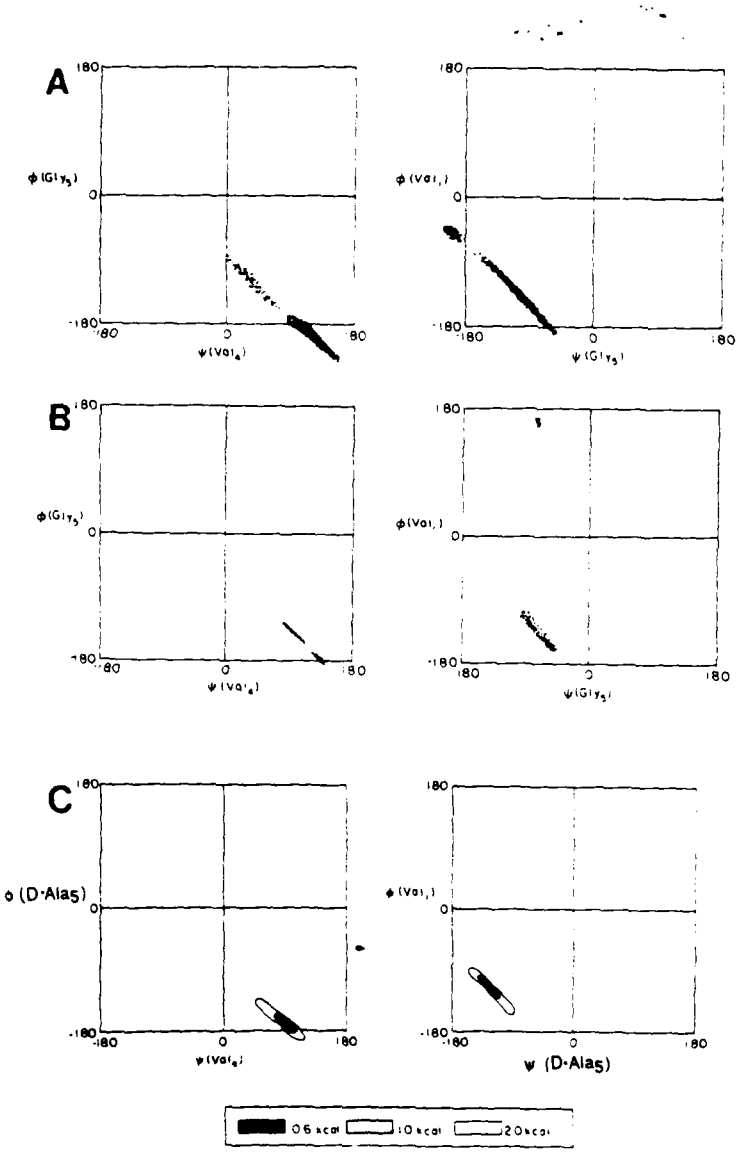

Fig. 18. Λ-plots of peptide moieties in the suspended segment of the β-spiral of the polypentapeptide of elastin in relaxed A and extended B states and for the D · Ala^5 analogue in relaxed state C. When the $\psi(Val_4)$ torsion angle is plotted versus the $\phi(Gly_5)$ torsion angle and similarly for the $\psi(Gly_5)$ versus the $\phi(Val_1)$, all the allowed conformational states within a 2 kcal/mole-residue cutoff energy fall on a 45° diagonal. Thus, these pairs of torsion angles are correlated and the peptide moieties undergo rocking motions, call librations. In the relaxed state, these librational motions can occur with large changes in torsion angle approaching 180°. When stretched to 130%, however, the amplitudes of the motion are greatly damped as shown in (B), where the magnitude of the changes in torsion angles within the same cutoff energy is now reduced to about one-third. For D · Ala^5-PPP in its relaxed state, the plots of $\psi(Val_4)$ versus $\phi(D \cdot Ala_5)$ and $\psi(D \cdot Ala^5)$ versus $\phi(Val_1)$ in (C), the range of allowed torsion angles is also markedly decreased over those for the PPP in (A). This decreased motion for the same 2-kcal/mole-residue cutoff energy obtained on the basis of the conformational energy calculations is observed in the dielectric relaxation studies (see text for discussion). (A and B from Urry *et al.*, 1982; Urry and Venkatachalam, 1983; C from Venkatachalam and Urry, 1986.)

coacervate, and the aggregation is irreversible (Urry *et al.*, 1983*a*). The D · Ala^5-PPP does form a viscoelastic coacervate (Urry *et al.*, 1983*b*). As noted in Fig. 12, the dielectric relaxation studies of PPP show an interesting low-frequency, intense, and localized relaxation demonstrating a peptide librational process within a regular structure (Henze and Urry, 1985). Dielectric relaxation studies on the D · Ala^5-PPP coacervate also demonstrate the relaxation, but the amplitude is much reduced (R. Henze and D. W. Urry, unpublished data).

Using a series of cutoff energies, 0.6, 1.0, 1.5, and 2.0 kcal/mole-residue, the dipole moment change for a pentamer was computed for both PPP and D · Ala^5-PPP (Venkatachalam and Urry, 1986). For example, at 1.5 kcal/mole-residue, the calculated dipole moment changes due to the librations were 3.78 Debye for PPP and 1.33 Debye for D · Ala^5-PPP. With these two values, it becomes of interest to use the Onsager equation for polar liquids (Onsager, 1931) to calculate the apparent dipole moment changes from the amplitudes of the dielectric relaxations. The dipole moment changes per pentamer approximated from the experimental data are 2 Debye for PPP and 0.7 Debye for D · Ala^5-PPP. Interestingly, the values of computed and approximated experimental dipole moment change due to the librations agree within a factor of 2. Perhaps even more noteworthy is that the computed ratio of dipole moments $3.78/1.33 = 2.8$, and the experimental ratio of the dielectric increments per mole pentamer at the 40°C coacervate concentrations, $(72/2)(33/2.5) = 2.7$, are essentially identical. Thus, the comparison of computed librational processes to experimental results is favorable. This provides the desired comparison of a *calculated and experimental* result on a property that is experimentally shown (Fig. 10) to be directly proportional to the magnitude of entropic elastomeric force.

5. SUMMARY

Prior to the studies briefly reviewed here on the polypentapeptide of elastin (see Section 3), entropic protein elasticity was considered to require networks of random chains (Hoeve and Flory, 1974), i.e., to be described by the classic theory of rubber elasticity. To have entropic elastomeric force exhibited by the more-ordered state (the higher temperature state) resulting from an inverse temperature transition, as demonstrated by the polypentapeptide of elastin studies, is contradictory to the random chain network requirement. Thus, to be able to vary the temperature for the development of elastomeric force by varying the hydrophobicity of a polypeptide sequence is a new concept. Clearly, as required for an inverse temperature transition, that an increase in the hydrophobicity of the polypeptide sequence causes the transition to shift to a lower temperature and that a decrease in the hydrophobicity causes the transition to shift to a higher temperature has been demonstrated. This means that at a fixed temperature it should be possible to turn elastomeric force exhibited by a protein on and off, by reversibly changing the polarity of such a polypeptide sequence. The polarity of polypeptide chains can be changed by means of a chemical process. Thus, the mechanism, called mechanochemical coupling of the first kind, becomes a new consideration for understanding protein mechanisms where elastic forces are involved.

Having demonstrated that protein elasticity does not require random chain networks and, in fact, having shown that thermal denaturation of an elastic protein results in dramatic loss of elastic modulus suggests that descriptions of protein elastic processes should not invoke random coils. Thermal denaturation of elastin and the polypentapeptide of elastin results in essentially complete loss of useful restoring forces. Thermally denatured polypentapeptide of elastin (random coil elastin) neither effectively resists nor recovers from deformation when denaturation is carried out while extended. Accordingly, in those cases in which the transition to the elastic state is a regular transition from a stiff lower entropy state to a higher entropy state with a useful elastic modulus, it now seems necessary to consider conformational states intermediate in entropy between α-helices for example and random coils. Consideration of the conformation of that intermediate entropy state, particularly when achieved using repeating peptide sequences, should include spiral conformations in which the repeating sequence as a conformational entity repeats on a helical axis. Whatever the conformation, it is argued that the entropy of the intermediate state should be described in terms of internal chain dynamics, i.e., librational motions, instead of networks of random chains with a random distribution of end-to-end chain lengths. As it would be a situation requiring that the same repeating sequence be compatible with two different nonrandom conformations, it would be a situation more demanding of the protein sequence. This more involved process for turning elastomeric force on and off will then be called mechanochemical coupling of the second kind. Again, a chemical process is sought that will shift the temperature of the transition as the mechanism to turn the elastomeric force on and off.

Important to the understanding of elastic processes in globular proteins is the demonstration that entropic elastomeric force is the result of internal chain dynamics and that a short peptide segment of but a few residues can exert an entropic elastomeric force. Because of this, the above considerations of obvious relevance to fibrillar proteins now become relevant to globular proteins. A short peptide segment can exhibit a large entropy due to internal chain dynamics with the ends of the segment fixed in space, and with the ends fixed in space, the internal motions become describable in terms of librations in which the change in one torsion angle is compensated by an oppositely signed change in one or more other torsion angles. This is called the librational entropy mechanism of elasticity. Any change in the segment that results in either a decrease in amplitude or an increase in the frequency of the motion will result in an entropic elastomeric force exerted at the points of attachment of segment to additional structure. The change could be a mechanical process such as stretching (an increase in end-to-end length of the segment), it could be a chemical process as in the mechanochemical coupling considered above, or it could be an electric process as the effect of an electric field on a membrane protein.

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OF MOLECULES, MOTION, MAN AND MACHINES*

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ABSTRACT:

As an historical point of departure, this lecture begins at the University of Padua in the period around 1600 A.D. Indeed the origins of the modern medical and physical sciences were profoundly influenced by three architects of the sciences: Vesalius, Galileo and Harvey, who either taught or were trained at the University of Padua in this period. It was a period of grand dimensions of cathedrals and of human anatomy and a product of this period was the description by Harvey of the contracting heart as a pump, as a machine.

Our window in time is recognized as a period of molecular dimensions and the molecular architecture of nature is seen as one of beautiful helical symmetries. The molecular architecture of particular interest in this lecture is that concerned with two events required for the contraction of the heart: a chemical event which triggers a structural event, the contraction. The Laboratory of Molecular Biophysics of the University of Alabama at Birmingham School of *Medicine has provided initial* fundamental understanding for both of these events giving rise to the motion of living organisms. The chemical event can be considered to be ions moving through an ion selective channel and the first described ion selective transmembrane channel is the Gramicidin A transmembrane channel, described by this Laboratory in 1971. Two helical molecules (β-helices) join together end-to-end to span the cell membrane and along the helix-axis is an ion selective channel. The structural event is the contraction of fibrillar protein and the first synthetic model protein to exhibit contraction was demonstrated by this Laboratory this year. As the result of a change in concentration of ions, the synthetic model protein, formed into a matrix, contracts and relaxes, which is an experimental finding shown publicly for the first time in this lecture. The contraction involves a relatively disordered polypeptide chain which as the result of a chemical signal wraps up into a new kind of dynamic helical structure called a β-spiral.

The mechanism of contraction is a new mechanism in which the change in hydrophobicity (water hating) or alternatively in hydrophilicity (water liking) character of the polypeptide chain changes the temperature of an inverse temperature transition. The unique feature about

an inverse temperature transition is that, as the temperature is raised, the polypeptide (model protein) becomes more ordered (e.g., *becomes helical*) with contraction as the consequence. For example, contraction occurs on raising the temperature from room temperature to body temperature. If the model protein is made less hydrophobic, i.e., more polar or more water-like by a chemical process, the transition shifts to higher temperature for example on going from 40°C to 60°C. This means that the contracted model protein at 37°C, when made more polar by a chemical process, simply unwinds; it relaxes. By reversing the chemical process, contraction again occurs. Through an understanding of structure and mechanism, an elastic molecular machine has been made that can be caused by chemical means to contract and relax in analogy to the contracting heart.

One medical relevance of this finding is that, when the elastic fiber of the lung is made less hydrophobic, i.e., more polar by oxidation, the fiber unwinds and losses its elastic recoil, as occurs in pulmonary emphysema. There are numerous biomedical implications ranging from contraction, cell motility, the chemical modulation of enzyme and channel activities and the physical changes preceding and following child birth to the sagging and wrinkling of skin in aging. A medical application of the capacity to synthesize these new elastomeric biomaterials is the development of synthetic arteries.

These developments, as those that have come before, are presented within the context of both past and contemporary worlds of diverse opinion, of tradition and of progress.

I. The University of Padua, circa 1600 A.D. (1):

A symbol of this period of greatness,

its architects of the sciences,

the first perspective of an organ as a machine, i.e., the heart as a pump,

and a period of grand dimensions of cathedrals and of human anatomy.

In the latter half of the sixteenth century and the early part of the seventeenth century, the University of Padua was at an apogee of greatness; husbanded within the Republic of Venice, it was the center of learning for Europe and the Mediterranean. For the medical sciences, a primary symbol of that greatness now nearly 400 years later is the anatomical amphitheater which yet stands where originally constructed in Il Bo, the first centralized structure of the University and yet today the central University building. This period epitomizes fundamental advances in knowledge and the conflicts that such breaks with tradition cause both from forces within the sciences and from without. One reminder of a principle conflict of the time is highlighted at the center of the anatomical amphitheater in Figure 1 where a trapdoor is seen on the dissection table through which the human cadaver would be dropped should antagonistic authorities arrive. Interestingly, the anatomical amphitheater of Figure 1 was being built as the dome of St. Peter's Basilica was under construction.

The scientific edifices constructed through the influence of the University of Padua parallel the building of the great cathedrals: many centuries later, these scientific advances stand as monuments of special meaning as do the cathedrals; just as century-long efforts were required in the building of cathedrals, so too are there century-long developments of concepts required of scientists to culminate a particular advancement in the body of science. This relationship was elegantly stated by G. N. Lewis and M. Randall a half a century ago in the Preface to their book on Thermodynamics (2):

"There are ancient cathedrals which, apart from their consecrated

purpose, inspire solemnity and awe. Even the curious visitor speaks of

serious things, with hushed voice, and as each whisper reverberates through the vaulted nave, the returning echo seems to bear a message of mystery. The labor of generations of architects and artisans has been forgotten, the scaffolding erected for their toil has long since been removed, their mistakes have been erased, or have become hidden by the dust of centuries. Seeing only the perfection of the completed whole, we are impressed as by some superhuman agency. But sometimes we enter such an edifice that is still partly under construction; then the sound of hammers, the reek of tobacco, the trivial jests bandied from workman to workman, enable us to realize that these great structures are but the result of giving to ordinary human effort a direction and a purpose. Science has its cathedrals, built by the efforts of a few architects and of many workers."

It is with the anatomical amphitheater built by Girolamo Fabrici d' Acquapendente in 1594 and what it symbolizes, and with three architects of the medical and physical sciences of the time - Vesalius, Galileo and Harvey - that this lecture begins (1,3-6).

Vesalius: "The father of modern anatomy"

His studies on human anatomy in conflict with the Galenist tradition of 13 centuries from within the practice of medicine and his acts of human dissection in conflict with religious authorities from without, Andreus Vesalius in 1543 completed what has been considered the first great book of modern science, Humana Corpora Fabrica. So severely was he attacked by the Galenists that he left his researches in anatomy for the practice of medicine only to attempt a return in the year of his death at age 50.

Galileo: "Father of the modern scientific method"

Galileo came to Padua in 1592 where he developed experimentation to be the central element in the search for knowledge; developer of the telescope, inventor, champion of the Copernican view of the universe that the earth rotates around the sun, teacher of the view that

the universe ran by natural laws rather than by miracles, Galileo particularly with publication of his book Dialogue on the Great World Systems came into conflict with Rome and twice threatened with the rack by the Inquisition was made to recant such views.

Harvey: "The father of modern biology"

William Harvey studied at Padua from 1599 to 1602; he was taught the anatomy of Vesalius; he learned of the valves in veins from their discoverer, his mentor Acquapendente; he learned of the work of Paduan Realdo Colombo describing the pulmonary circulation and at Padua was influenced by the scientific method of Galileo. On receiving his medical degree from the University of Padua, Harvey returned to England to develop his theory of the circulation of the blood culminating in publication of De Motu Cordis in 1628, just as Galileo's contributions were being suppressed by the Inquisition. With the environment of the Protestant Reformation, Harvey came to view the heart as a pump, as a machine, making analogy to the manpowered pump of the fire engine of the time. The work, that has rightfully earned Harvey the title of "the father of modern biology", was the result of the focused culmination of concepts, a century in development, by those architects of the sciences: Vesalius, Galileo, Acquapendente, Colombo and others.

It is here with the view of the heart as a pumping machine that I choose to introduce elements of our own work on processes responsible for motion of living organisms. The two primary events required for the pumping of the heart are a chemical event followed by a structural event (a contraction). It is with these two elements necessary for locomotion, for the beating heart for example, which has so come to symbolize animal life that our research addresses each in a fundamental way.

Before proceeding, however, it is useful to attempt to place in perspective the conflicts with which these three architects of the sciences were faced. As stated in more modern times by Maurice Maeterlinck (7),

> "At every crossway on the road that leads to the future, tradition, has placed against each of us, 10,000 men to guard the pass."

Tradition, as the embodiment of the wisdom of centuries and the force that keeps a society intact, should be breached only with the greatest care. Science has its traditions; they are the theories and bodies of thought that have been developed over the centuries; neither should they be breached without great care. For it is easy to recall the agronomist Lysenko's views of acquired characteristics in heredity which dominated this body of science in the Soviet Union for a quarter of a century because it was in tune with political thought. This dogma had devastating effects on the development of the biological sciences in the Soviet Union. When approaching the unknown and in the process of developing new perspectives, the role of the guards at the pass must not solely be decried but must also be valued in order to prevent passing into devastating periods due to new dominance of unsound perspectives. Progress requires the solomonic balance between the new and the traditional in order that the pass lead to the truths of an improved future. With the quest for an understanding of nature being one of the highest expressions of the uniqueness of man (as our challenge) with a mission to alleviate suffering and to improve (added quality of life (as our charge) and with the competitions between societies of the world as our (added reality, there is no satisfactory option but with all vigor to explore those passes and to find an ever more complete understanding of nature.

II. Our Window in Time - a Period of Molecular Dimensions

Jacob Bronowski has recently said (3),

> "The kind of man who is interested in the architecture of nature today is the kind of man who made this architecture (the cathedral of Rheims) nearly eight hundred years ago."

But the architecture of nature has many dimensions; these may be stated as the macroscopic (gross anatomical), the microscopic (cellular), the molecular and the submolecular. The great period in Padua 400 years ago was a period of the macroscopic when gross anatomical studies were at the forefront of the development of medical knowledge. The microscopic or cellular level was made possible by the development of the microscope with which Malpighi dis-

covered the capillaries necessary for completing the understanding of Harvey's circulation of the blood and with which Hooke first observed cells in cork (4). The molecular level in regard to the architecture of nature began with the accidental laboratory synthesis of urea by Freiderich Wöhler in 1828. Prior to this, man's composition was thought to be beyond man's comprehension. From this, came the synthesis of many natural products and the demonstration that these biological molecules were characterizable by the laws of chemistry and physics. And with the applications of the laws of physics and chemistry to natural products came the application of quantum chemistry to biomolecules which was popularized by Albert Szent-Györgyi in his small book on submolecular biology (8) and which has become now for a quarter of a century the focus of Per-Olov Löwdin's Sanibel Symposia on Quantum Biology and Quantum Pharmacology (9). By studying the distribution of electrons and location of nuclei which comprise biomolecules, quantum chemistry seeks to describe the properties and behavior of molecules.

Today, all of the dimensions of the architecture of nature are active areas in the practice of medicine and in medical research. The traditional anatomy departments are becoming Departments of Anatomy and Cell Biology, and within cell biology there is increasing emphasis on cellular movement and the fibrillar protein structures responsible for migration and on the ultrastructure of cellular and extracellular matrices. In physiology departments, now often called Departments of Physiology and Biophysics, there is great interest in the protein channels of cell membranes; these channels are the basis of cellular excitability and channels provide the chemical signal that triggers intracellular contraction. Departments of Pharmacology and Biochemistry have from their origins focused on molecules, the biological responses they engender, their mechanism, their production and elements of their structure. Currently, biochemistry and microbiology departments share a common focus in the study and utilization of recombinant DNA methodologies in genetic engineering. And it is with the relatively recent technology facilitating DNA sequencing that the sequence of amino acids in proteins is becoming routinely determined. Interestingly, pharmaceutical houses (the drug companies) are utilizing genetic engineering approaches to produce as pharmaceuticals - proteins, polypeptides and hormones -

and to produce more effective variants they are relying more heavily on quantum chemistry, on understanding molecules at the submolecular level, to assist in the design of new pharmaceuticals. Also within the Departments of Medicine, there is for example the area of molecular cardiology with major focus on proteins in membranes, on proteins that pump sodium ions and calcium ions out of the cell and potassium ions into the cell and on proteins that function as channels through which the excitatory currents of sodium ions and of calcium ions flow back into the cell; and there is the area of pulmonary medicine where there is concern for example with the processes whereby the elastic protein of lung loses its elastic recoil which is a central element in the disease pulmonary emphysema.

There is now general appreciation as stated for example by M. W. Shaw (10) that,

"Understanding the molecular basis of a disease is a necessary step in its rational treatment."

It seems apparent for our window in time that the molecular dimension is at the forefront of developments in the medical sciences.

III. The Molecular Architecture of Nature

A. The Molecular Structure of DNA

The centerpiece of the molecular architecture of nature and indeed a symbol of science today is the double stranded spiral structure of DNA. It is a structure of artistic beauty and yet it is more. As stated so simply in 1896 by Louis Sullivan (11), the great Chicago architect and mentor of Frank Lloyd Wright,

"Form ever follows function"

The form, in this case the molecular structure of DNA, contains a ready understanding of function (12). By the base pairing that is central to the molecular structure, it is apparent that this molecule has the information for self-duplication, hence the basis for genetic transmission of biology. Also, the sequence of the bases can become translated into a sequence of amino acids resulting in protein and it is the proteins that are the primary players in the functioning cell.

The DNA of the cell contains the blueprint whereas the cell or the integrated set of cells and their products is the completed functional living edifice. The proteins perform the functions of the cell and give rise to the behavior of living organisms, behavior such as excitability and motion.

B. Two Key Events Required for the Properties of Excitability and Motion of Living Organisms

There are now recognized two primary events which are necessary for the excitability and motion of living organisms. These are a chemical event, an ionic trigger, which is the sudden flow of positive ions into the cell and a resulting structural event which is a molecular contraction causing visible motion. In a particular sense, this is called excitation-contraction coupling. In a more general sense, life and death are defined in terms of these two fundamental events. The presence or absence of a pulse, detectable by touch but also at locations visually seen as motion, is the most ordinary structural event for assessing state of life. In more sophisticated circumstances, the electrocardiogram, reporting on the electrical activity of the heart, and the electroencephalogram, reporting on the electrical activity of the brain, provide the most definitive information on the state of life. The state of the heart or brain, whether healthy, diseased or dead, is assessed by the shape and periodicity of the electrical waves, or in the latter case, by the absence of electrical activity. The electrical activity is due to the chemical event of the flow of ions passing through cell membranes. These currents of life pass through channels in cell membranes and are the chemical event that can result in the structural event of motion. And it is fibrillar proteins that bring about a decrease in length as a result of the chemical event that are responsible for the structural event of motion. The research of the Laboratory of Molecular Biophysics of the School of Medicine at the University of Alabama at Birmingham has had the great good fortune and the excitement of providing firsts in understanding for both of these fundamental events of living organisms. Even so, it needs to be stated that our understanding is yet rudimentary. For as scientists have been chided by Mark Twain, one of the more entertaining guardians of the pass (13),

"In the space of one hundred and seventy-six years the Lower Mississippi has shortened itself two hundred and forty miles. That is an average of a trifle over one mile and a third per year. Therefore, any calm person, who is not blind or idiotic, can see that in the Old Oölitic silurian period, just a million years ago next November, the Lower Mississippi was upward of one million three hundred thousand miles long, and stuck out over the Gulf of Mexico like a fishing rod. And by the same token any person can see that seven hundred and forty years from now the Lower Mississippi will be only a mile and three quarters long, and Cairo and New Orleans will have joined their streets together, and be plodding comfortably along under a single mayor and a mutual board of Aldermen. There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact."

With this humorous but sobrietous note duly considered, I hope to demonstrate in what follows that basic processes of excitability and motion are now describable at a molecular level. These can provide the point of departure for our understanding of excitability and motion in living organisms, for the molecular basis of related diseases, and for helpful interventions. It is the sense of excitement in the scientific quest for an understanding of the architectural elements of the living organism and their functions that this lecture is intended to convey.

Particularly with the presence of the "guards at the pass", the perspective of my mentor, Henry Eyring, is relevant; he is reported to have said,

"I've never felt that approval was as exciting as the scientific quest, itself."

Of course, today approval or at least acceptance in certain quarters is required to continue the scientific quest. Research work must find a publishable outlet, and to occur, research work must be funded. Because of this, our society is well-served by having multiple routes to publication and multiple sources of funding. In this way, tradition as exercised by man with all his foibles, for example as exercised by those who may have helped to shape a par-

ticular tradition or by those whose fortunes are favored by maintaining a particular tradition, cannot unduly suppress innovation.

IV. A Chemical Event that Triggers Motion in Living Organisms: The Ion Channel

The nerve impulse that excites the muscle cell is initially the inward flow of sodium ions across the nerve cell membrane through sodium channels (14), and the excitation of the muscle cell that results is characterized by the inward flow of sodium ions and of calcium ions across the muscle cell membrane through sodium and calcium channels, respectively, and the calcium ion released to the inside of the cell is the direct trigger of the contractile event (15). In this way, channels provide the chemical event that results in the motion of contraction. While there are additional elements and circumstances wherein contraction is achieved by a chemical event, that chemical event can often be related either directly or indirectly to channel function.

The detailed structural information that is currently known for channels is due to a channel from lower organisms called Gramicidin A. An understanding of the molecular architecture of the Gramicidin A channel was developed by the Laboratory of Molecular Biophysics in November of 1970, within months after its move from the American Medical Association's Institute for Biomedical Research to the School of Medicine at the University of Alabama at Birmingham (16,17). Two molecules come together to span the cell membrane with a structure which contains a continuous channel that spans from one side to the other of the cell membrane as shown in Figure 2 (18,19).

Demonstrated by studies on the Gramicidin channel is the remarkable observation of single molecular events; it is possible "to see" a single channel "turn on", as the result of two molecules coming together to span the cell membrane, and it is possible "to see" that channel "turn off" as the result of two molecules separating. This is shown in Figure 3 where a step up in electrical current is the formation or opening of a channel and a step down in electrical current is the closing, or dissociation, of the channel. During the period of time in which the channel is on, some ten million ions per second (10^7 ions/sec) pass through the channel. In

nerve and muscle cell membranes, these events are occurring sporadically all of the time but it is the coordinated turning on and off of channel currents that gives rise to the electrical activity of the heart as monitored by the electrocardiogram.

The molecular structure of the Gramicidin channel that is seen here is the first described ion selective channel; it stands today seventeen years after its description, confirmed by seventeen years of tests and by the laying to rest of alternate opposing structures, as the accepted molecular structure of the channel. This molecular architecture is the demonstration that channels do exist (14); it provides numerous new concepts on the mechanism of ion transport through channels and it has been the proving grounds for new methodologies for characterizing channels (18,19). Both the new concepts and the new methodologies are being applied to channels from higher organisms. With the above description is seen the basis of the chemical event responsible for excitability and motion.

V. A Structural Event of Chemically Triggered Motion: Contraction

The capacity of living things to initiate motion seemingly spontaneously from within, as opposed to motion being imparted from some external force, is a central fascination of life. The first person to separate that biological motion from the living cell was Albert Szent-Györgyi (20). He isolated a protein preparation from muscle, made it into artificial fibers as one might do by extruding it through the needle of a syringe, and on adding the required chemicals, he saw the fibers contract. What memorable excitement reading of the Szent-Györgyi experiment gave me as an undergraduate student. For the first time outside of a living organism Szent-Györgyi had observed the motion of contraction so identified with life. Perspective can be gained by knowing how this man viewed his craft in a quotation ascribed to him,

> "Research is to see what everyone else has seen,
> but to think what no one else has thought"

and it is, of course, thinking what no one else has thought that can bring the researcher by design of experiment to see what no one else has yet seen.

It is the primary objective of this lecture to impart to you the sense of excitement of such discovery. To do so with the work of the Laboratory of Molecular Biophysics it is necessary to journey through our development of the understanding of the elasticity of proteins and the story can begin with the pumping heart as first perceived by William Harvey (6).

Elasticity and Motion

When the blood of the heart is impelled by the contraction of the left ventricle into the arteries, it first passes into the aortic arch which on receiving the blood expands much as a rather stiff balloon expands. Because of this stretching, there continues a force on the volume of blood after the contraction of the ventricle has ceased and after the aortic valve has closed. The stretched wall of the aortic arch continues to impel the blood further into the arterial tree much as the partially filled balloon on removing the filling pressure expels its contents as the stretched elastic walls relax. It is a remarkable fact that elastic fibers with which we are born continue to function until death which means in the aortic arch and in the arterial tree in general that the elastic fibers of man by the age of 60 years will have undergone 2 billion stretch-relaxation cycles. This is an unmatched durability for elastomers and the Laboratory of Molecular Biophysics for the last fifteen years has been studying the nature of this elasticity.

As is outlined below, the study of the nature of the passive elastic forces developed in the arteries in response to the chemical triggering of elastic forces in the heart will bring us through an understanding of the nature of elasticity to an understanding of a basic mechanism whereby elastic forces may be triggered chemically (21-23). This sojourn is depicted in Figure 4. The first step is to describe the molecular architecture of the elastic component. In the reverse process of the architect, e.g., of Sullivan's building of skyscrapers where desired function dictate structure, we first determine structure and then deduce function. Then with an understanding of the molecular function of the elastic component, it should become possible in the role of the architect to design a chemically driven elastic molecular machine. As with any purported new understanding, a new concept requires testing and that testing is most effectively achieved by the demonstration of some new capability.

Molecular Structure of the Elastic Component of Elastic Fibers

In cattle, at the heart of the amino acid sequence of the single protein that forms purified elastin fibers and the longest sequence between the cross-links that make a single protein into an insoluble elastic matrix is the repeating amino acid sequence (L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5)$_n$. This is abbreviated (VPGVG)$_n$ or it may be called the polypentapeptide of elastin abbreviated as PPP. This sequence has now been observed in pig, in chicken and in man (25,26).* When very large polypeptides are made of this repeating sequence, for example when it repeats one hundred times or more, we have found in water that the structure would unwind at room temperature; but when the temperature is raised to body temperature, this model protein coils up into a new kind of helix (see Figure 5) (27,31). It is with the formation of this new kind of helix, which we have called a β-spiral, that the elastic properties of the cross-linked protein are enhanced. Here we are told by the guardians of the pass that this cannot be, that the biological elastic protein must be like an ordinary rubber; it must be a random network of chains. Nonetheless, when a synthetic cross-linked matrix is formed in water on raising the temperature from room temperature to body temperature, it will contract. While common stretched rubber shows this property of decreasing length and lifting a weight on raising the temperature, this effect is only a few percent for the indicated temperature change whereas it is ten times greater for the model protein material. In spite of the declarations of the guardians of the pass that the biological elastic fiber functions just like rubber, this observation requires a new mechanism, a mechanism different than that described for rubber (see Figure 6) (32).

While this new mechanism at this stage of description clarifies the passive development of elastic forces, further knowledge is required for an understanding of biological motion. This is because living organisms do not function like a steam engine or the engine of our automobiles

* While the commonness of protein sequences in man and other animals is clear demonstration of man's evolutionary ties to lower animals, the uniqueness of man among the animals of course is just as clear. All recognize a uniqueness in man's art, in his language, in his music and in his social structure and we who are in research particularly value his unique capacity to discover and utilize the laws of nature and of his own physical nature.

by using temperature changes to achieve motion. Accordingly, this cannot directly be the contractile event that we seek to understand; we seek to understand a contractile event that is the result of a chemical event. Nonetheless, as is shown below, a proper understanding of the mechanism of this heat elicited contraction can result in the design of an elastomer that can contract at body temperature by using a chemical process.

The Temperature Range of the Contraction Depends on the Hydrophobicity of the Model Protein

The winding up of the structure into a β-spiral noted above depends on the presence of the oil-like side chains of the Val and Pro residues and the temperature range over which the β-spiral forms and over which the contraction occurs depends on the relative oil-like properties of the side chains. Oil is made up of CH_2 moieties. If we add one CH_2 group to the Val in position one to give what is labelled Ile^1, it becomes more hydrophobic (more water hating) and instead of the contraction occurring in the 20° to 40°C temperature range as it does for the natural sequence, the contraction now occurs on going from 0° to 20°C (see Figure 7) (33). Furthermore, if we remove an oily side chain by removing the Val^4 residue altogether, then the model protein becomes less hydrophobic and more hydrophilic (more water loving). Contraction now occurs at a higher temperature on raising the temperature from 40° to 60°C (see Figure 7) (34). These observations and an understanding of their cause allow us to think what no one else has thought. If there were a way to change the hydrophobicity reversibly at 37°C, then it would be possible to bring about a contraction at body temperature. Maintaining body temperature it would be possible, for example, to start with the relaxed, less hydrophobic model protein and by a chemical process, which would make the model protein more hydrophobic, to cause the protein to contract.

Chemically Driven Contraction

Now that we are thinking what no one else has thought, can we devise an experiment to see what no one else has seen?

In order to do this, the model protein is designed to include a few residues per hundred of an amino acid which can reversibly be made more or less hydrophobic by a chemical process.

The amino acid we first chose was glutamic acid and it replaces the Val^4 residue in one out of each five or ten pentamers. The side chain of a glutamic acid residue contains a carboxyl group, a COOH, which can be made even less hydrophobic by removal of the proton to give COO^-. By making the solution more acidic, the less hydrophilic (more hydrophobic) COOH group is formed and by making the solution less acidic, the less hydrophobic (more hydrophilic) COO^- is formed. When this model protein is made into a cross-linked matrix, when it is placed at body temperature in a less acidic solution with a suspended weight attached and when the solution is made more acidic, the contraction occurs, as is shown publicly here for the first time (see Figure 8A and B) (23). Seemingly spontaneously from within a contraction occurs; a weight is lifted; work is done. A synthetic model protein has now been made to contract by a chemical event. The synthetic contractile protein is based on a new principle of mechanochemical coupling using as a basic concept a nature of elasticity declared impossible by the guardians of the pass. Having carried our research to the point where we have thought what no one else has thought, it has been possible to devise an experiment to see what no one else has yet seen. Produced in a wholly synthetic system is the motion of a contracting model protein, the seemingly spontaneous type of motion that we have come to associate with life. A chemically driven elastic molecular machine has been made using new principles for mechanochemical coupling that we propose to be relevant to the heart, to the chemically driven pump of Harvey, wherein there is analogous active development of elastic forces.

VI. Medical Implications and Applications

There are a number of proposed applications of these concepts to protein mechanisms. Some have been recently reviewed and are to be published in the Second1988 issue of Journal of Protein Chemistry (22). Here will be briefly mentioned one medical implication and one developing medical application.

With the above background information, the medical implication is a simple statement. It was just shown above that making the elastic protein less hydrophobic causes it to unwind and

to lose its elastic recoil. One way to make the elastic protein less hydrophobic is to oxidize it. Oxidants in smoke and in toxic atmospheres entering the lung would oxidize, would make less hydrophobic, the elastic protein of lung and cause it to unwind and to lose its elastic recoil. This proposal was made at the international Micromechanics of the Lung Workshop at the National Institutes of Health this past March to be a primary etiology in the development of the disease pulmonary emphysema and we have since shown oxidants such as superoxide, hydrogen peroxide and hypochlorite (the primary ingredient of chlorox) to cause lengthening and loss of elastic recoil in both synthetic and natural elastic fibers (22, unpublished data).

The medical application under development is the making of synthetic arteries. They are to be formed from the model elastic protein shown above but they are to be modified in ways specific to their proposed role. The elastic properties of the natural artery can be matched, and peptides are being identified which cause directed migration of the natural arterial wall cells into the synthetic elastic artery (see Figure 9). The planned scenario is for endothelial cells and smooth muscle cells as well as fibroblasts of the natural artery to migrate into the synthetic artery, to become attached there and to restructure the synthetic artery in the process of regenerating a natural artery.

These developments with this new class of biomaterials, elastomeric polypeptide biomaterials, call to mind another quotation due to Bronowski (3),

> "In effect, the modern problem (of biomaterials research) is no longer to design a structure from the materials (available) but to design materials for a structure."

From the Book of Psalms 139:14 in the King James version is read,

> "I will give thanks to Thee for I am fearfully and wonderfully made."

It is now said that more recent findings of old transcripts have altered this favored quotation, but surely it is wondrous that the universe runs by natural laws and that man is made that he may come to understand those laws and his own nature, and in doing so, each of us draw from the past and with our transitions through the passes, hopefully we provide to the future.

Motion coming seemingly spontaneously from within

not the motion of the trees in the wind
not the motion of the surf and the tide

but motion coming somehow from deep down inside

for many, this is a central fascination of life.

The foregoing contains an effort to impart our understanding of the molecular origins of that motion.

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FIGURE LEGENDS:

Figure 1 This 1986 poster commemorating 700 years of medical sciences at the University of Padua has chosen as its subject the anatomical amphitheater constructed by Girolamo Fabrici d'Acquapendente in 1594. In this anatomical amphitheater, the students would stand behind the bannisters in elliptical tiers (two of five tiers are shown) and they would observe the dissection on the table at center. The dissection table also served as a trapdoor through which the human cadaver could be quickly removed should those opposed to human dissection approach the amphitheater where the human cadaver would be disposed of. In the photograph, the trapdoor is removed allowing one to see below the amphitheater. With 700 years of contributions from which to choose, it is noteworthy that what was featured were this symbol of the contributions to human anatomy in the period near 1600 A.D. and the conflict that this progress experienced. It is with architects of the sciences of the period - Vesalius, Galileo and Harvey - and with an awareness of the conflicts their advances engendered that this lecture begins.

Figure 2 Molecular structure of the gramicidin A transmembrane channel shown in space filling model

A. Side view of this first-described ion selective transmembrane channel. It is comprised of two molecules in β-helical conformation attached by six hydrogen bonds at the middle. This structure spans the lipid layer of a cell membrane and allows passage of monovalent cations such as sodium ions and potassium ions with a preference for the latter.

B. Channel view showing an approximately 4Å diameter and 26Å long channel through which monovalent cations selectively pass. Anions and multivalent cations do not pass through this channel. This structure is representative of the selective passage of ions through transmembrane channels which is the chemical event that can trigger muscle contraction and the resulting

motion of living organisms. Reproduced with permission from Urry, Long, Jacobs and Harris, Ann. NY Acad. Sci., 264, 203-220, 1975.

Figure 3 Single channel current tracings below of the naturally occurring Gramicidin A, HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-NHCH$_2$CH$_2$OH, and above of synthetic L-Ala^7-Gramicidin A in which residue L-Val^7 has been replaced by L-Ala^7. This synthetic analog was designed to gain an understanding of the variable single channel current steps seen in Gramicidin A below; note that in the synthetic analog (above), all of the step heights are the same whereas the step heights vary with the natural molecule.

Each "step up" represents the current resulting from a single channel forming by the association of two molecules as seen in Figure 2A which span the membrane and each "step down" is due to the turning off of the current as the channel dissociates into two molecules. While the channel is formed, a current of approximately 10^7 ions/sec flows through the channel when the applied potential is 100 mV and the temperature is near body temperature. In the cell membrane, such channel events are occurring randomly until there is a wave of membrane depolarization which causes many channels to open or close synchronously and it is this synchronous turning on and then off of many channels that is responsible for the electrical signals of the electrocardiogram and the electroencephalogram.

Figure 4 Beginning with the actively contracting heart of Harvey which is describable as a chemically driven pump (machine) in which there is an active development of elastic forces, consideration is then given to the aortic arch which stretches elastically as the left ventricle of the heart empties its contents into the arteries. The stretched aortic arch then continues to impel the blood on into the arterial tree. This is an example of the passive development of elastic forces, i.e., of the

development of elastic forces from without the aortic arch due to the application of the external force of the contracting heart.

An elastic component of the aortic arch is then studied and its molecular structure is determined (see Figure 5). Having arrived at a molecular structure, the function of the elastic component can then be understood.

Because the molecular structure is found experimentally to form as the temperature is raised from room temperature (25°C) to body temperature (37°C) with a resulting elastic contraction, this is called thermomechanical transduction and the elastic component is a thermomechanical transducer. The driving force for the contraction is the increase in the number of configurations of molecules on return to the unstretched state for the elastic component and its aqueous solvent. For the elastic component itself, the increase in number of states for the unstretched as opposed to the stretched state is depicted in Figure 6. An understanding of the driving force for the contraction and for the stretched elastic component to return to the unstretched state is obtained by means of the Boltzmann relation, $S = R\ln W$, where W is the number of states that can occur in a low energy range, R(=1.987 cal/mole·deg) is the gas constant and S is the entropy. (The development of this equation, which is as important to biological function as $E = MC^2$ is to nuclear energy, and the conflict which this caused Boltzmann to endure, which contributed to his suicide just a few years before his views triumphed, constitute a compelling chapter in the history of science.)

With the understanding that elastic forces and contraction occur as the result of an inverse temperature transition (see Figure 7), it becomes possible to design and to synthesize a chemically driven elastic molecular machine (see Figure 8) which brings us full circle to the fundamental description of the contracting heart.

Figure 5 Molecular Structure of the Elastic Component

The elastic component is a repeating pentamer, (Val^1-Pro^2-Gly^3-Val^4-Gly^5), and the molecular structure of the pentamer is shown in A. The hydrogen bond between the C-0 of residue one and the NH of residue 4 forms the U-shaped structure (inverted as shown) called a β-turn. At low temperatures, these β-turns are in relative disorder, one with respect to the other, and the length of the chain tends to be extended, but on raising the temperature, as part of the contraction, the β-turns wind up into helical array as schematically shown in B and in greater detail in C where the β-turns are seen to function as spacers between the turns of the helix. The helical structure, called a β-spiral, is shown in atomic detail in axis view in D. and in side view in E. and in both cases as stereo pairs. As shown in F. and G., several β-spirals supercoil to form the basic elastic filament. Reproduced with permission A. from reference 27, B. C. and D. from reference 29, E. from reference 28, and F. and G. from reference 30.

Figure 6 A. Three pentamers in the β-spiral conformation (approximately one turn of helix) of Figure 5 D. and E. This is the relaxed, unstretched state. In this dynamic relaxed state, segments of the polypeptide chain can undergo large rocking motions with little change in internal energy. This means that W of the Boltzmann relation in Figure 4 is large causing the entropy, S, to be large for the unstretched state.

B. On stretching (on extension), the amplitudes of the rocking motions are damped such that W and consequently S are decreased. It is the increase in S on returning to the relaxed unstretched state that provides the driving force (the decrease in free energy) for elastic retraction. Reproduced with permission from reference 32.

Figure 7 Plots of increase in force (Δ force) or decrease in length (-Δ length) of matrices of the polypentapeptide of elastin, PPP or $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, and

two analogs: one in which the valyl 1 residue is replaced by isoleucyl, i.e., Ile^1-PPP, making it more hydrophobic (more oil-liking or more water hating) and a second in which the hydrophobic Val^4 residue is deleted, i.e., PTP (polytetrapeptide) or $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$, making it less hydrophobic (more hydrophilic, more water liking). When the model protein is made more oily (as in Ile^1-PPP), it contracts on raising the temperature in the 0° to 20°C temperature range rather than in the 20° to 40°C temperature range as occurs for PPP. When the model protein is made less hydrophobic (as in PTP), it contracts on raising the temperature through a higher temperature range from 40°C to 60°C. Now if it were possible to keep the temperature at 37°C and by a reversible chemical process to make PPP more like PTP, then the model protein would be contracted when like PPP and it would relax by the chemical process that made it like PTP, that is, less hydrophobic. One relatively easy way to do this is to include in PPP an occasional residue that has a titrateable functional group like a carboxyl. When as the less hydrophobic, more polar COO^-, the structure would be unwound and relaxed but by the reversible chemical process of protonation, the functional group would become the less hydrophilic COOH, causing the structure to wind up with contraction and shortening as the result. This is demonstrated in Figure 8.

Figure 8 Contraction and relaxation of the model protein $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}\phi^4\text{-}Gly^5)_n$, in which ϕ^4 is Val or Glu at a ratio of 4:1. This means four glutamic acid residues per 100 residues. The glutamic acid side chain is $CH_2\text{-}CH_2\text{-}COOH$ at low pH and is the less hydrophobic $CH_2\text{-}CH_2\text{-}COO^-$ at high pH. As shown, when the pH is 7, the elastomeric matrix is relaxed at the start in A. When the pH is lowered to 3, most of the side chains are converted to COOH and the sample contracts with time lifting the weight. Now in B, when starting at the contracted state and changing the pH to 7, the sample with time relaxes by lengthening and lowers the weight.

This material is able to pick up and set down weights that are one thousand times greater than the weight of the contracting elastomer.

Figure 9 Synthetic artery made from the elastic component (the repeating pentamer) and another repeating sequence (a hexameric repeat). This tube matches the elastic properties of a natural artery. Importantly, it can be modified in way that will favor migration of arterial wall cells of the natural artery into the synthetic artery and that will provide cell attachment. The perspective is one of making a synthetic artery that will provide a temporary scaffolding for the restructuring of a natural artery.


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IN COLLABORAZIONE CON: UNIVERSITÀ DEGLI STUDI DI PADOVA - REGIONE VENETO - C.N.R.

FIGURE 1

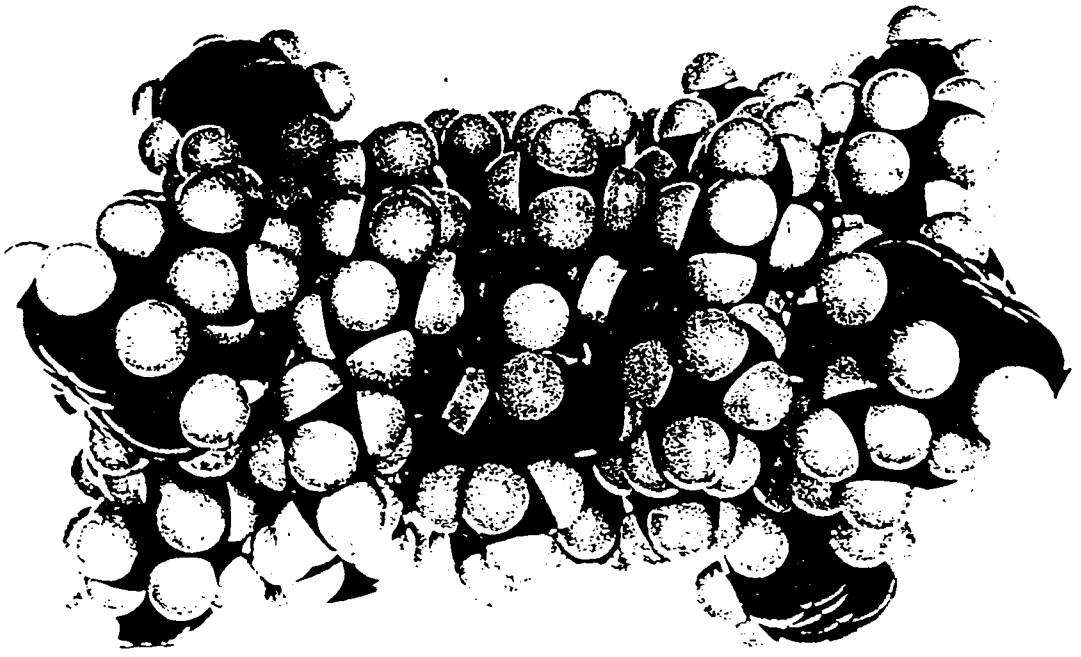


FIGURE 2A

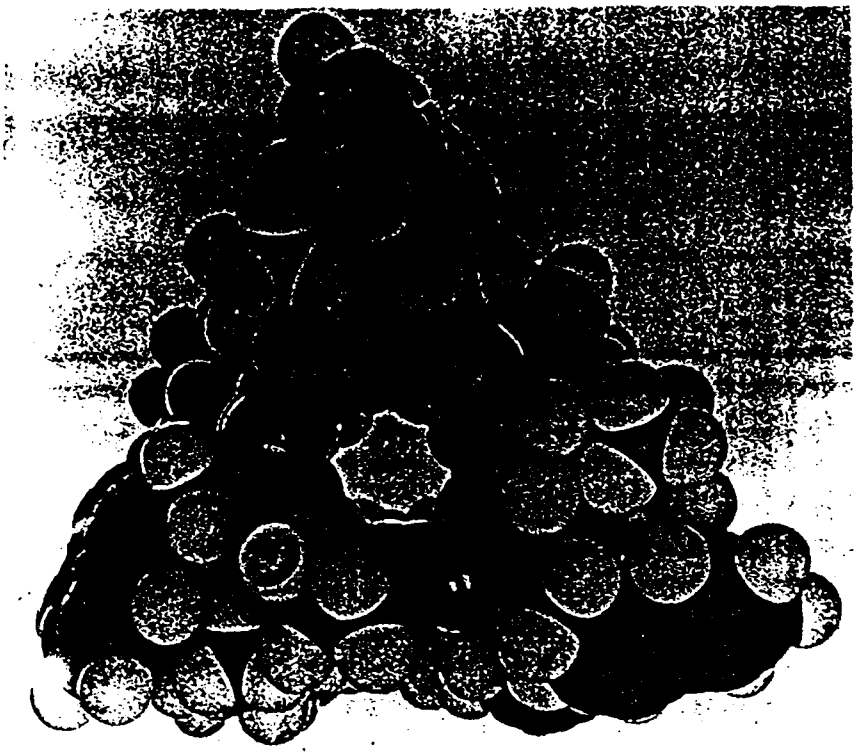


FIGURE 2B

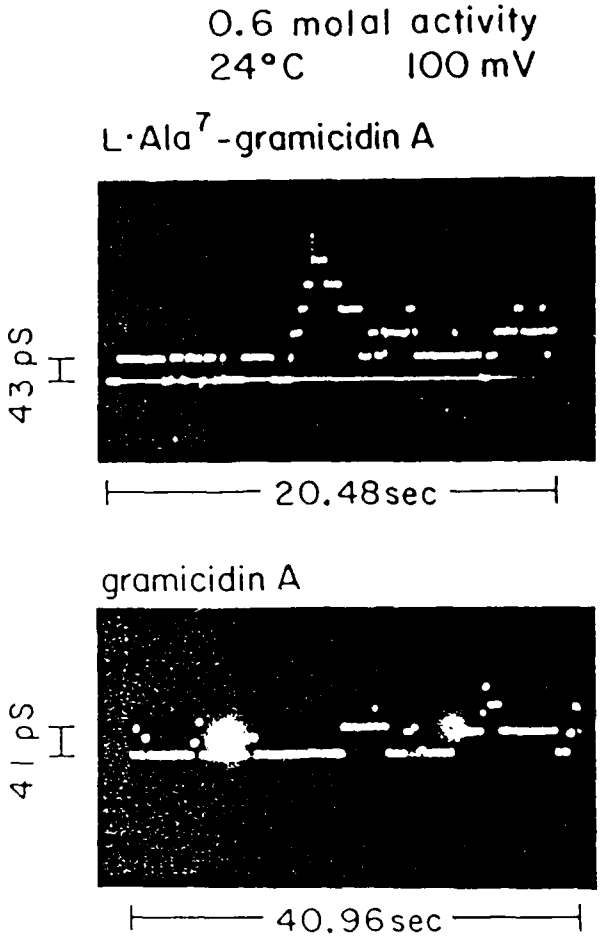

0.6 molal activity
24°C 100 mV
L·Ala⁷-gramicidin A
43 pS
20.48sec
gramicidin A
41 pS
40.96sec

FIGURE 3

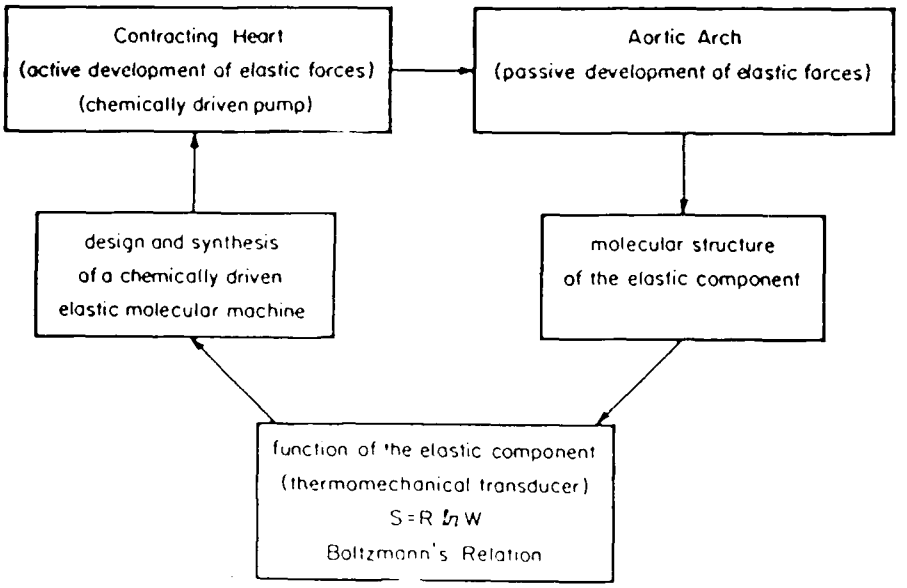

Contracting Heart
(active development of elastic forces)
(chemically driven pump)
Aortic Arch
(passive development of elastic forces)
design and synthesis
of a chemically driven
elastic molecular machine
molecular structure
of the elastic component
function of the elastic component
(thermomechanical transducer)
S=R ln W
Boltzmann's Relation

FIGURE 4

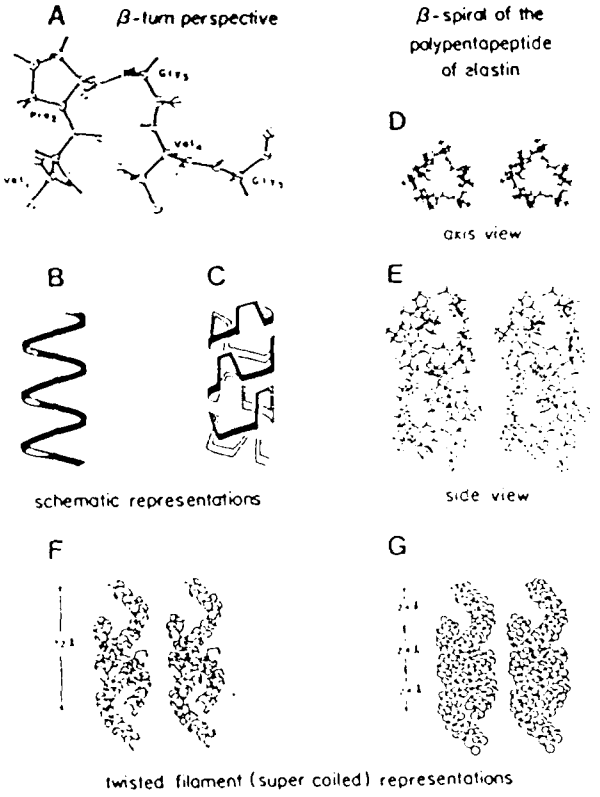

A
β-turn perspective
β-spiral of the polypentapeptide of elastin
D
axis view
B
C
E
schematic representations
side view
F
G
twisted filament (super coiled) representations

FIGURE 5

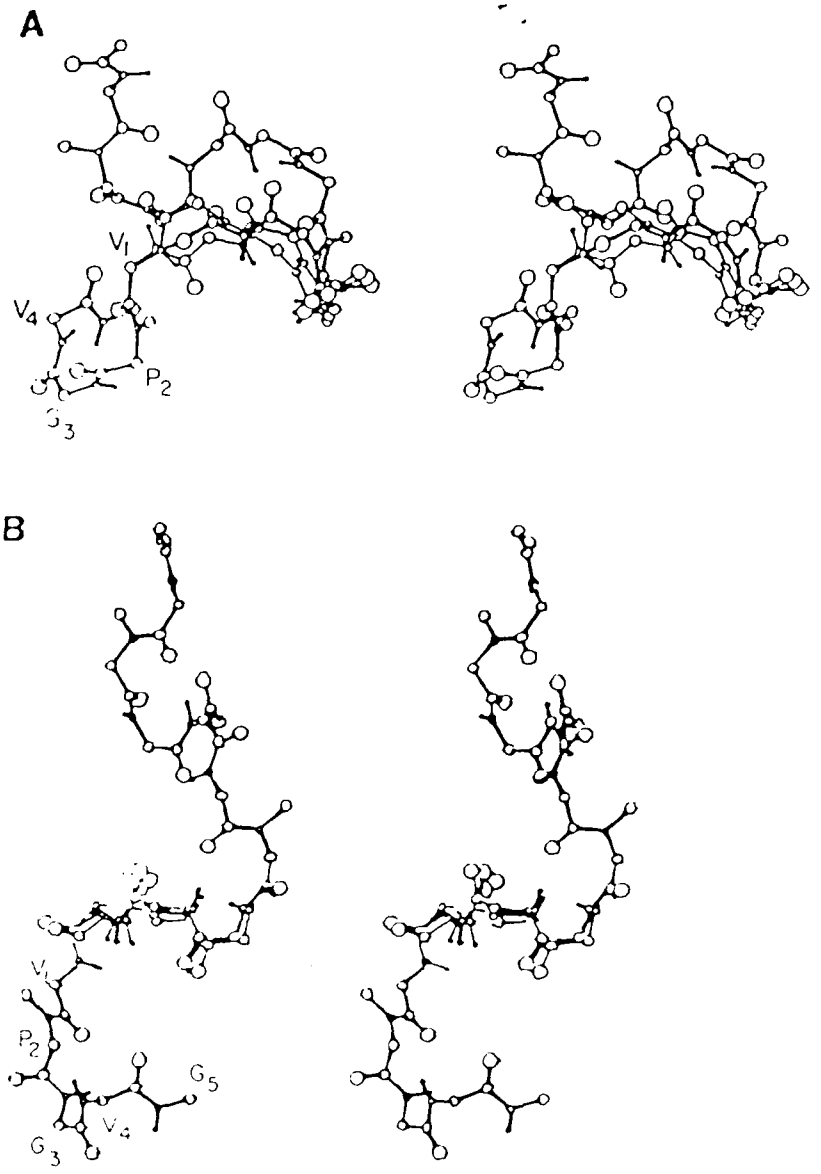
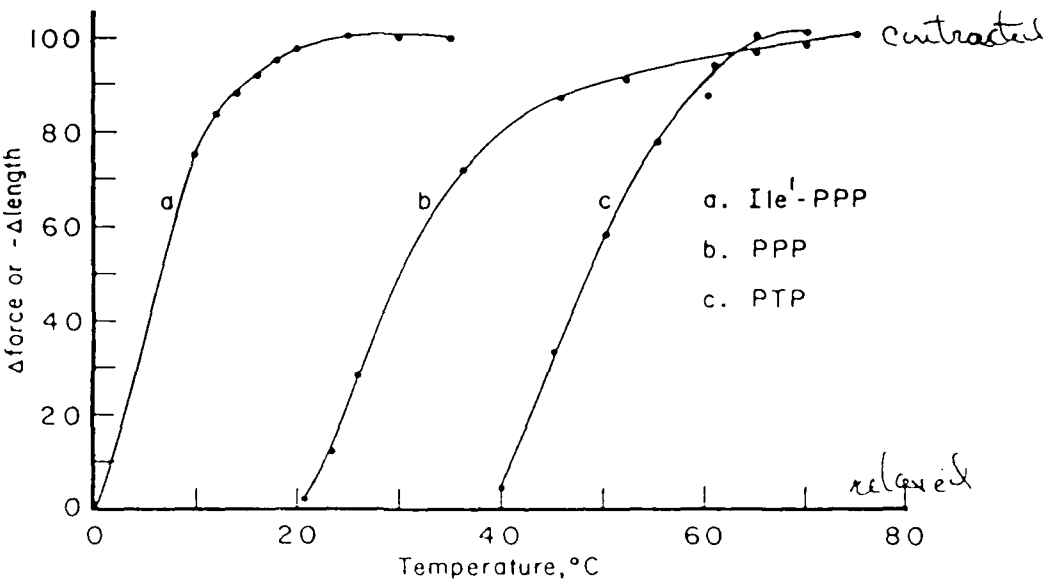


FIGURE 6


100
80
60
40
20
0
Δforce or -Δlength
0
20
40
60
80
Temperature, °C
a
b
c
a. Ile¹-PPP
b. PPP
c. PTP
contracted
relaxed

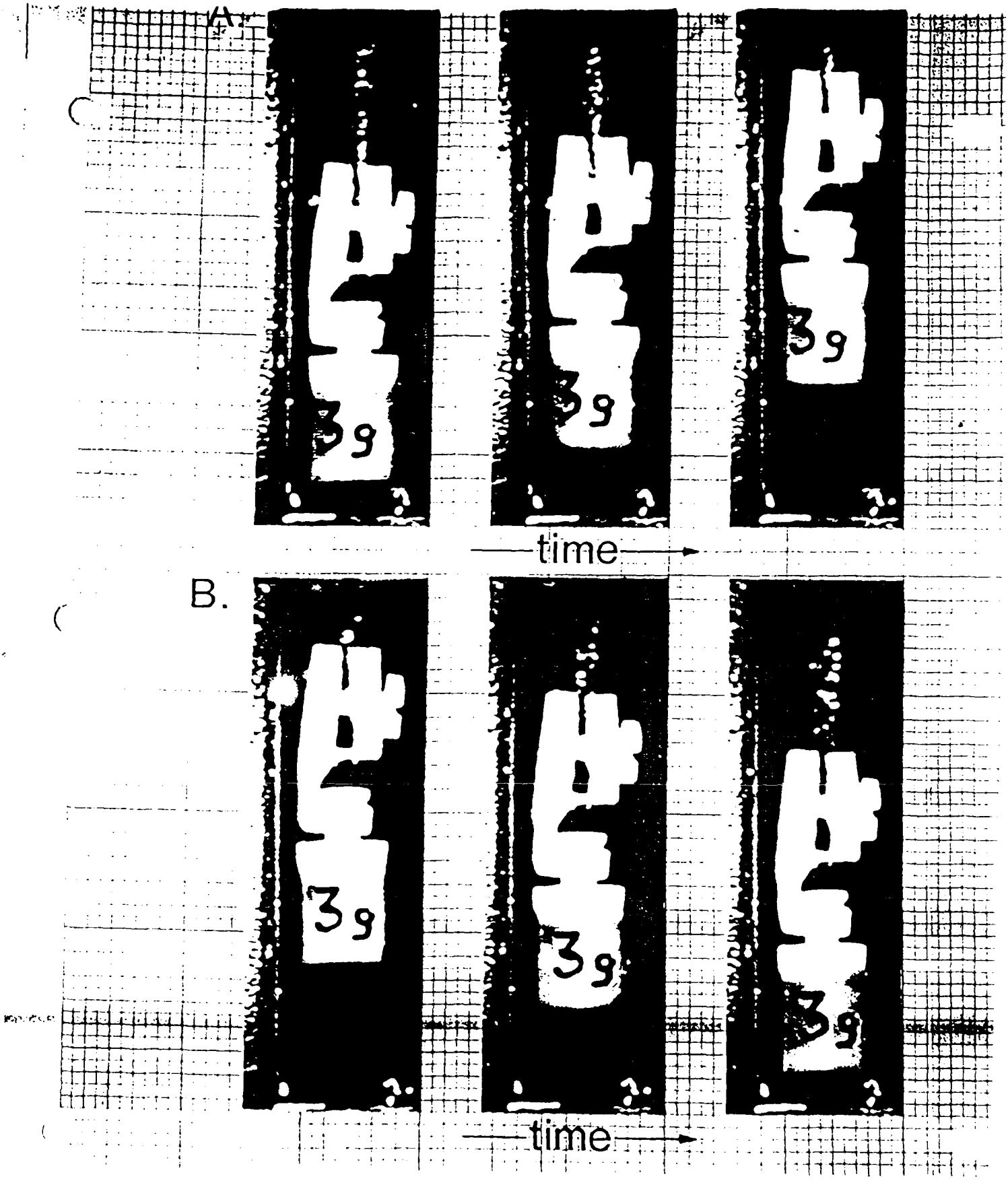


FIGURE 8

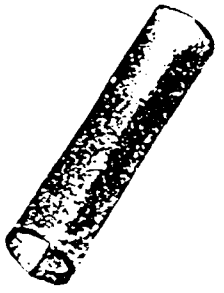


FIGURE 9

"BIOELASTICS:"

A NEW DIMENSION IN BIOMATERIALS APPLICATIONS

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"In effect, the modern problem (of biomaterials research)
is no longer to design a structure from the materials (available)
but to design materials for a structure"

J. Bronowski
Ascent of Man

I. Overview

Certain polypeptides comprised of repeating sequences are the basis of a new class of biomaterials. These elastomeric polypeptides are life-like biomaterials. They can be made to match the compliance of natural biological tissues and when useful can be modified to elicit desirable tissue reactions at the cellular and enzymatic levels. They can be designed to contract and to relax and to do work as the result of a change in chemical potential in a manner analogous to the production of motion in all living organisms. They are functional in an aqueous environment and can be optimized for physiological temperatures making biomedical applications all the more appropriate.

In terms of industrial applications these biomaterials have the properties to function as sensors based on demonstrated physical properties of thermomechanical and chemomechanical transduction. With this class of biomaterials, contraction can be achieved by reversible chemical modulation of an inverse temperature transition such that stretching becomes the free energy input for reversing the chemical process, and in one design the biomaterial would become a mechanochemical engine which on driving in reverse would achieve desalination. Among the biomedical applications under development and consideration are synthetic arteries, a material to prevent post-surgical and post-trauma adhesions, burn cover, synthetic ligaments and targeted drug delivery.

II. Elastomeric Matrices of Sequential Polypeptides

General preparation of Elastomeric Matrices

The polypentapeptide, $(L\text{-}Val^1\text{-}L\text{-}Pro^2\text{-}Gly^3\text{-}L\text{-}Val^4\text{-}Gly^5)_n$, may be considered as the parent sequential polypeptide of this new class of biomaterials. Abbreviations for this polypentapeptide are $(VPGVG)_n$, poly(VPGVG) or simply PPP. This sequence repeats eleven times in bovine and porcine elastic fibers and constitutes the longest sequence between cross-links (1,2). This polypentapeptide and its analogs have been prepared by solution and solid phase peptide synthesis and microbial syntheses are underway. Poly(VPGVG) is soluble in water in all proportions below 25°C but on raising the temperature to 37°C of concentrations more dilute than 400 mg/ml, a phase separation occurs resulting in an overlying equilibrium solution and a more dense viscoelastic phase called a coacervate. By a suitable mold the coacervate can be formed into a desired shape. When very high molecular weight polymers are made, for example with n in the range of 100 to 200, γ-irradiation can be used to cross-link the coacervate and to result in an elastomeric matrix of the desired shape. For characterization of the elastic properties, elastomeric bands with initial dimensions at 37°C of 25 mm x 10 mm x 1 mm are commonly made (for more detail on preparation see reference 3)..

Mechanical Characterizations

The most elemental mechanical characterization of an elastomer is provided by its *stress/strain curve* which is a plot of force required to extend (stress) versus the extent of elongation (strain). The slope of the curve at a particular extension divided by the cross-sectional area gives an elastic modulus also referred to as the Young's modulus. At 37°C the elastic modulus of X-PPP (where X- denotes cross-linked) varies over the range of 10^5 to 10^7 dynes/cm^2 depending on the γ-irradiation dose, with 20 Mrad giving a value of 10^6 dynes/cm^2. The elastic modulus may in addition be increased by including a repeating sequence, VAPGVG, which functions in a manner analogous to the hard segments of polyurethane (see biomedical applications below).

For purposes of elucidating mechanism of elasticity traditional characterizations of elastomers involve thermoelasticity studies in which the elastomer is stretched to a fixed extension and then the elastomeric force is monitored as a function of temperature. When this is done for 20 Mrad cross-linked polypentapeptide, X^{20}-PPP, and the data are plotted as ln[force/temperature(°K)] versus temperature as in the solid curve of Figure 1, a dramatic rise in elastomeric force occurs on going from 20° to 40°C, and above 40°C the curve is essentially flat. The rapid rise in force is the result of an inverse temperature transition, the temperature of which is dependent on the hydrophobicity of the polypeptide, and the near zero slope above 40°C reflects the dominantly entropic, or near ideal, nature of the elastomer (4).

III. Basic Physical Properties (4).

Thermomechanical Transduction

The thermoelasticity experiment discussed above follows force as a function of temperature at constant length; experiments may also be carried out by following length as a function of temperature at constant force. When the latter is done, a dramatic contraction occurs between 20° and 40°C. On attaching a fixed weight (f = mg = mass x gravitational force), raising the temperature a few degrees can cause the weight to be lifted through a substantial distance, Δl, and mechanical work ($f\Delta l$) is performed. This is most obviously thermomechanical transduction. When carried out at constant length, the extension can be set in such a way that a dramatic rise in force can occur with but a small change in temperature.

As noted above, the dramatic contraction at fixed force or steep force development at fixed length occurs as the result of an inverse temperature transition and the temperature range over which such a transition occurs is inversely dependent on the hydrophobicity of the polypeptide chain. Accordingly when the sequential polypeptide is made more hydrophobic, as in $(L\text{-}Ile^1 - L\text{-}Pro^2 - Gly^3 - L\text{-}Val^4 - Gly^5)_n$ which when cross-linked at 20 Mrad is designated as X^{20}-Ile^1-PPP, the contraction or development of elastomeric force occurs between 0° and 20°C. When the sequential polypeptide is made less hydrophobic, as in $(L\text{-}Val^1 - L\text{-}Pro^2 - Gly^3 - Gly^4)_n$, which when cross-linked can be designated as X^{20}-desVal^4-PPP or simply X^{20}-PTP

(for polytetrapeptide), the contraction or development of elastomeric force occurs between 40° and 60°C. These data are given in Figure 2. Therefore by changing the hydrophobicity the thermally induced contraction can be made to occur over any desired temperature range from subzero to 60°C. Relaxation involves a swelling process whereby the volume of X^{20}-PPP in water increases by 10 fold on going from 40° to 20°C.

The rationale for understanding the process of an inverse temperature transition resides in the hydrophobic effect demonstrated above. When there are hydrophobic side chains of polypeptides exposed to water such as the side chains of the Ile, Val and Pro residues, the water surrounding the hydrophobic side chains is more-ordered than bulk water and is called clathrate-like water. On raising the temperature through the temperature of the inverse temperature transition, the clathrate-like water destructures and becomes less-ordered bulk water as the hydrophobic side chains interact intramolecularly in the formation of a more-ordered polypeptide. The entropy of the total system, polypeptide plus water, increases with temperature as required by the second law of thermodynamics but the entropy of the polypeptide alone decreases.

Chemomechanical Transduction (Chemical Modulation of an Inverse Temperature Transition)

Polymer-Based: With the principle that contraction can occur as the result of an inverse temperature transition, which allows that changing the hydrophobicity of the sequential polypeptide can change the temperature range over which the transition occurs, an exciting capability now becomes possible. A polypeptide can be designed in which hydrophobicity can be changed by a reversible chemical process such as protonation and deprotonation (5). In the more polar (less hydrophobic) state, for example involving the carboxylate anion (COO^-), the transition would occur over a higher temperature range as in the dashed curve of Figure 1 whereas in the less polar (more hydrophobic) state, for example on shifting to a protonated carboxyl (COOH), the transition would occur over a lower temperature range as in the solid curve of Figure 1. Importantly, with awareness of this molecular biophysics, it now becomes

possible at a fixed temperature, that is at a temperature intermediate between the temperature ranges for the transitions of the less and the more polar polypeptides, to have the elastomer relaxed at high pH where the COO^- species is dominant and to cause the elastomer to contract on lowering the pH to where the COOH is the dominant species. This is chemomechanical transduction. And this new mechanism of free energy transduction, achieved by chemical modulation of an inverse temperature transition, was first demonstrated with these elastomeric polypeptides as discussed below.

By synthesis of poly[(VPGVG),(VPGEG); 4:1] where E is the glutamic acid residue, Glu, a polypeptide is obtained with 4 Glu residues per 100 residues of polypeptide which is designated as 4%-Glu-PPP. In phosphate buffered saline (PBS), which is 0.15 N NaCl and 0.01 M phosphate, this polypentapeptide coacervates in a similar temperature range as does PPP in water when the pH is 3.0. On raising the pH to 7 the temperature of the transition systematically rises to 70°C with a pK of about 4.5 (6). On cross-linking, an elastomeric band of X^{20}-4%-Glu-PPP is obtained. This biomaterial is contracted at pH 3 and is relaxed at pH 7. At constant force, this biomaterial is seen in Figure 3A to contract lifting the weight at pH 3.3 and to relax lowering the weight at pH 4.3. At constant length using an unnecessarily wide pH range in Figure 3B, it develops force as shown at pH 2.1 and turns off force at pH 7.4, and it can repeatedly raise and lower a weight 1000 times its own dry weight. As in living organisms, a change in chemical potential can bring about contraction and motion and chemical modulation of an inverse temperature transition is relevant both to protein function and to protein pathology (5).

Solvent-Based: In the preceding example of chemomechanical transduction, changes in chemical potential altered the physical properties of the polypeptide, e.g., changed the state of ionization of a side chain functional group. When the mechanism is chemical modulation of an inverse temperature transition, where there is clathrate-like water surrounding the hydrophobic side chains at temperatures below the transition, there is also a solvent-based (in addition to the above polymer-based) process whereby the temperature of an inverse

temperature transition may be shifted. In both processes the issue can be stated as the change in free energy of the solvent as compared to the change in free energy of the polymer. Thus if the clathrate-like water surrounding the hydrophobic side chains were destabilized by changes in the chemical potential of the medium, even without any change in the chemical nature of the polypeptide, then the temperature of the inverse temperature transition would be lowered. The perspective is taken that raising the salt concentration is a change in chemical potential that destablizes clathrate-like water. Ions, by tenaciously drawing water molecules into their solvation shells, shift the equilibrium of possible water structures away from that of clathrate-like water. This is the rationale whereby increases in salt concentration can lower the temperature for coacervation of PPP and lower the temperature of the inverse temperature transition of X^{20}-PPP (7). In this case there is essentially no effect of changing pH. Accordingly at the appropriate temperature, which is 25°C for X^{20}-PPP, it is possible at constant load to have X^{20}-PPP largely relaxed at 25°C, and, on changing to phosphate buffered saline (PBS), to bring about a contraction. Alternatively at constant length, raising the salt concentration causes the development of elastomeric force. The process is readily reversible as shown in Figure 4.

Electromechanical Transduction

Electromechanical transduction has not yet been demonstrated but the potential exists for a high frequency piezoelectric effect because of the development, as the result of the inverse temperature transition, of a very intense dielectric relaxation near 10 MHz. The magnitude of the dielectric increment, $\Delta\varepsilon$ on going from 20° to 40°C has been reported to be approximately 70 and it is nearly a pure Debye-type relaxation with a distribution of frequencies, α, of less than 0.1 (8). Therefore X^{20}-PPP exhibits both the dielectric coefficient and the elastic coefficient required for a piezoelectric effect (9). The expectation is that the application of an electric field oscillating at a frequency below 10 MHz would cause an extended elastomer to contract and to develop force.

IV. Industrial Applications

The durability of the biological elastic protein, elastin which contains the polypentapeptide $(VPGVG)_n$ as the most striking primary structural feature, is remarkable. The half-life of this protein in the body is 50 to 60 years. This means in the aortic arch and the descending thoracic aorta where there is twice as much elastin as collagen that, by the age of 60 years, individual elastin fibers will have survived some two billion stretch/relaxation cycles. This durability is due to the dominantly entropic nature of the elasticity, that is, resistance to extension and the development of a restoring force on extension (elastic recoil) is not the result of the straining of bonds that could lead to increased probability of bond breakage but rather is due to the decrease in the number of equivalent energy states accessible to the fiber on extension. The increase in free energy due to extension is not due to an increase in internal energy but rather it is due to a decrease in entropy. This remarkable potential for durability is a worthwhile point to keep in mind when considering industrial applications of these and related elastomeric polypeptides.

In considering industrial and biomedi al applications of this new class of biomaterials, the industrial applications will be briefly noted first as these relate more directly to the fundamental physical properties of these elastomeric sequential polypeptides.

Sensors

Discussed above were four basic processes: thermal, chemical, mechanical and electrical(oscillatory). Within limits of experimental conditions all four processes are reversible, and depending on experimental conditions any pair or all may be operative in the transduction process utilized in a particular sensor. For these polypeptides the usable temperature range is from below 0°C (to the extent that freezing point depression is achievable without seriously affecting elastic properties) to about 60°C, above which temperature thermal denaturation occurs. The chemical range depends on the chemical function introduced with the PPP or the salt concentration range over which significant shifts in transition temperature are achieved. The mechanical range involves forces resulting from weights in the range of 1 to

1000 times the dry weight of the elastomer and can utilize length changes up to a factor of about 2. The electrical range requires high frequency oscillating electric fields at strengths that have yet to be defined experimentally.

When operating within the limits of the above ranges, the four reversible processes allow for six classes of transducers to be designed. The application for which the sensor would be utilized determines the conditions. If for example it is a change in chemical potential, e.g., a change in pH, salt concentration, oxidizing or reducing equivalents, etc., that is to be monitored, then in the absence of an oscillating electric field and at constant temperature, an appropriate chemomechanical transducer would be designed. Obviously there are a great number of applications that might be considered, for example as sensing elements for controlling pH, ionic strength, temperature, oxidative or reductive potential (singly or in combination). A particularly intriguing example follows.

Reversible Mechanochemical Engines

Katchalsky and coworkers (10) some years ago designed a mechanochemical engine with rotary motion which utilized a belt of collagen that could be made to contract on introduction into a medium of high salt concentration. The effect of the high salt concentration was to lower the transition temperature for thermal denaturation. As might be required for the relatively high heats of denaturation, the concentration gradients that were required were large; and the two baths that were used were 11.25 N LiBr and 0 or 0.3 M LiBr. In our case, presumably because the heats of coacervation are small, ~1cal/gram, relatively small concentration gradients can be effective in bringing about contractions as shown in Figure 4 where the concentrations were 0.15 N and 0 N NaCl. Significant also is that sodium chloride is an effective salt. One possible application of such an engine, other than the obvious uses of an empowered rotating shaft, would be to drive the shaft in reverse and thereby to achieve desalination, i.e., to transfer salt up a concentration gradient.

Desalination: The physical basis for desalination may be stated as follows: When X^{20}-PPP is stretched, hydrophobic groups become exposed; the elastomer takes up water in an

exothermic reaction as the exposed hydrophobic groups become surrounded with clathrate-like water. This has analogy to the swelling that occurs on lowering the temperature from 40° to 20°C where there is a 10 fold increase in volume for X^{20}-PPP. The more hydrophobicity expressed by a polypeptide, the less favorable is the situation for ions. This is demonstrated by the raised pK_a of the Glu side-chain for the more hydrophobic X^{20}-4%-Glu-Ile^1-PPP and by the raised pK_a on stretching X^{20}-4%-Glu-PPP. In both of these cases the increased expression of hydrophobicity results in the free energy for formation of the carboxylate anion being less favorable. Therefore stretching X^{20}-PPP in a high salt solution results in the uptake of solution into the elastomer which is low in ions; the excess solution which is drained off while X^{20}-PPP is stretched, is at a higher salt concentration. When the fiber is relaxed it gives off a solution which is at a lower salt concentration. By appropriately repeating this process with directed solution flows, the result would be the splitting of a salt solution into two solutions one higher and the other lower in salt concentration.

For a belt comprised of elastomeric polypeptide in which the mechanism of contraction involves the chemical modulation of an inverse temperature transition, however, there are many more processes that can be used to drive the engine. Any reversible chemical process that would make chemical moieties attached to the polypeptide chain either more or less polar could be used to drive the mechanochemical engine or when driven externally in reverse could be used in a preparative manner (i.e., to prepare the chemical species which caused contraction). Conceivably the chemical to be prepared could be the reduced species of a redox couple or, as in the pH driven mechanochemical engine, the couple is protonation/deprotonation or the couple could be phosphorylation/dephosphorylation. Driving the system in reverse by stretching could result in the pumping of protons or electrons against their concentration or electrical potential gradients.

In general when contraction is achieved in a reversible manner, as it can be in the chemical modulation of the inverse temperature transition of our elastomeric polypeptides, then stretching becomes the free energy input with which to reverse the chemical process. The

reversed chemical process can be a means whereby a chemical moiety attached to the elastomeric polypeptide is made less polar (relatively more hydrophobic) or whereby the free energy of the clathrate-like water is increased.

V. Biomedical Applications

In man elastic protein is essential in arteries, skin, ligaments, lung and muscle. In the former three, synthetic replacements and preferably transition biomaterials would be of great value. As for the latter, muscle is a mechanochemically coupled macromolecular machine and such a machine was first demonstrated in a polypeptide or a model protein by the new class of biomaterials under review here (as shown in Figure 3). While there are many potential biomedical applications of elastomeric polypeptides, only two will be briefly considered here. These are the development of synthetic arteries and a material to prevent adhesions following surgery and other trauma. Before proceding, however, the issue of biocompatibility needs to be addressed. Studies, as yet limited, have been carried out which show X^{20}-PPP to be biocompatible in both soft tissue and hard tissue sites in rabbits and show PPP to be innocuous in cell cultures of human fibroblasts.

Synthetic Vascular Materials

In the design of elastomeric polypeptide vascular materials, there are a number of aspects to be considered. 1) The compliance of the synthetic vessel is to match the vessel that it replaces within the normal dynamic operational range. This means having the appropriate elastic modulus in the relevant extension ranges, exhibiting minimal hysteresis in the stress/strain curves, and being a material with low fatiguing characteristics. 2) The consideration of synthetic vessel wall layering for example with an intimal layer specialized for endothelial cells, a medial layer designed for smooth muscle cells and, when of interest, an adventitial layer which would provide for covalent attachment to the surrounding extracellular matrix (as shown in Figure 5). 3) The inclusion within the polypeptide sequence of cell attachment sequences specialized for example in an intimal layer for endothelial cells and in a medial layer for smooth muscle cells. 4) The inclusion within the particular layer of diffusible

chemotactic peptide sequences for the vascular wall cell of interest and 5) the material needs to be examined for its blood-materials interactions.

The general approach is to use the elastomeric polypentapeptide, (Val-Pro-Gly-Val-Gly)$_n$ as the fundamental matrix which is then to be modified in particular ways to add desired properties. The polyhexapeptide, (Val-Ala-Pro-Gly-Val-Gly-Val)$_n$, is to be added either in parallel or in sequence to increase strength, elastic modulus and ease of handling. Cell attachment sequences for vascular wall cells, whether found in fibronectin, laminin, collagen or elastin, are to be added covalently in sequence to provide for the desired cell adhesion. When of interest as in the adventitial layer, lysine could be added occasionally in position four to provide for covalent cross-linking to the extracellular matrix. And chemotatic peptides can be added to the low temperature, swollen matrix such that on raising the temperature, they become incorporated within the matrix at a concentration appropriate to allow diffusion outward to provide the appropriate concentration gradient and chemotatic peptides can be included in the primary structure which would lead to their release during biodegradation. A synthetic vessel comprised of polyhexapeptide and polypentapeptide in a 1:6 ratio is shown in Figure 6.

With the design of the synthetic vascular wall being to induce vascular cell migration into and attachment to the synthetic vascular biomaterial, it is central to note that vascular wall cells respond to the cyclic stretching of the matrix to which there is a attachment by synthesizing new vascular wall. With the slow biodegradation of the synthetic elastomeric vascular wall, the intended result is regeneration of natural arterial wall.

Prevention of Adhesions

Adhesions accompanying the healing of wounds, whether due to surgery or other trauma, are well known detrimental sequelae. Peritoneal cavity adhesions leading to intestinal obstruction and necessitating recurring operations, the added difficulties that adhesions themselves present during a recurrent operation, tendon adhesions considered to be factors foremost compromising tendon surgery and repair, etc., are among many of the serious problems presented by adhesions. Clearly desirable would be a material that could function as

an insulator isolating wound repair sites; it should be a material that would match the compliance of the soft tissue site of application; it should be biodegradable and hence disappear after adhesion-free repair is complete; it should be obtainable in different states, e.g. elastomeric sheets or foams, that would provide sufficient ease of handling for each particular application; it should be readily sterilizable; and it should be biocompatible eliciting insignificant immunogenic and antigenic response in the host. Such a material could be expected to result in a more salutary wound healing process.

Having potential for the desirable properties in the control of adhesions are the elastomeric polypeptide biomaterials addressed here. Under physiological conditions the biomaterial is comprised of about 40% peptide and 60% water and the biomaterial is elastomeric with an adjustable elastic modulus. It is biodegradable, biocompatible and readily sterilizable, and it can be formed in cross-linked sheets or strips varying from a gelatinous to a teflon-like consistency and it should be possible to make it in a deformable foam-like state with or without cross-linking. The elastomeric polypeptide biomaterial of particular interest in the prevention of adhesions would be comprised of repeating peptide sequences that occur naturally within the elastic fiber of biological connective tissue. The sequential polypeptides are as above, $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ and $(Val\text{-}Ala\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$. The polypentapeptide has been shown to be biocompatible and biodegradable as a soft tissue implant and, also as a natural component of the connective tissue, a similar result is expected for the polyhexapeptide. The material is sterilizable since it withstands autoclaving conditions and primarily because cross-linking to form elastomeric matrices is characteristically achieved by γ irradiation at 10-20 Mrad. It is the appropriate combination of polypentapeptide and polyhexapeptide that allows the elastic modulus (compliance) to be varied and the material consistency to be changed from gelatinous to teflon-like.

Acknowledgement:

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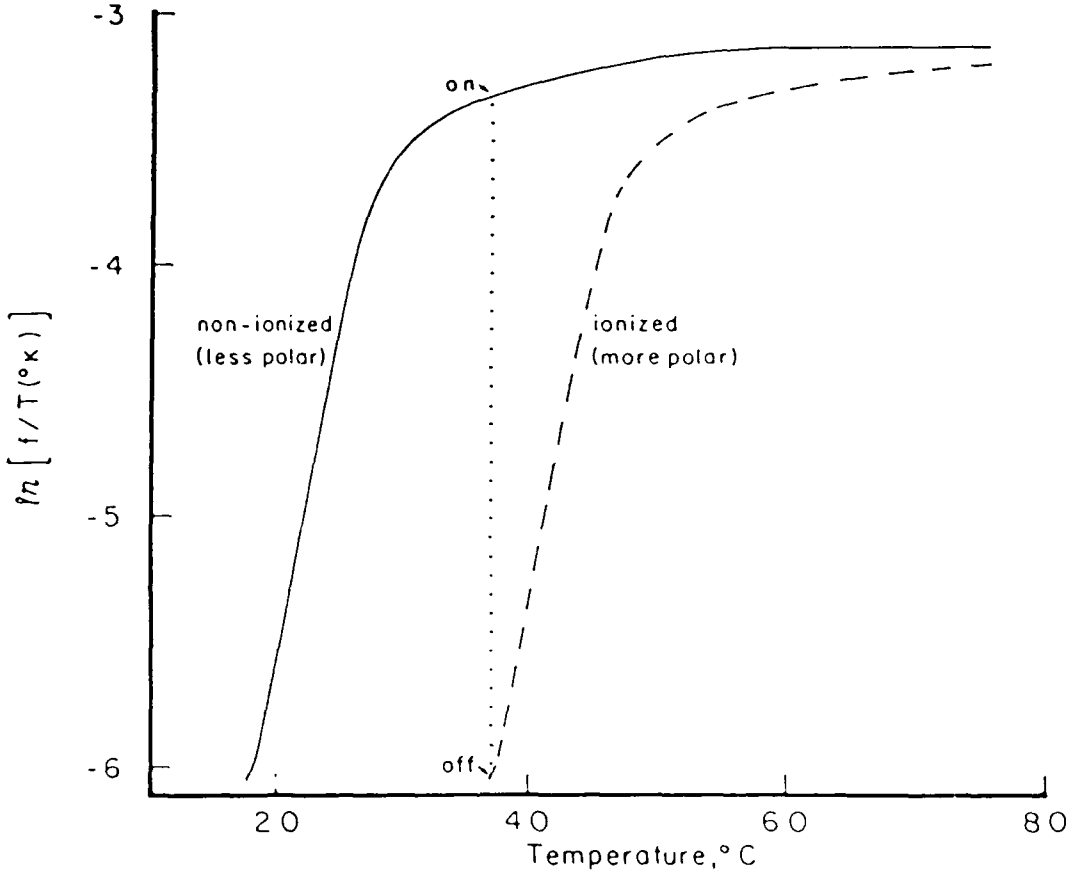
-3
-4
-5
-6
ln [f / T (°K)]
on
off
non-ionized
(less polar)
ionized
(more polar)
20
40
60
80
Temperature, °C

FIGURE 1

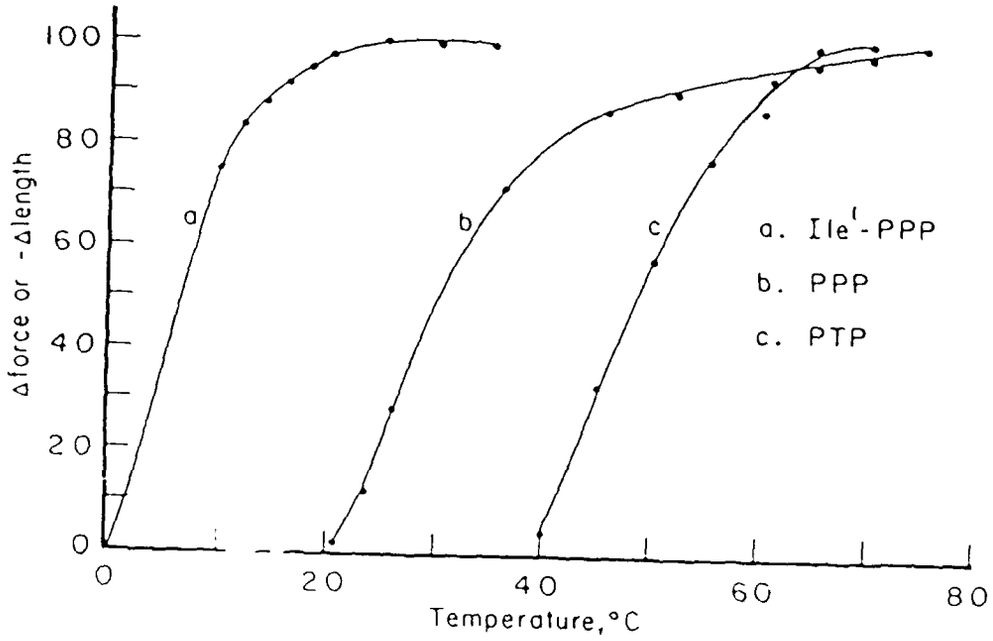
100
80
60
40
20
0
Δforce or -Δlength
0
20
40
60
80
Temperature, °C
a
b
c
a. Ile¹-PPP
b. PPP
c. PTP

FIGURE 2

Mechanochemical Coupling in Cross-linked 4% Glu-Polypentapeptide
due to Changes in pH at 37°C

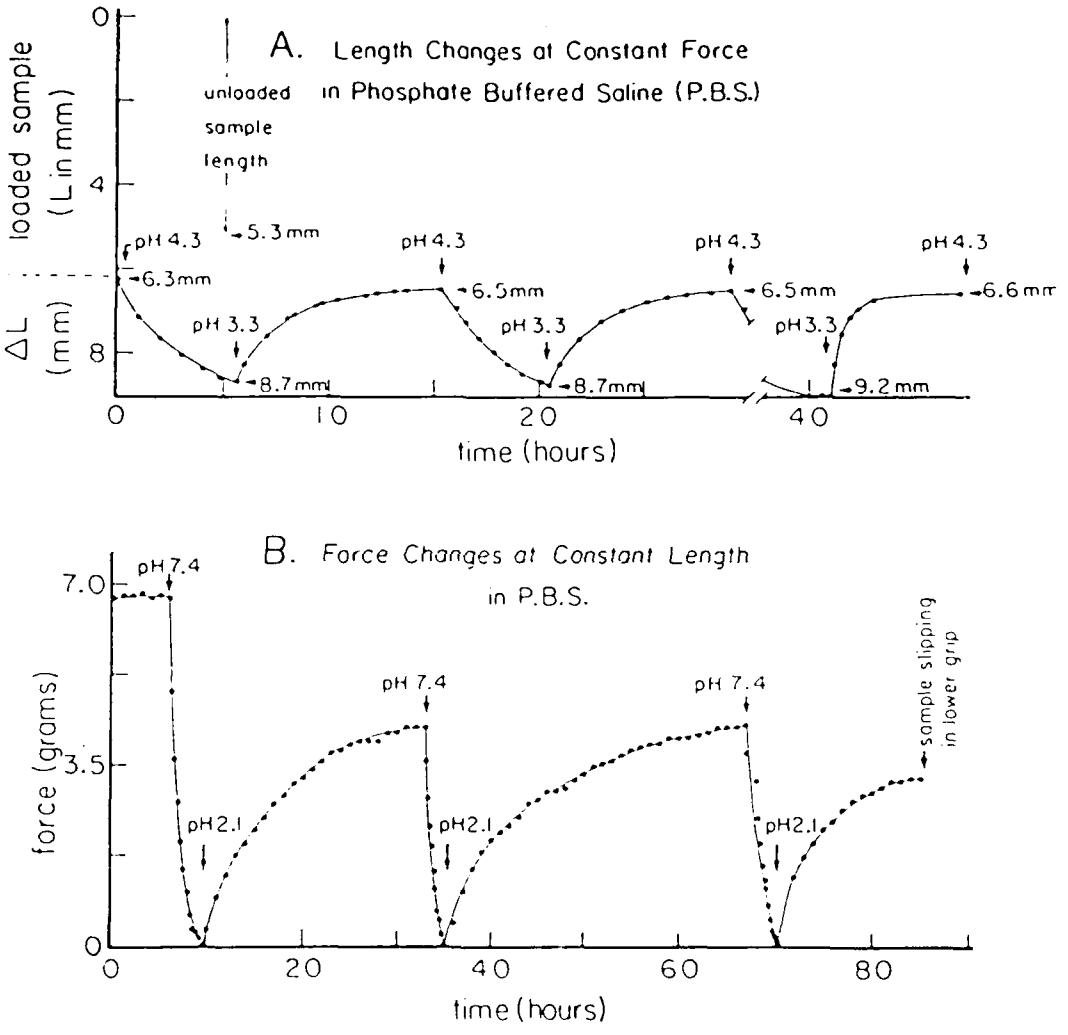

FIGURE 3

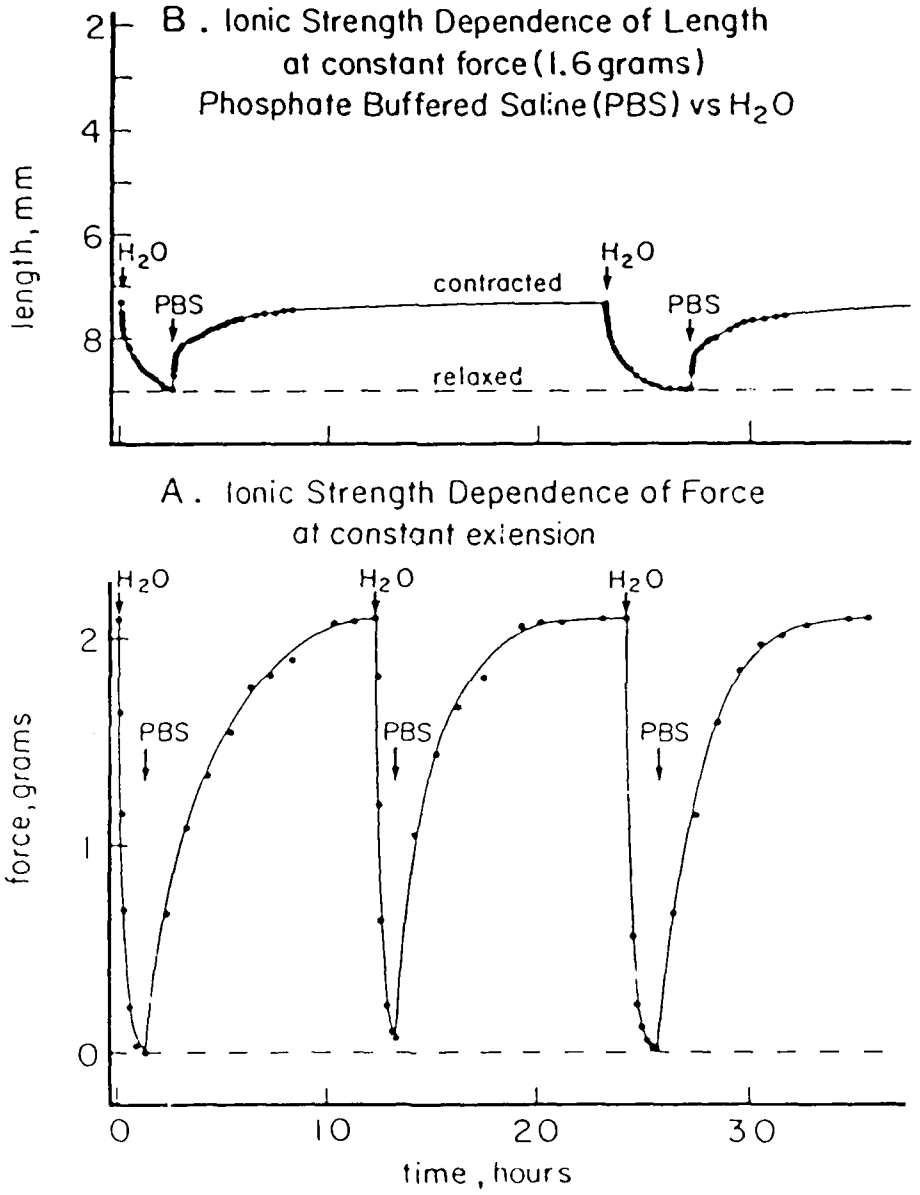
B . Ionic Strength Dependence of Length
at constant force (1.6 grams)
Phosphate Buffered Saline (PBS) vs H_2O
length, mm
2
4
6
8
H_2O
PBS
contracted
H_2O
PBS
relaxed
A . Ionic Strength Dependence of Force
at constant extension
force, grams
2
1
0
H_2O
H_2O
H_2O
PBS
PBS
PBS
0
10
20
30
time , hours

FIGURE 4

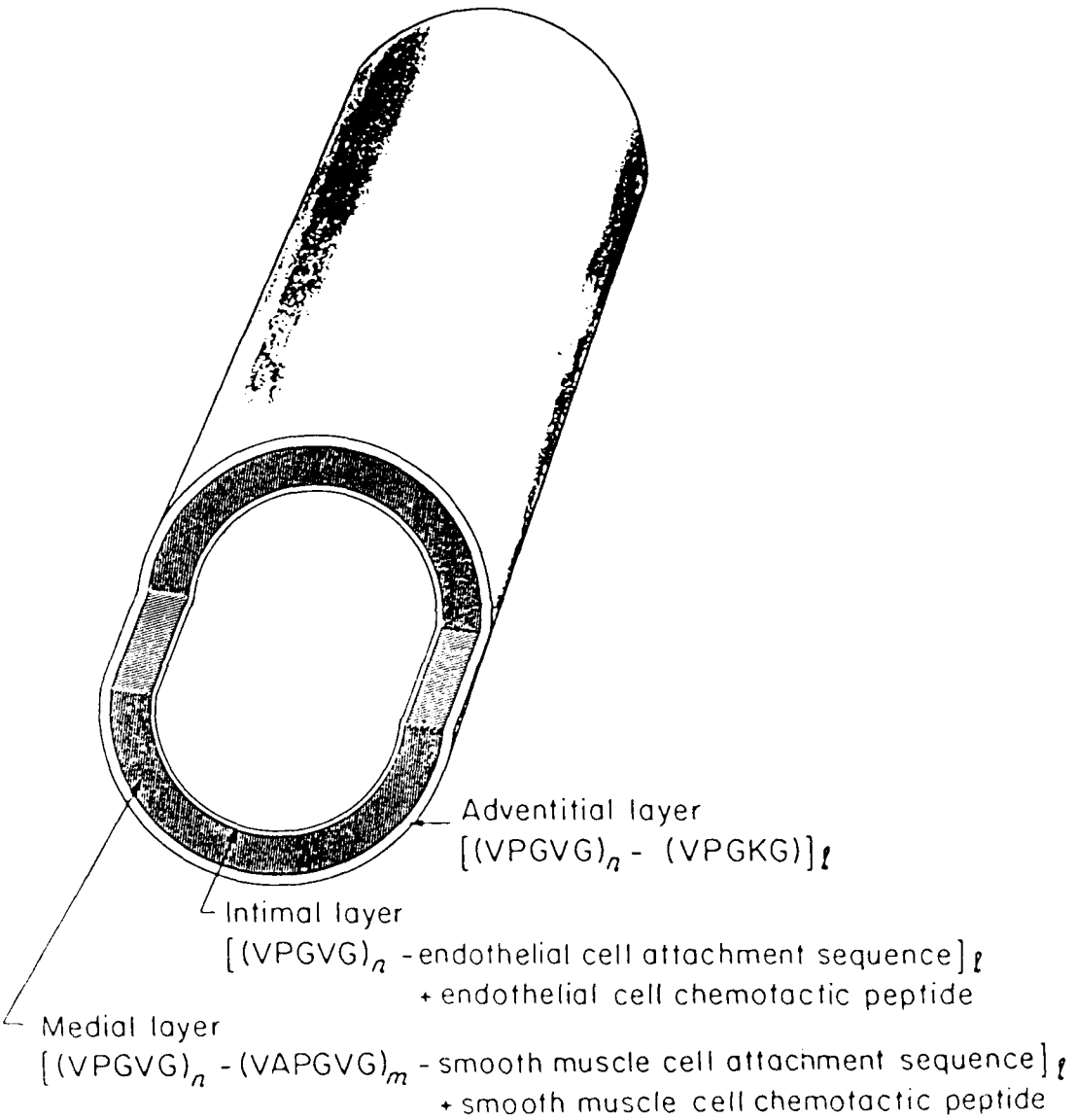
Adventitial layer
[(VPGVG)$_n$ - (VPGKG)]$_\ell$
Intimal layer
[(VPGVG)$_n$ - endothelial cell attachment sequence]$_\ell$
+ endothelial cell chemotactic peptide
Medial layer
[(VPGVG)$_n$ - (VAPGVG)$_m$ - smooth muscle cell attachment sequence]$_\ell$
+ smooth muscle cell chemotactic peptide

FIGURE 5

X20 - (PHP:PPP ' 6)

FIGURE 6

Reprinted from The Journal of Physical Chemistry, 1988, 92, 511.

Dielectric Relaxation Studies on Analogues of the Polypentapeptide of Elastin

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Dielectric measurements of the complex permittivity of coacervate concentrations of two analogues of the polypentapeptide of elastin, $(Xxx^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, where Xxx is Val for the elastin polypentapeptide and Ile and Leu for the two analogues, were taken over the frequency range 1–1000 MHz and over the temperature range 0–60 °C. Two relaxation processes were observed in each polypentapeptide. One relaxation has a frequency centered in the low megahertz frequency range, which has been attributed to a low-frequency librational mode within the polypeptide. The other relaxation is located near the gigahertz frequency range. The magnitude of the dielectric increment, $\Delta\epsilon$, of the librational mode of each polypentapeptide analogue increases with increasing temperature from near zero at 0 °C to approximately 40 at 60 °C, showing an inverse temperature transition to a more ordered structure. Conversely, the magnitudes of the dielectric increment of the high-frequency relaxation decrease with increasing temperature and differ in approximate proportion to the hydrophobicity of the pentamer for the polypentapeptide of elastin and the two analogues at temperatures below the inverse temperature transition. It is suggested that clathrate-like water surrounding hydrophobic side chains contributes to the high-frequency relaxation.

Introduction

Fibrous elastin from aorta occurs at 5–6-μm diameter fibers.[1] These fibers are comprised of a single protein, which as the soluble precursor is called tropoelastin.[2,3] A key development in deriving the molecular mechanism of biological elasticity was the finding by Sandberg and colleagues of repeating peptide sequences. The most striking repeating sequence is the polypentapeptide $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, where n is 11 or greater.[4] It has been demonstrated that this polypentapeptide of elastin, also referred to as $(VPGVG)_n$ or PPP, on increasing the temperature in water undergoes an inverse temperature transition with the proposed development of a dynamic β-spiral conformation within which occurs a $Val^4\text{-}Gly^5\text{-}Val^1$ suspended segment capable of large-amplitude, low-frequency rocking motions (for a review see ref 5). The repeating pentamer in an unrolled perspective of the β-spiral is given in Figure 1

Previous relaxation studies on the polypentapeptide of elastin[6] and on α-elastin,[7] a 70000-D chemical fragmentation product from elastin,[8] have demonstrated a single Debye-type relaxation with a relaxation time of about 7 ns at 40 °C for the polypenta; eptide and about 8 ns at 40 °C for α-elastin. This relaxation has been attributed to the internal dynamics of the polypentapeptide, primarily arising from the rocking motion of peptide moieties in the suspended segment $Val^4\text{-}Gly^5\text{-}Val^1$.[6,9]

Below the temperature of the transition for the polypentapeptide, which is centered at 31 °C and begins at 24 °C, the polypentapeptide of elastin is soluble in water in all proportions. Above the temperature of the transition at concentrations less than 37% peptide by weight, two phases are observed. One is due to the more dense, viscoelastic coacervate state of the polypentapeptide, and the other is due to the transparent equilibrium solution. The polypentapeptide coacervate is a two-component system which contains by weight 63% water and 37% polypentapeptide at 30 °C.[10]

Dielectric measurements have been proven to be useful for studying water in protein. A sound discussion of this method is found in several books.[11,12] Recently, the hydration of lipoprotein,[13] protein,[14] ocular tissue,[15] brain tissue,[16,17] hemoglobin,[18] polyadenine,[19] and Na–DNA gels[20]) has been studied by dielectric relaxation measurements. (For earlier work, see ref 11 and 12.) One of the challenges of dielectric measurements is to determine

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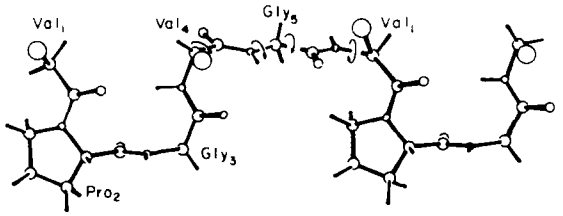

Figure 1. Unrolled perspective of the molecular structure of the β-spiral of the polypentapeptide of elastin. Reproduced with permission from *Ultrastruct. Pathol.* **1985**, *4*, 227–251.

the contributions to the so-called δ dispersion, which occurs in the range 0.1–3 GHz. This relaxation could be due to rocking motions of polar side chains or to reorientation of protein-bound water molecules. Of particular interest are the different kinds of water which could contribute in different ways to the dielectric properties and to the δ dispersion.[18] In a water–protein system, possible kinds of water would be water hydrogen bonded to dipolar and to charged groups in protein, bulk water, and (as discussed below) clathrate-like water surrounding hydrophobic side chains of the protein.

In order to examine further the rocking motion of the polypentapeptide moieties and the interaction of water within the polypentapeptide in relation to the inverse temperature transition, we have determined for comparison with PPP the dielectric properties over a frequency range 1 MHz to 1 GHz and a temperature range 0–60 °C of the two polypentapeptides: (Leu[1]-Pro[2]-Gly[3]-Val[4]-Gly[5])$_n$ and (Ile[1]-Pro[2]-Gly[3]-Val[4]-Gly[5])$_n$ also referred to as (LPGVG)$_n$ or Leu[1]-PPP and (IPGVG)$_n$ or Ile[1]-PPP, respectively.

Methods

Preparation of the Polypentapeptides. The preparation of Ile[1]-PPP was reported earlier.[22] The synthesis of Leu[1]-PPP was carried out by the classical solution methods. The polymer (LPGVG)$_n$ was synthesized by using the two monomer permutations LPGVG and GVGLP. As reported previously,[23] the sequence with Pro as the C-terminal amino acid for activation gave a higher molecular weight polymer, and the details of the synthesis of this approach (Scheme I of ref 23) will be discussed here.

As an overview, Boc-GVG-OH (III), prepared by the mixed anhydride (MA) method, was coupled with H-LP-OBzl (II) also prepared by the MA method.[24] In order to suppress the urethane byproduct formation, 1-hydroxybenzotriazole (HOBt) was added during the MA reaction.[25] Boc-GVGLP-OBzl (IV) was hydrogenated to the free acid (V) and was converted to *p*-nitrophenyl ester (ONp) by using bis(*p*-nitrophenyl) carbonate.[26] After the Boc group was removed, the peptide–ONp was polymerized in dimethyl sulfoxide (DMSO) in the presence of *N*-methylmorpholine (NMM) for 20 days. The polypeptide was taken into water and dialyzed against water by using 50-kD molecular weight cutoff dialysis tubing and lyophilized. The purity of the intermediate and final products was checked by carbon-13 nuclear magnetic resonance spectroscopy, thin-layer chromatography (TLC), and elemental analysis.

Elemental analyses were carried out by Mic Anal, Tucson, AZ. Melting points were determined with a Thomas Hoover melting point apparatus and are uncorrected. TLC was performed on silica gel plates obtained from Whatman Inc., Clifton, NJ, with the following solvent systems: (R_f^1) ethyl acetate/acetic acid/ethanol (90:10:10); (R_f^2) chloroform/methanol/acetic acid (95:5:3); (R_f^3) chloroform/methanol (5:1); (R_f^4) chloroform/methanol/acetic acid (85:15:3). Boc amino acids were purchased from Bachem Inc., Torrance, CA. HOBt was obtained from Aldrich Chemical Co., Milwaukee, WI. 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide (EDCI) was obtained from Sigma Chemical Co., St. Louis, MO. All amino acids are of L configuration except for glycine.

Boc-Leu-Pro-Obzl (I). Boc-Leu-OH (18.69 g, 75 mmol) in dimethylformamide (DMF; 75 mL) was cooled to 0 °C, and NMM (8.25 mL) was added. The solution was further cooled to −15 °C, isobutyl chloroformate (IBCF) (9.72 mL) was added drop by drop while the temperature was maintained, and the mixture was stirred for 10 min at which time HOBt (11.48 g, 75 mmol) was added and the stirring continued for 10 min more. A precooled solution of HCl-H-Pro-OBzl (18.12 g) in DMF (65 mL) and NMM (8.25 mL) was added to the above reaction mixture. After about 20 min an additional 1 equiv of NMM was added, and the completeness of the reaction was followed by TLC. The pH of the solution was adjusted to 8 with saturated $KHCO_3$ solution, the mixture was stirred for 30 min and poured into 90% saturated NaCl solution, and the product was extracted into $CHCl_3$. After the solvent was removed, 25.5 g of I was obtained as an oil: yield 81%; R_f^1 0.96, R_f^2 0.46, R_f^3 0.95. Anal. Calcd for $C_{23}H_{34}N_2O_5$: C, 66.0; H, 8.19; N, 6.69. Found: C, 65.87; H, 8.48; N, 6.64.

Boc-Gly-Val-Gly-Leu-Pro-OBzl (IV). Boc-GVG-OH (III) (5.41 g, 16.3 mmol) and HOBt (2.75 g, 18 mmol) in DMF (50 mL) were cooled to 0 °C, EDCI (3.44 g, 18 mmol) was added, and the mixture was stirred for 20 min. To this a precooled solution of H-LP-OBzl (II) (5.8 g, 16.3 mmol), obtained by deblocking I with HCl/dioxane, and NMM (1.8 mL) in DMF (30 mL) was added, and the mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure. The residue was taken in $CHCl_3$ and extracted with acid and base. After the solvent was removed the peptide was precipitated by adding EtOAc, filtered, washed with EtOAc, and dried to obtain 8.2 g of the product: yield 79%; mp 132–134 °C; R_f^1 0.75, R_f^2 0.2. Anal. Calcd for $C_{32}H_{49}N_5O_8$: C, 60.83; H, 7.82; N, 11.08. Found: C, 60.35; H, 7.97; N, 10.85.

Boc-Gly-Val-Gly-Leu-Pro-OH (V). IV (7 g, 11 mmol) in glacial acetic acid (70 mL) was hydrogenated in the presence of 10% Pd/C (0.8 g). The catalyst was filtered with the aid of Celite and solvent removed under reduced pressure. The precipitate obtained by the addition of EtOAc was filtered, washed with EtOAc and petroleum ether, and dried to give 5 g of V: yield 83.3%; mp 130–136 °C dec; R_f^3 0.2, R_f^4 0.41. Anal. Calcd for $C_{25}H_{43}N_5O_8 \cdot {}^1/_2H_2O$: C, 54.52; H, 8.05; N, 12.71. Found: C, 54.49; H, 8.38; N, 12.3.

Boc-Gly-Val-Gly-Leu-Pro-ONp (VI). V (4.5 g, 8.3 mmol) in pyridine (35 mL) was reacted with bis(*p*-nitrophenyl) carbonate (BNPC) (3.8 g, 12.46 mmol) for several days while the progress of the reaction was followed by TLC. Two additional 0.5 equiv of BNPC were added during that time. After pyridine was removed, the peptide was precipitated by added ether. VI was filtered, washed with ether, dilute citric acid, and water, and dried to obtain 4.25 g: yield 77.3%; mp 122–124 °C dec; R_f^2 0.21, R_f^4 0.7. Anal. Calcd for $C_{31}H_{46}N_6O_{10}$: C, 56.18; H, 6.99; N, 12.68. Found: C, 55.79; H, 7.02; N, 12.48.

H-(Gly-Val-Gly-Leu-Pro)$_n$-OH (VIII). The Boc group was removed from VI (3.5 g, 5.28 mmol) by treatment with trifluoroacetic acid (TFA, 30 mL) for 30 min. TFA was removed under reduced pressure, triturated with ether, filtered, washed with ether, and dried to give 3.5 g of VII. The TFA salt (VII) (3.5 g, 5.17 mmol) in DMSO (5.2 mL) was stirred for 20 days in the presence of NMM (0.91 mL, 8.3 mmol). The reaction mixture was diluted with water and dialyzed against water by using 50-kD cutoff dialysis tubing, changing the water daily for 15 days. The retentate was lyophilized to obtain 1.8 g of Leu[1]-PPP (yield 79.3%). In order to remove any unreacted ONP groups present on different chains of polymer, VIII was treated with base, neutralized, dialyzed, and relyophilized.

Dielectric Relaxation Measurements. The dielectric relaxation studies were carried out by means of a coaxial line cell/vector

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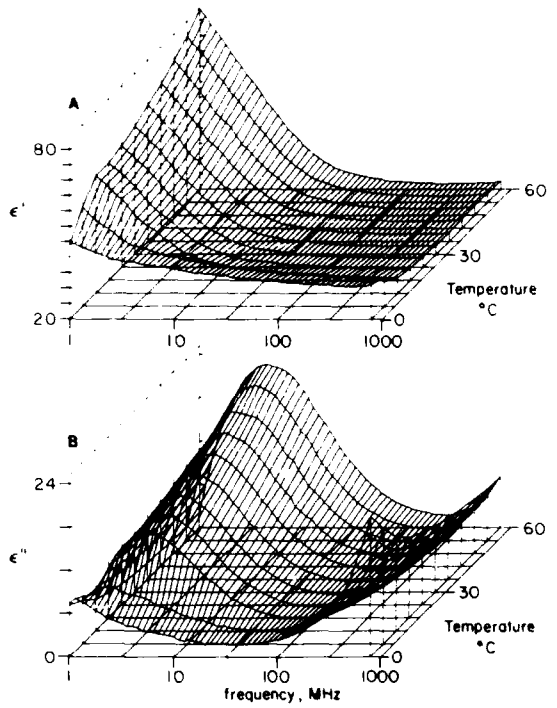

Figure 2. Real part ϵ' (A) and the imaginary part ϵ'' (B) of the dielectric permittivity of the Ile^1-PPP over the frequency range 1–1000 MHz and over a temperature range 0–60 °C. From the imaginary part of the dielectric permittivity the conductivity term has been subtracted, which represents mainly the dielectric property of the free ions present in water. The conductivity values used are listed in Table I.

analyzer method using the Polarad Model ZPV vector analyzer with the Model E2 tuner. The signal generator used was the Wavetek Model 3510, covering the frequency range 1–1040 MHz. Both instruments were controlled by a Tektronix Model 4054 computer graphics system using an IEEE-488 Bus. The dielectric cell and the method are described elsewhere.[27,28] The temperature was controlled by a refrigerated circulating bath, Neslab Endocal RTE-5DD, and monitored by a thermocouple placed in contact with the cell using an Omega Model 410A.

The polypeptide sample was dissolved in water, coacervated at 40 °C in an injection syringe, and allowed to stand for several days at 40 °C to complete the phase separation. The coacervate concentration was carefully injected from below into the cell at a temperature below that required for the onset of coacervation where the viscosity is less. The sample was allowed to equilibrate overnight at 60 °C, a temperature above the transition temperature. Then the cell temperature was decreased to 0 °C in the period of 1 h. The cell temperature was raised in 6 °C steps from 0 to 60 °C. After each temperature was reached, the sample was allowed to equilibrate for 30 min before data collection began. The polypentapeptide of elastin, $(VPGVG)_n$ or PPP, and its two analogues, $(I.PGVG)_n$ or Leu^1-PPP and $(IPGVG)_n$ or Ile^1-PPP, were studied.

Results

Figure 2 shows the real part (at the top) and the imaginary part (at the bottom) of the dielectric permittivity of the Ile^1-PPP over the frequency range 1–1000 MHz and over a temperature range 0–60 °C. At each temperature, 61 logarithmic scale data points were collected for the frequency range. For the three-dimensional plots, straight line segments connected each pair of experimental data points.

Clearly, two relaxation processes can be observed. As can be seen in Figure 2, the first one is seen as an increasing relaxation process near 5 MHz when raising the temperature, and the second one shows a decreasing relaxation process within the gigahertz range when raising the temperature. Qualitatively similar results have been obtained for PPP and α-elastin.[6,7] It was suggested for PPP and α-elastin that the first relaxation is intrinsic to the polypentapeptide. More precisely, this relaxation was assigned to a peptide librational process arising primarily from the recurring suspended segment Val^4-Gly^5-Val^1 within the polypentapeptide. This segment, more accurately defined from the Val^4 α-carbon to the Val^1 α-carbon, is capable of low-frequency rocking motion while the other segment Val^1-Pro^2-Gly^3-Val^4, from the Val^1 α-carbon to the Val^4 α-carbon, is less flexible (see Figure 1). This latter segment of polypentapeptide has a β-turn conformation involving a Val^1 C=O···H—N Val^4 hydrogen bond. The substitution of Val^1 of PPP by Ile (Ile^1-PPP) does not modify this hydrogen bond and retains β-branching in the side chain. Thus the conformation is expected to be the same for PPP and for Ile^1-PPP. Circular dichroism studies, in fact, show identical spectra before and after the transition.[22]

While not altering conformation, this substitution of Ile^1, however, has an important effect on the temperature of the phase transition. The phase transition temperature of the PPP coacervate is centered near 30 °C while the phase transition temperature of Ile^1-PPP is centered near 10 °C.[22,29] As Ile is more hydrophobic than Val,[30,31] the phase transition temperature for coacervation therefore is inversely related to the hydrophobicity of the pentamer side chains.[22,29] In this perspective, the dielectric behavior of the PPP analogues provides an interesting opportunity to examine the interaction of water within these polypentapeptides. This is particularly the case because of the unusually high pentapeptide to water ratio, which provides that a greater fraction of the total water will be the unique water surrounding the hydrophobic side chains.

The second relaxation process (located within the gigahertz range) was previously attributed to water (see Figure 2). These two relaxation processes behave inversely with the temperature. Without polar side chains in the pentamers only two species, water and backbone peptide moieties, could contribute to the dielectric permittivity changes. One may wonder if other conformational states of the Ile^1-PPP could contribute to this second process. This relaxation is observed below the phase transition, where the polymer is less ordered and the pentamers would exhibit a dispersity of conformational states with a resulting broad range of relaxation times. The polypeptide therefore is not a good candidate for the relatively intense relaxation near 1 GHz. Thus it is necessary to consider if the high-frequency relaxation process may be due to water.

In this system, at least three kinds of water are present. Free or bulk water which is not bonded to the polypeptide has a relaxation process located at 25 GHz.[11] Another kind should be water hydrogen bonded to the polar groups of the polypeptide backbone, and yet another kind, as suggested by Urry et al.[22,29] should be due to clathrate-like water associated with the hydrophobic side chains of these polypeptides which undergo inverse temperature transitions. In this regard it is now useful to consider another pentamer with yet a different hydrophobicity. Figure 3 shows the real and imaginary part of the dielectric permittivities as a function of temperature for Leu^1-PPP. The results are very similar to Ile^1-PPP as presented in Figure 2. Leu^1-PPP also exhibits two relaxation processes which can be considered in the same way as previously done for Ile^1-PPP. The phase transition for Leu^1-PPP is centered at 15 °C, intermediate between that of Ile^1-PPP and PPP, and the substitution of Leu instead of Val should have little effect on the Leu^1 C–O···H–N Val^4 hydrogen bond. Thus the β-spiral conformation should be the same for

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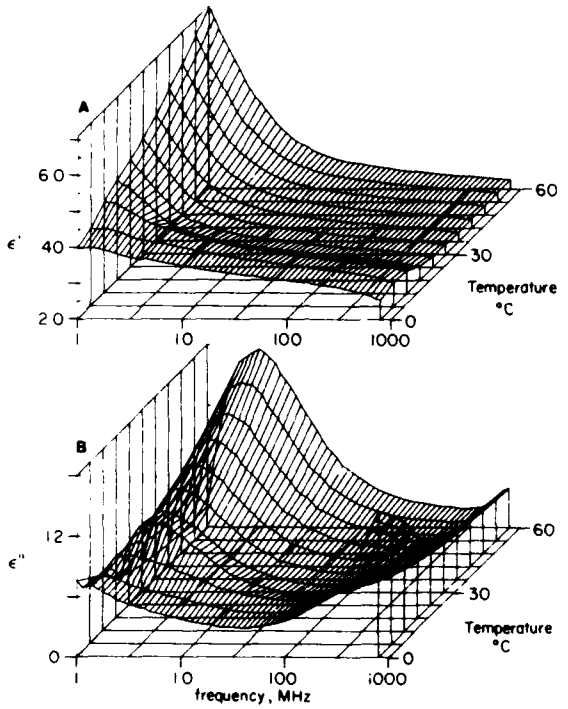

Figure 3. Real part ϵ' (A) and the imaginary part ϵ'' (B) of the dielectric permittivity of the Leu[1]-PPP over the frequency range 1–1000 MHz and over a temperature range 0–60 °C. From the imaginary part of the dielectric permittivity the conductivity term has been subtracted, which represents mainly the dielectric property of the free ions in water. The conductivity values used are listed in Table II.

Leu[1]-PPP and for PPP. The PPP results, previously reported[6] and not shown in this paper, are very similar to those of Leu[1]-PPP and Ile[1]-PPP.

A more detailed comparison of the dielectric results of the analogues shows interesting features concerning the water–polypeptide interaction which further demonstrate the origins of the near-gigahertz relaxation.

Figure 4 gives the temperature dependence of the imaginary part of the permittivity at 794 MHz for the polypeptides studied in this paper. For comparison, the temperature dependence of the permittivity (imaginary part) of pure water is shown. One can see very well the accentuated drop of the dielectric loss as a function of temperature for the polypeptides. The origin of this dielectric loss can be further considered. Free water contributes little to the dielectric loss within the polypeptide–water system at 1 GHz. It is possible that the water hydrogen bonded to the polar groups of the polypeptide backbone could give a contribution to the dielectric loss. However, another fact should be considered. Raman spectroscopy has shown that the β-turn in the polypentapeptide (with hydrogen bond) is relatively unchanged over the temperature range from 20 to 40 °C.[32] Therefore, a change in hydrogen bonding is not expected through the transition. Within the pentamer, nine polar groups are capable of hydrogen bonding (five carbonyl oxygens and four peptide hydrogens); two of these polar groups are inaccessible to water due to the intramolecular hydrogen bond of the β-turn (see Figure 1). Thus we could estimate seven sites where water molecules could hydrogen bond within the pentapeptide, but these are not expected to change significantly over the temperature range. Thus the number of polar groups hydrogen bonded to water is essentially constant. One might briefly also consider ice-like structures within the protein. However, ice has a relaxation process within the 10-kHz range,[11] and thus such highly ordered water would not contribute to the dielectric loss observed at 1 GHz.

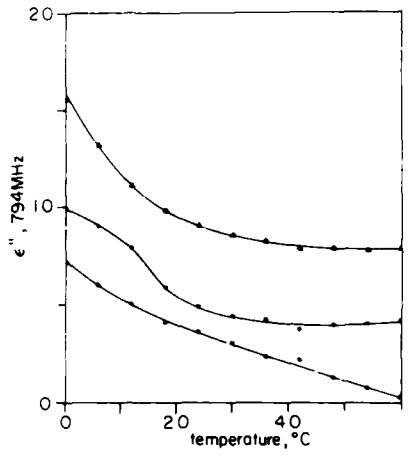

Figure 4. Temperature dependence of the imaginary part of the dielectric permittivity at 794 MHz of water (○), Leu[1]-PPP (●), and Ileu[1]-PPP (▲).

Two important facts must be noted: the first one is the high concentration of polypeptide in water (about 40%); the second one is the high content of hydrophobic groups. Thus, before the inverse temperature transition, there must be a large proportion of water associated with hydrophobic side chains. At the coacervate concentration there are approximately 30–35 water molecules per pentamer. With some 7 water molecules hydrogen bonded below the phase transition, about 25 water molecules would be either free bulk water or clathrate-like water in association with the hydrophobic side chains. It is not unreasonable that most of the 25 water molecules would be in association with the three bulky hydrophobic side chains of the pentamer of Ile[1]-PPP.

We propose that this water located in association with hydrophobic side chains of the polypeptide contributes to the dielectric loss observed at high frequency. This water should have a similar structure to water surrounding methane in clathrates,[33] and perhaps clathrate-like water therefore is an appropriate term for describing this kind of water. As all three polypentapeptides have the same number of polar groups and similar conformations, one expects that the number of water molecules hydrogen bonded to the backbone should be similar for each polypentapeptide derivative. The contribution of hydrogen-bonded water to the relaxation in the near-gigahertz range should be similar for each polypentapeptide derivative. In Figure 4, it is seen, however, that each polypentapeptide has a different magnitude of dielectric loss. It could be suggested that the number of hydrogen-bonded water molecules would not be the same for each polypentapeptide due to a restriction of access to binding sites. However, it is unlikely that Ile[1]-PPP and Leu[1]-PPP, each of which differ from PPP by a single additional CH_2 moiety, should differ from each other or even for that matter have more restricted hydrogen-bonding access than PPP. If it were a matter of steric blocking of sites, PPP would be expected to have the greater number of water molecules hydrogen bonded, and if the near-gigahertz dielectric loss were due to water molecules hydrogen bonded to the backbone, PPP should exhibit the more intense high-frequency dielectric loss. What is observed is exactly the inverse situation. Thus water hydrogen bonded to polypeptide backbone could not explain the dielectric loss. On the basis of these considerations, it is proposed that clathrate-like water contributes to the near-gigahertz dielectric loss.

Figure 5 gives the variation of the real part of permittivity at 794 Hz as a function of temperature for the polypeptides studied in this paper. Differences are apparent between the data for the polypeptides and water. While water presents a linear decrease of permittivity with increasing the temperature,[11] this is not the case for the polypeptides. A transition curve is seen. The high-frequency permittivity could reflect mainly the permittivity con-

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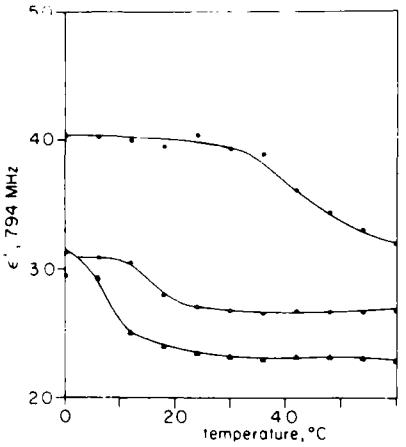

Figure 5. Real part of the permittivity ϵ' of Ileu[1]-PPP (▲), Leu[1]-PPP (○), and PPP (●) at 794 MHz as a function of the temperature.

TABLE I: Ile[1]-PPP Dielectric Relaxation Data

temp, °C	$\Delta\epsilon$	τ, ns	α	σ, mS/m	ϵ^∞ at 100 MHz ± 0.5
0[a]	26 ± 3	139 ± 6	0.36 ± 0.08	1.2 ± 0.2	32.3
6[a]	26 ± 4	87 ± 10	0.3 ± 0.1	1.8 ± 0.6	30.8
12	37 ± 2	49 ± 3	0.11 ± 0.03	0.5 ± 0.1	26.3
18	34 ± 2	44 ± 3	0.08 ± 0.02	0.6 ± 0.1	24.7
24	37 ± 3	44 ± 3	0.08 ± 0.07	0.6 ± 0.1	23.7
30	38 ± 2	38 ± 2	0.08 ± 0.02	0.6 ± 0.1	23.2
36	42 ± 3	36 ± 2	0.09 ± 0.02	0.6 ± 0.1	23
42	43 ± 3	35 ± 2	0.09 ± 0.02	0.7 ± 0.1	22.7
48	48 ± 3	39 ± 2	0.10 ± 0.02	0.7 ± 0.2	22.6
54	50 ± 3	35 ± 3	0.10 ± 0.02	0.9 ± 0.2	22.6
60	55 ± 4	31 ± 2	0.11 ± 0.03	1.0 ± 0.2	22.4

[a] The dielectric parameters of the sample at 0 and 6 °C are only suggestive since the relevant dielectric relaxation is not yet very well resolved.

TABLE II: Conductivities of Leu[1]-PPP as a Function of Temperature

temp, °C	conductivity, mS/m	temp, °C	conductivity, mS/m
0	0.3	36	1.25
6	0.27	42	1.40
12	0.57	48	1.55
18	0.67	54	1.73
24	0.96	60	1.90
30	0.9		

tribution of the water solvent. Figure 5 shows an increasing permittivity at 794 MHz from Ile[1]-PPP to Leu[1]-PPP and PPP. Thus the water content within the polypentapeptide derivative should increase from Ile[1]-PPP to Leu[1]-PPP and PPP.

Discussion

In Table I are presented the results of a curve-fitting analysis on the Ile[1]-PPP data. The dielectric permittivity was analyzed by means of one Cole–Cole function plus the conductivity term:

$$\epsilon = \epsilon^\infty + \frac{\Delta\epsilon}{1 + (i2\pi\nu\tau)^{1-\alpha}} + \frac{\sigma}{i2\pi\nu\epsilon_0}$$

where ϵ is the complex permittivity, ϵ^∞ is the high-frequency limit permittivity, $\Delta\epsilon$ is the dielectric decrement, ν is the frequency, τ is the relaxation time, α is the Cole–Cole parameter which indicates a spread of relaxation times centered about τ, σ is the conductivity, and ϵ_0 is the vacuum permittivity.

When the temperature is decreased, α of the Cole–Cole term becomes larger, indicating a spread of relaxation times which could arise from several conformational states. Above the phase transition temperature, Ile[1]-PPP develops a nearly pure Debye relaxation with α being close to zero. When the temperature is raised, the dielectric decrement becomes greater and α becomes

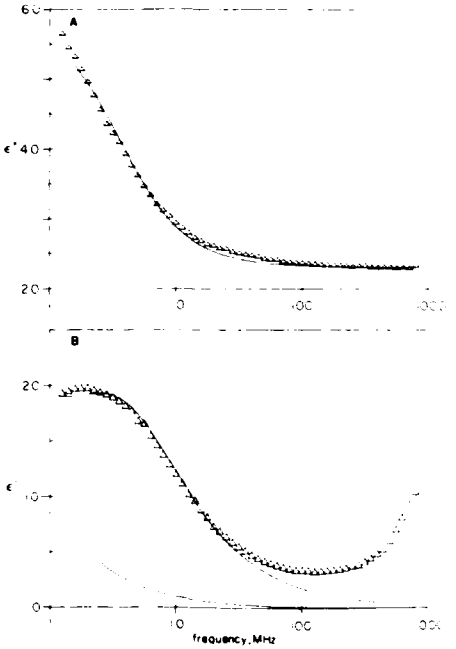

Figure 6. Example of the quality of curve fitting obtained on the Ile[1]-PPP data. The solid line represents the calculated fit, and the symbols represent experimental data. At the top (A) is the real part and at the bottom (B) the imaginary part of the dielectric permittivity of Ileu[1]-PPP at 24 °C. The other solid line curve seen at the bottom of the figure represents the conductivity.

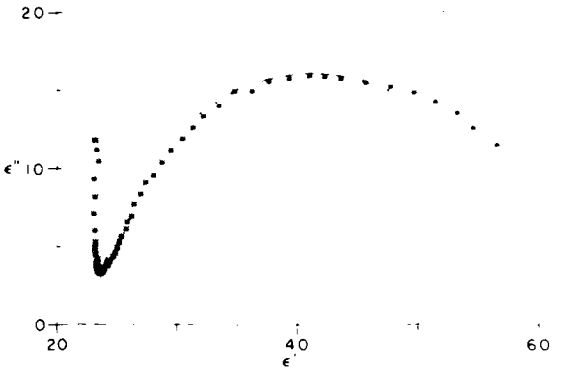

Figure 7. Cole–Cole plot of the Ile[1]-PPP sample at 24 °C. The full line is the theoretical curve, and the symbols represent the experimental data plots. The conductivity term at 24 °C (listed in Table I) has been subtracted from the dielectric loss ϵ''.

smaller, indicating an inverse temperature transition resulting in the formation of a regular structure, as can be seen qualitatively in Figures 2 and 3. As had already been reported, PPP[6] and elastin[7] develop an increasing dielectric decrement when the temperature is raised. The circular dichroism spectra of Ile[1]-PPP and PPP[22] indicate an increase of intramolecular order on raising the temperature. Thus the dielectric measurements further characterize this as an inverse temperature transition.

Figure 6 shows a typical example of the quality of curve fitting obtained on the Ile[1]-PPP data. The full line represents the best fit and the dotted points are experimental data. No attempt has been made to fit the high-frequency relaxation, since we do not know the maximum of this dielectric relaxation. The quality of the fit at low frequency (close to 1 MHz) becomes slightly less satisfactory than in the higher frequency range. Perhaps another dielectric relaxation begins to contribute at the low-frequency end of the observed range. It is also quite possible that electrode polarization would not be negligible in this frequency range. Figure 7 shows the Cole–Cole plot of the Ile[1]-PPP sample at 24 °C. At the left of the figure, the dispersion due to the clathrate-like

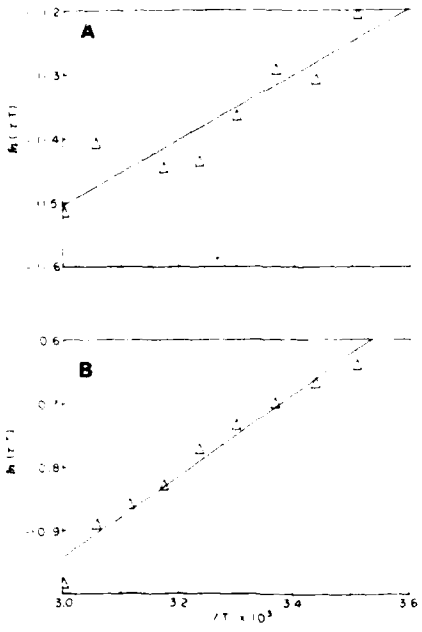

Figure 8. Temperature dependence of the correlation time (τ) from 12 to 60 °C, plotted at τT (K). The straight lines in A and B correspond to the least-squares best fit yielding an enthalpy of activation of 1.1 ± 0.2 kcal/mol (for Ile[1]-PPP in A) and of 1.3 ± 0.1 kcal/mol (for leu[1]-PPP in B). When the data point for the second highest temperature is deleted in A, the value is 1.2 ± 0.1 kcal/mol.

water is again well demonstrated.

Since the conductivity and the electrode polarization could contribute to the dielectric background, the value of the relaxation time could vary somewhat. It is still possible nonetheless to utilize the temperature dependence of the correlation time to give an estimate of the enthalpy of activation. The values from the data plotted in Figure 8 are 1.1 ± 0.2 kcal/mol for Ile[1]-PPP and 1.3 ± 0.1 kcal/mol for Leu[1]-PPP, which are similar to the value for α-elastin (1.7 kcal/mol).[7] These low energy barriers to the polypentapeptide backbone motions indicate that a dynamic structure results from the inverse temperature transition, and on γ-irradiation cross-linking the many states accessible due to the low activation energy for backbone motion provides the basis for the entropic elastomeric force exhibited by these polypentapeptide elastomers.[5,22]

Next, the high-frequency spectrum of the permittivity can be used to estimate the fractional volume of the polypeptide by means of the Maxwell–Fricke equation (usually considered appropriate for protein in solution[34-37]):

$$\frac{\epsilon - \epsilon_w}{\epsilon + x\epsilon_w} = P_f \frac{\epsilon_f - \epsilon_w}{\epsilon_f + \epsilon_w}$$

where ϵ is the measured high-frequency permittivity, ϵ_w is the high-frequency permittivity of water, ϵ_f is the high-frequency permittivity of the polypentapeptide derivative, x is the shape factor which varies between 1 and 2, and P_f is the fractional volume of the polypentapeptide derivative. For the present calculations, we chose a value of $x = 1$, which corresponds to a rigid rod shape. This choice was made because the polypentapeptide forms anisotropic fibers rather than isotropic spheres. The ϵ_f value has been computed by using the PPP data. The partial volume of PPP, which is 0.47,[10] has been used. We have found a value of $\epsilon_f = 5$. This value has been taken for Ile[1]-PPP and Leu[1]-PPP in order to compute the partial volume. As pointed out by Dawkins,[37] a 20% uncertainty in ϵ_f results in an uncertainty of less than 4% in the calculated value P_f.

TABLE III: Comparison of the Volume Fraction of the Polypentapeptide Derivative in Water (60 °C) Computed by Means of the Maxwell–Fricke Equation[a]

	PPP	Leu[1]-PPP	Ileu[1]-PPP
volume fraction of PPP analogue	0.31 ± 0.02	0.50 ± 0.02	0.54 ± 0.03
mass fraction of PPP analogue	0.37 ± 0.02	0.57 ± 0.02	0.61 ± 0.03

[a] The errors correspond to the errors on the ϵ values only. The parameters are $X = 1$; $\epsilon_f = 5$; $\epsilon_w = 67$.

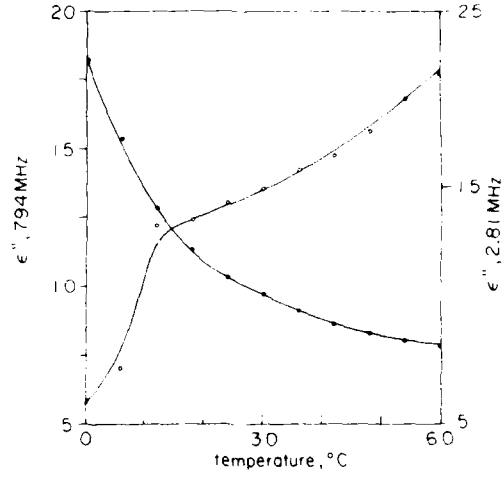

Figure 9. Imaginary part of the permittivity ϵ'' of the Ileu[1]-PPP at 794 MHz (●) and at 2.81 MHz (○) as a function of temperature. The conductivity term from the dielectric loss ϵ'' has been subtracted. The values used are listed in Table I.

The estimation of the volume fraction of the polypentapeptide derivative is presented in Table III. The water content within the polypentapeptide derivative increases in this order: Ile[1]-PPP < Leu[1]-PPP < PPP. This estimation of water content is only approximate since we do not know the exact values of the parameters. However, the data clearly demonstrate an increasing water content within the polypeptide in the order given. Interestingly, at 60 °C composition studies have shown a similar sequence for water content.[38]

Conclusions

The dielectric properties of polypentapeptide analogues of elastin in aqueous solutions provide interesting insight into the mechanism of biological elasticity and into the nature of the water within the polypentapeptide derivative.

When temperature is increased, the aqueous solutions of each polypentapeptide analogue form coacervates. By dielectric measurements a nearly simple Debye-type relaxation is observed to develop on raising the temperature, which has been attributed to peptide rocking motions of the polypentapeptide. Thus during the increase in temperature the polypentapeptide analogues become more ordered. An increase in intramolecular order on raising the temperature has also been observed by circular dichroism spectroscopy.[22]

In the high-frequency range, there is observed a dielectric relaxation which has been attributed to hydrogen-bonded water and to clathrate-like water. Since the values of the dielectric loss of PPP, Leu[1]-PPP, and Ileu[1]-PPP are not similar, water hydrogen bonded to the polypeptide backbone itself cannot explain the dielectric loss. Instead the magnitude of the dielectric loss increases with increase in hydrophobicity of the pentamer. For this reason,

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clathrate-like water is proposed to contribute to this high-frequency relaxation (within the gigahertz range). This high-frequency dielectric relaxation exhibits a normal phase transition, which indicates an increasing disorder on raising temperature. Thus considering the behavior of these two relaxations (one arises from the polypentapeptide backbone and the other one from the clathrate-like water or hydrogen-bonded water), there emerges an interesting picture of the temperature dependence of the water–polypentapeptide system. When the temperature is increased, the polypentapeptide derivative becomes more ordered and the clathrate-like water becomes less ordered. This inverse behavior is demonstrated in Figure 9. Thus hydrophobicity plays a role in strucutre formation in these bioelastomers, which correlates with development of elastomeric force as has been reported.[22,29]

The high frequency (1 GHz) of the dielectric permittivity of these polypentapeptide derivatives seems to correlate with the hydrophobicity of the polypentapeptide. Perhaps one of the most interesting applications of this two-component system could be the establishment of a hydrophobicity scale based on the relative amounts of observable clathrate-like water.

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Registry No. I, 83610-44-0; II, 83610-64-4; III, 96847-92-6; IV, 111583-44-9; V, 111583-45-0; VI, 111615-37-3; VII, 111583-48-3; VIII, 111583-46-1; LPGVG, homopolymer, 111583-50-7; LPGVG, SRU, 111583-51-8; IPGVG, homopolymer, 106855-57-6; IPGVG, SRU, 106855-24-7; BNPC, 5070-13-3; VPGVG, 52231-42-2; VPGVG, SRU, 111793-53-4; BOC-Leu-OH, 13139-15-6; H-Pro-OBzl·HCl, 16652-71-4.

ELASTIC MOLECULAR MACHINES AND A NEW MOTIVE FORCE IN PROTEIN MECHANISMS

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Abstract

It is demonstrated that the polypentapeptide, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)n$ when γ-irradiation cross-linked, can perform work on raising the temperature from 20° to $40^{\circ}C$. This is due to an inverse temperature transition leading to a regular helical structure called a dynamic β-spiral, which exhibits entropic elastomeric force. Processes which alter the hydrophobicity of a peptide segment can shift the temperature of an inverse temperature transition.[4] When the hydrophobicity is changed reversibly as is possible with 20% Glu^4-poly-pentapeptide, the temperature for the onset of the inverse temperature transition can be reversibly shifted from being initiated at $37^{\circ}C$ at pH 2 (COOH) to being initiated at $50^{\circ}C$ at pH 7 (COO^-). Presumably therefore once a synthetic elastomeric matrix is formed from 20% Glu^4-polypentapeptide, it should be possible at $50^{\circ}C$ to turn "on" elastomeric force by changing the pH from 7 to 2 and to turn "off" elastomeric force by returning the pH to 7. This is called mechanochemical coupling of the first kind, and, in addition to ionization and deionization, it should be possible similarly to turn off and on elastomeric force by phosphorylation and dephosphorylation, respectively.

When the elastomeric state is arrived at by means of a regular transition from a more ordered state (e.g., α-helix) to a less ordered state (e.g., a spiral) on raising the temperature and a chemical process can change the temperature of the transition, this is referred to as mechanochemical coupling of the second kind. Mechanochemical coupling ongoing from an ordered state to a disordered state has often been considered. The studies on the polypentapeptide bring consideration of an inverse temperature transition for mechanochemical coupling of the first kind and of a less-ordered but nonrandom state for mechanochemical coupling of the second kind. It is proposed that these new considerations are relevant to mechanisms for the turning on and off of elastic forces in protein mechanisms as varied as those of enzymes and muscle contraction.

The Polypentapeptide of Elastin as an Elastic Molecular Machine

By definition a machine is a device for doing work and work is performed when a force acts against resistance to produce motion in a body. Consider as a specific example a weight suspended from the synthetic elastomeric polypentapeptide band at $20^{\circ}C$ in water (see Figure 1A).[5] The band is formed on γ-irradiation of $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)n$ where n is greater than 100, and the composition is approximately 40% peptide, 60% water by weight (1,2). On raising the temperature to

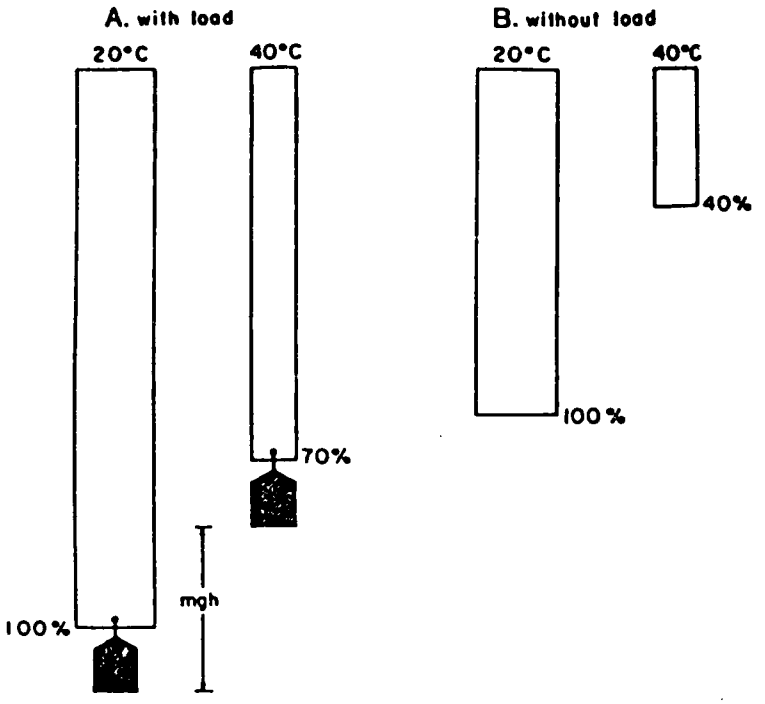

Figure 1: The polypentapeptide of elastin as an elastic molecular machine. High molecular weight $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ with n greater than 100 is γ-irradiation cross-linked when in a viscoelastic state of 40% peptide, 60% water by weight to form an insoluble band of material.

A. A weight of 300 gms/cm^2 of band cross-sectional area measured at 40°C is applied. At 20°C in water, the length is taken as 100%. On raising the temperature to 40°C, the band shortens to 70% lifting the weight. The work performed is mgh. B. A band of cross-linked polypentapeptide is depicted at 20°C in water in the absence of any load. On raising the temperature to 40°C, the sample contracts to approximately 40% of its original length. The heat absorbed during this inverse temperature transition is approximately 1 cal/gm polypentapeptide. This shortening of the sample is due to the winding up of the polypentapeptide chain into a helical structure, termed a β-spiral, as shown in Figure 2.

40°C, the weight (300 grams/cm^2 elastomer cross-section) is raised against gravity as the synthetic elastomeric band shortens to 70% of its 20°C length (3). For a band 10 cm long, the weight would be raised 3 cm against the pull of gravity. The same qualitative result is obtained with the entropic elastomer, latex rubber, but for this classical rubber the length change is much less, only about 5% instead of 30% (3). Both elastomers are molecular machines but the polypentapeptide elastomer is a more effective machine for moving an object when changing the temperature from 20° to 40°C. What occurs as the result of this temperature change in the polypentapeptide is an enhanced effect due to an inverse temperature transition wherein the polypentapeptide wraps up into a helical structure, i.e., a β-spiral, on raising the temperature from 20°C to 40°C (4). The helical structure is the result of optimizing intramolecular hydrophobic interactions. The heat absorbed during this inverse temperature transition occurring between 20° to 40°C is approximately 1 cal/gram of the polypentapeptide in water. The class of β-spirals to which the elastomeric polypentapeptide belongs is shown in Figure 2 (5-8) and the length change under zero load is from 100% to 40% on going from 20°C to 40°C (see Figure 1B). At fixed length, development of elastomeric force (f) correlates with structure development (4) and as shown in Figure 3, the structure so formed exhibits entropic elastomeric force (9). As will be further discussed below, the polypentapeptide forms an entropic anisotropic elastomer.

Entropic Elastomeric Force Resulting From an Inverse Temperature Transition

Previously entropic elastomeric force has been taken to require that the polymeric system be a network of random chains in adherence to the classical theory of rubber elasticity (10). When elastin fibers were shown to give a result like that in Figure 3 for the polypentapeptide of elastin in the temperature range above 40°C, the conclusion was "A network of random chains within elastin fibers, like that in a typical rubber, is clearly indicated" (11). In a typical rubber, the decrease in entropy on deformation is taken to be due to the displacement from a random distribution to end-to-end chain lengths (12,13). But a random distribution of end-to-end chain lengths is not the product of an inverse temperature transition. An inverse temperature transition involves an increase in polymer order on increasing the temperature. For the polypentapeptide of elastin, it is demonstrated in Figure 3 that entropic elastomeric force occurs above 40°C on completion of the inverse temperature transition, that is, once the increase in order has occurred. This is because the elastomeric force, f, is the sum of two components: f_e, an internal energy component and f_s, the entropic component. When ln[elastomeric force/T(°K)] is plotted versus temperature, a zero slope is taken to mean that $f_e/f = 0$, that is, the elastomer exhibits dominantly entropic elastomeric force (14). As shown in Figure 3 (solid curve), the cross-linked polypentapeptide exhibits a dominantly entropic elastomeric force above 40°C (15, 16).

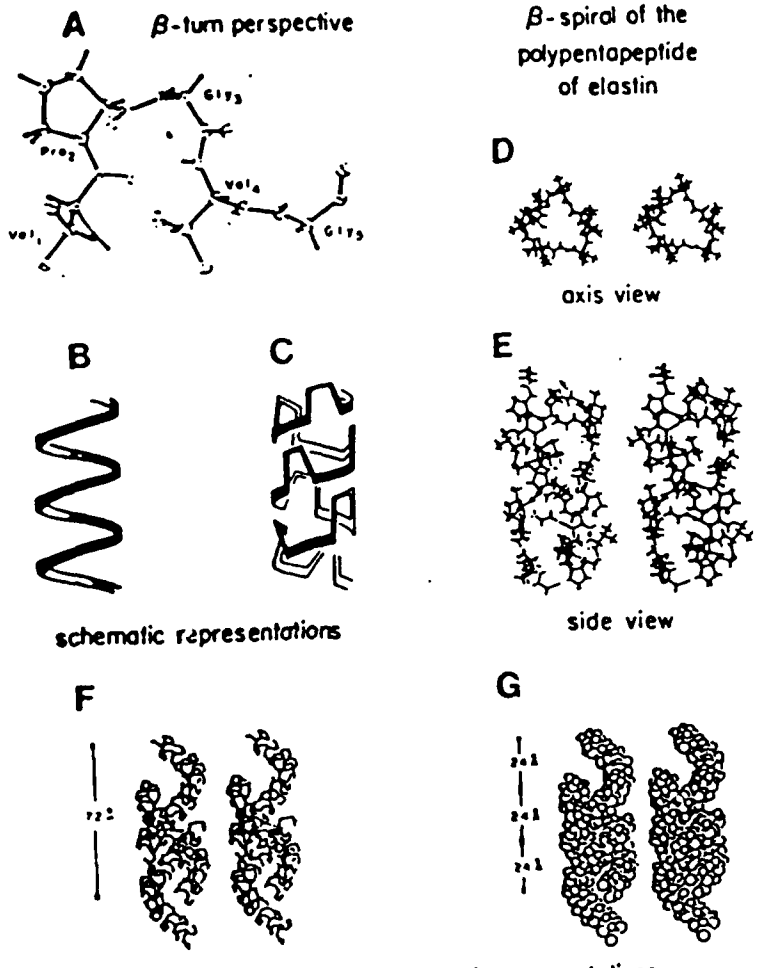

Figure 2:

Molecular conformation of the 40°C state of the polypentapeptide of elastin in water.

A. The β-turn perspective of the pentamer, showing the Val^1 C-O...HN Val^4 ten atom hydrogen bonded ring with Pro^2-Gly^3 at the corners. Adapted with permission from reference 5. Raman studies indicate that the β-turn is present before and after the transition observed in Figure 1B on going from 20° to 40°C (6). B. On raising the temperature, the largely extended series of β-turns wrap up into a helix in which the β-turns function as spacers between turns of the helix as shown in C. The helix with β-turns spacers is called a β-spiral and it is the wrapping up into a β-spiral that is responsible for the dramatic contraction of size seen in Figure 1B. The length change for the depicted β-spiral to become an extended series of β-turns is close to a factor of 3; interestingly, a hot a unreasonable factor of 2.5 is observed (see Figure 1B). (B., C. and D. reproduced with permission from reference 7.) D. and E. are stereo pair perspectives of the detailed β-spiral seen in axis view (d) and side view (E). The interturn contacts are hydrophobic and result from the optimization of intramolecular hydrophobic contacts developed during the inverse temperature transition. Between the β-turns are suspended segments running from the Val^4 α-carbon to the Val^1 α-carbon in which the peptide moieties can undergo large amplitude, low frequency librational motions. On stretching these librational motions become damped which is a decrease in entropy that provides the resistance to and the restoring force from deformation. This is called the librational entropy mechanism of elasticity (7). E. is reproduced with permission from reference 8.

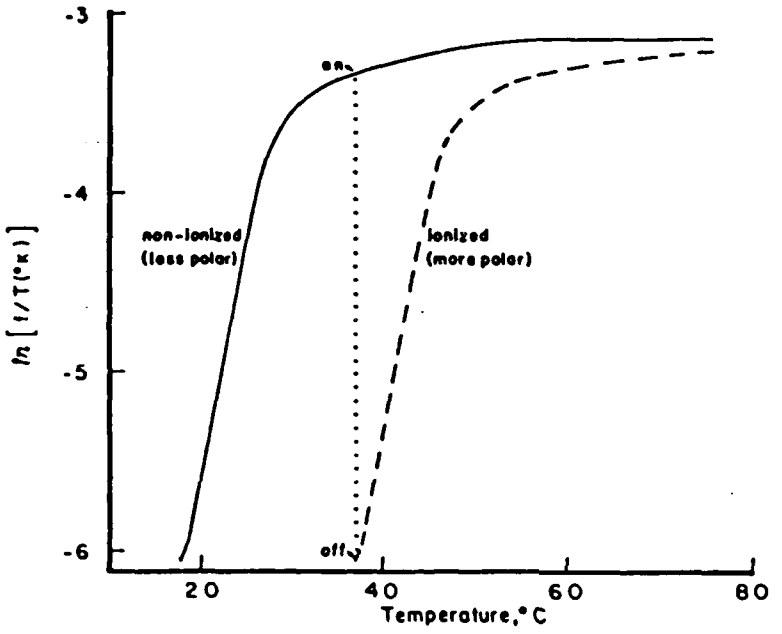

Figure 3: Thermoelasticity data on the γ-irradiation cross-linked polypentapeptide of elastin (solid curve). The observation that the slope of the ln[f/T°K)] versus temperature curve at fixed extension is near zero above 40°C is the basis for arguing that the elastomeric force is dominantly entropic. The data (9) is used in the present context to demonstrate the effect of decreasing the hydrophobicity, i.e., increasing the hydrophilicity, as for example by ionization of a residue within the polypentapeptide such as an occasional glutamic acid residue or by a phosphorylation of a serine or threonine residue. The effect of making this polymer more polar would be to raise the temperature midpoint of the inverse temperature transition that is responsible for the observed development of elastomeric force. The expected result is the dashed curve. The perspective is, therefore, that the elastomeric force is turned on at 37°C when the polypeptide is neutral and is turned off when the polypeptide is more polar or charged.

Numerous physical characterizations of the polypentapeptide of elastin in water have shown that on raising the temperature from 20° to 40°C, there is an increase in molecular order. Those physical characterizations include: i. light and electron microscopy demonstrating on increasing the temperature a self-assembly into fibers, comprised of parallel aligned fibrils, which in turn are comprised of parallel aligned filaments (7,17,18); ii. light scattering following the aggregation with increase in temperature (2); iii. circular dichroism showing an increase in intramolecular order with occurrence of regularly recurring β-turns (19); iv. the nuclear Overhauser effect demonstrating the intramolecular hydrophobic interactions attending the inverse temperature transition (20); v. composition studies showing the phase transition to a unique composition of polypentapeptide plus water (2); vi. nuclear magnetic resonance relaxation studies showing a decrease in backbone mobility on raising the temperature through the inverse temperature transition (21,22); vii. dielectric relaxation studies showing the development of an intense, low frequency, high amplitude, localized, Debye-type relaxation on raising the temperature through the inverse temperature transition (23,24); viii. and the above noted temperature dependence of elastomer length (3). Having demonstrated an increase in order on arriving at 40°C, thermal denaturation can be demonstrated on raising the temperature above 60° to 80°C (2) and by circular dichroism showing the decrease in intramolecular order on standing at 80°C (2); and thermal denaturation has been demonstrated directly in the loss of elastomeric force and elastic modulus on heating at 80°C (25). These are not the properties of random chain networks. Accordingly a new understanding is required for the decrease in entropy on deformation and it is one of a damping of internal chain dynamics on deformation called the librational entropy mechanism of elasticity (9,26,27). A new understanding of entropic elastomeric force has emerged from which interesting new possibilities arise.

Effect of Changing Hydrophobicity of Polypeptide Elastomers

The fact that the elastomeric force development occurs with shortening by means of an inverse temperature transition (3,4) gives interesting new potential to the polypentapeptide and like elastomers as molecular machines. It has been shown (28,29) that changing the hydrophobicity of the repeating unit in the elastomeric polypeptide changes the temperature range of the inverse temperature transition, which gives rise to regular structure; that changing the hydrophobicity changes the temperature range over which elastomeric force develops, and that changing the hydrophobicity changes the range over which the elastomer shortens. For example, increasing the hydrophobicity of the polypentapeptide (VPGVG)n as in the Ile^1-polypentapeptide, (IPGVG)n, analog (28) lowers the temperature range over which the transition occurs by some 20°C from a midpoint of near 30°C for (VPGVG)n to near 10°C for (IPGVG)n. Furthermore when the hydrophobicity is decreased as when the Val^4 residue is removed as in the polytetrapeptide (VPGG)n, the product is an elastomer but the

development of elastomeric force is now shifted to 50°C (29). Thus by changing the hydrophobicity, the midpoint temperature of the inverse temperature transition for the development of elastomeric force has been shifted over the temperature range from 10°C to 50°C. Increase the hydrophobicity and the inverse temperature transition occurs at a lower temperature; decrease the hydrophobicity and the inverse temperature transition occurs at a higher temperature. Even the magnitudes of the shifts are calculable from the change in hydrophobicity (29,30,31).

When temperature is limited as a variable, therefore, as an example in the case of living organisms, varying the hydrophobicity would be a useful way to perform work. Decreasing hydrophobicity which, of course, is equivalent to increasing hydrophilicity can be achieved by hydroxylation. Accordingly, it has been shown, by chemically introducing hydroxyproline in place of proline and by direct hydroxylation of (VPGVG)n using the enzyme prolyl hydroxylase, that the temperature range of the inverse temperature transition can be raised in proportion to the amount of replacement or conversion of proline to hydroxyproline (32). When the ratio of (Val-Pro-Gly-Val-Gly) to (Val-Hyp-Gly-Val-Gly) was 9:1 in the polymerizing mixture, the resulting polymer exhibited a transition midpoint that was shifted 7°C to higher temperature; for (Val-Hyp-Gly-Val-Gly)n itself the transition midpoint was above 65°C (32). What would be of particular interest would be to shift reversibly the temperature of the inverse temperature transition and thereby to turn "on" and "off" the elastomeric force. One means would be protonation or deprotonation of a functional side chain; another might be enzymatic reactions wherein there is an interconversion between charged and uncharged states of a side chain; and yet another could be the phosphorylation and dephosphorylation, for example, of a serine or threonine side chain. The use of pH is briefly considered below.

Reversibly Changing Hydrophobicity of the Polypentapeptide as a Means of Turning Elastomeric Force "On" and "Off"

As shown in Figure 4, the inverse temperature transition can be followed by means of the temperature profiles for aggregation for the polypentapeptide and its 20% Glu^4 analog. Changing one in five Val^4 residues to a Glu^4 residue, when the pH is 2 where the side chain is the carboxyl, changes the onset of the inverse temperature transition from 25°C to 37°C. On ionization of the side chain to form the carboxylate anion at pH 6, the onset of the inverse temperature shifts further to 49°C. Once the 20% Glu^4-polypentapeptide is cross-linked to form the elastomeric matrix, it is expected that the elastomer can most effectively at 50°C be turned "on" at pH 2 and "off" by changing the pH to 7. This would be a chemomechanical transducer. If 50°C were not the desired temperature, for example, if lower temperature were desired, then more hydrophobic residues could be used in place of Val^1 and Val^4.

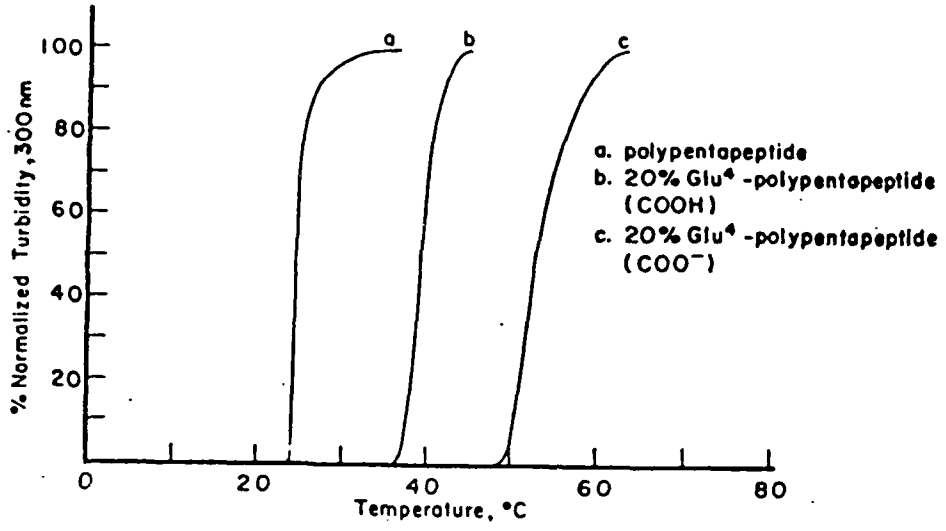

Figure 4: Temperature profiles of aggregation showing the temperature dependence of the intermolecular aspect of the inverse temperature transition. This also coincides with the intramolecular component of the inverse temperature transition. Curve a: the polypentapeptide of elastin; curve b: 20% Glu^4-polypentapeptide at pH 2 where the polar side chain is the COOH moiety; and curve c: 20% Glu^4-polypentapeptide where the side chain is ionized (COO^-) at pH 6. The solid curve in Figure 3 showing the development of force with temperature corresponds to curve a. On the basis of this, it is expected that the 20% Glu^4-polypentapeptide cross-linked matrix would develop elastomeric force with a midpoint temperature near 40°C when at pH 2 and that force development would shift to a midpoint temperature of near 55°C when at pH 7. Therefore at 50°C, changing the pH from 2 to 7 should turn off elastomeric force and the reverse should turn on elastomeric force.

Starting with Ile^1-polypentapeptide, which has a transition midpoint of about 10°C (28), inclusion of a more polar residue, such as Glu, Asp, His, Lys, or Tyr, for example in every third pentamer at position four would raise the temperature of the transition toward 30°C for the nonionized state. The appropriate mix of Ile^1 and Val^1 and of Val^4 and the more polar side chain at position four would allow the midpoint of the transition to be selected over a temperature of 10°C to above 30°C. On ionization the transition would shift to a higher temperature yet. Suppose that the non-ionized analog exhibited a transition midpoint near 30°C and that the β-spiral structure were formed and the development of elastomeric force were essentially complete by 37°C, as in the solid curve of Figure 3, then on ionization (e.g., on raising the pH above the pK of the ionizable function) the transition midpoint would shift to a higher temperature; the structure would unwind and the elastomeric force would be turned off as in the dashed curve of Figure 3. Lowering the pH to below the pK would cause the elastomeric force to turn back on. A change in the activity of the hydrogen ion becomes the switch. A number of other switches could be devised.

Mechanochemical Coupling

Previously, developments of elastomeric force with increase in temperature have been conformed to considerations of the classical theory of rubber elasticity with consideration of an order to disorder transition. In one proposed mechanism for the power stroke of muscle contraction in the S-2 fragment of myosin, an α-helix to random coil transition provides an interesting possibility to consider (33,34). The polypentapeptide data, in which the elastomeric state is a nonrandom β-spiral structure, indicate that random coil is not a necessary consideration and even suggests that it could be incorrect since the elastic modulus on thermal randomization of the polypentapeptide of elastin and of elastin itself becomes so low as to be of little relevance to the elastic forces of muscle contraction (16,25). Accordingly, analysis in terms of an α-helix to spiral transition seems warranted. Thus the situation could be one as shown in Figure 5 in which an α-helix with 1.5Å/residue converts to a spiral with about half the translation per residue, e.g., 0.7Å/residue as in the β-spiral of Figure 2. This would be consistent with the pitch estimated for the β-spirals of the polypentapeptide with about 15 residues per turn of spiral (8), of the polytetrapeptide with about 16 residues/turn (35), of the polyhexapeptide with about 12 residues/turn (36) and of the polynonapeptide with about 18 residues per turn (Chang, Trapane and Urry, in preparation). With heptamer repeats in myosin grouped as 28mers (37) some fourteen residues per turn would be reasonable for a spiral structure. Whatever the details of the situation, one looks for a chemical process to shift the temperature of the transition such that at a given temperature the elastic contraction could occur as the result of a chemical process.

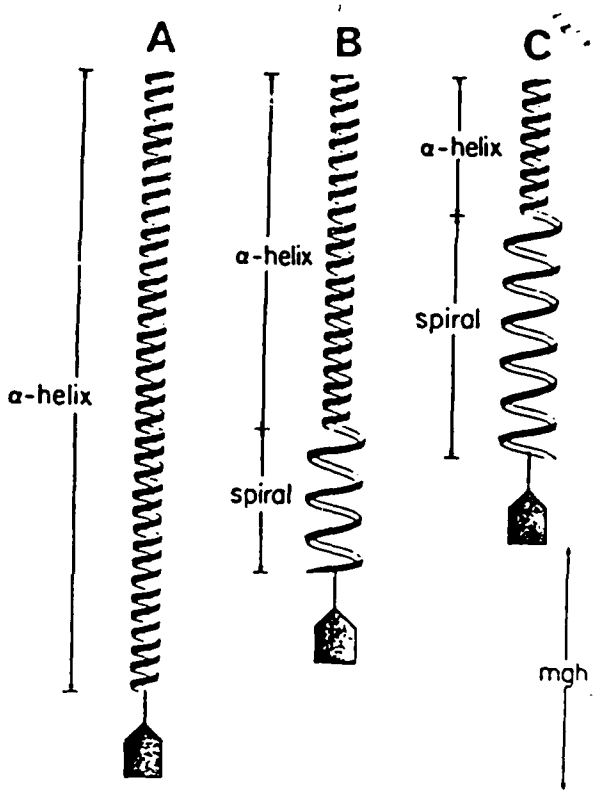

Figure 5: Proposed α-helix to spiral structural transition for the turning on of elastomeric force with the result of lifting a weight. The α-helix is shown with a graded instability such that the lower end would first convert to spiral on raising the temperature as in B and then as the temperature increased further, the conversion would continue on up the chain as shown in C. The graded instability with respect to temperature would facilitate reversibility in a condensed matrix.

There have now been discussed two kinds of mechanochemical coupling. Mechanochemical coupling of the first kind in which an inverse temperature transition from a higher to a lower entropy structure is the transition for the development of elastomeric force, and mechanochemical coupling of the second kind, which utilizes a standard transition from a lower entropy state to a higher entropy state. The structural transitions for these two kinds of coupling are shown schematically in Figure 6. A key element of these considerations is that there exist structures intermediate in entropy between the α-helix, β-sheet and collagen triple stranded (ordered and relatively rigid) structures on the one hand the random chain networks or random coil structures on the other hand. That such regular structures of intermediate entropy exist has been shown with the sequential elastomeric polypeptides of elastin as well as in the series of helical structures that can exist for the polydipeptide gramicidin A (9).

Entropic Motive Force in Protein Mechanisms

Having demonstrated entropic elastomeric force to be due to internal chain dynamics in nonrandom polypeptide systems, it seems appropriate to note situations in which protein elastic processes may be viewed in terms of this new perspective. Several examples will be briefly noted below.

Elastin: The most striking primary structural feature of porcine and bovine elastin is the polypentapeptide considered above. It is not suprising therefore that similar physical characterizations of elastin have shown it to be an entropic elastomer which forms its elastic structure by means of an inverse temperature transition (15,16,38). Accordingly, it is expected that increased hydrophilicity such as prolyl hydroxylation would raise the temperature of the inverse temperature transition and limit fiber formation. This process has been observed in cell culture (39) and has been proposed to be the reason for the near absence of elastic fibers in the scar tissue of wound repair (40). Of further medical significance is that by the foregoing reasoning, any oxidative process should shift the inverse temperature transition, that gaves rise to elastin fibers, to higher temperature and cause an unwinding of the spiral structures in elastin. A loss of elastic recoil and elongation has been looked for and observed using a superoxide generating system with bovine ligamentum nuchae elastin (38). Elastin fiber oxidation has been proposed in the initiation of pulmonary emphysema (38), which is characterized by disrupted elastic fibers and loss of elastic recoil of the lung (41). Oxidation loss of elastic recoil can also be considered in the sagging and wrinkling of skin with age.

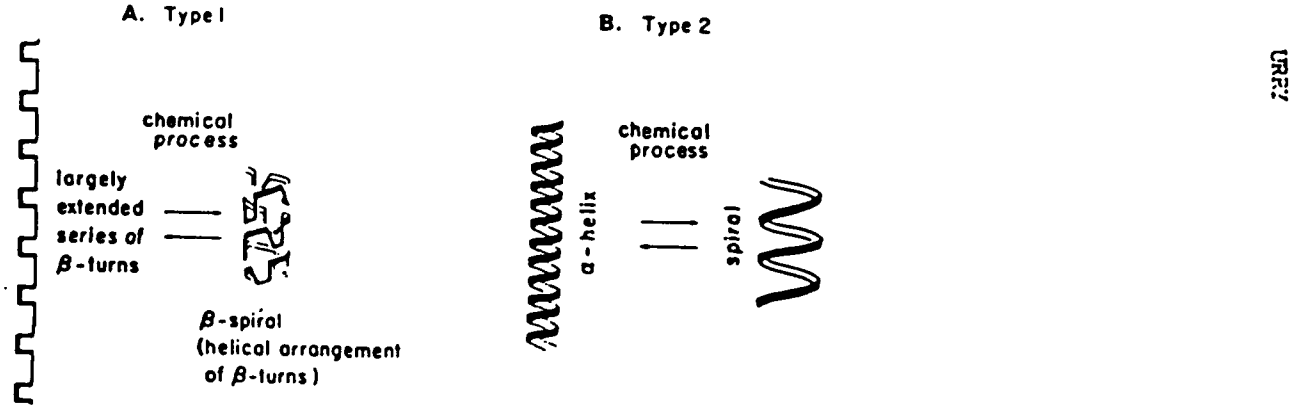

Figure 6: Structural Transitions for Mechanochemical Coupling

A. Type 1. The structural transition is from a higher entropy to a lower entropy state with increase in temperature, that is, it is an inverse temperature transition. The example involves a largely extended high entropy series of β-turns that wrap up to form an elastic β-spiral. A β-spiral is defined as a helical arrangement of β-turns. The result of the inverse temperature transition is the optimization of intramolecular, interturn hydrophobic interactions. Increasing hydrophobicity lowers the temperature of the transition and decreasing hydrophobicity increases the temperature of the transition. The development of elastomeric force at fixed extension occurs as the result of the inverse temperature transition and therefore by this analysis, elastomeric force can be turned on and off by varying the temperature of the transition, that is, by reversibly changing the hydrophobicity of the polarity of the polymer, for example by ionization of a side chain; the result is that the temperature for the transition is raised above the fixed temperature and elastomeric force is turned off. Deionization would lower the temperature of the transition to below the fixed temperature and would turn elastomeric force back on. Enzymatic phosphorylation and dephosphorylation at constant temperature and constant pH would be another means of reversibly shifting the temperature of the transition by reversibly changing the hydrophobicity of the polypeptide. B. Type 2. The structural transition is from a lower entropy state to a higher entropy state with increase in temperature, that is, this is a standard transition. The example is the conversion of an α-helix to an elastic spiral wherein changes in internal chain dynamics give rise to an entropic elastomeric force. The relaxed state of the spiral has a lower axis translation per residue (0.5Å/residue) than the α-helix (1.5Å/residue) such that the structural conversion results in a contraction. Any chemical process which shifted the temperature of the transition by changing the relative free energies of the structure could turn on or off the elastomeric force of the spiral structure. Possible chemical processes would be changes in pH, changes in calcium ion concentration or phosphorylation/dephosphorylation. The mechanism, Type 2, has been generally considered to be mechanochemical coupling. The element introduced by the work on polypeptide elastomeric biomaterials is the consideration of a spiral structure instead of a random coil.

Summarizing Comments

The general implications of the foregoing considerations are several: i. Elastic processes in polypeptides and proteins need not be described in terms of networks of random chains or random coil structure; indeed it may be incorrect to do so. ii. Entropic elastomeric force can be exhibited by a short anisotropic peptide segment due to internal chain dynamics (the librational entropy mechanism of elasticity). Random chain networks are not required. iii. Whether in a short peptide segment of a globular protein or in a fibrillar protein, it is expected that elastomeric force can be turned "on" and "off" by reversibly changing the hydrophobicity of the polypeptide. A most obvious means of doing so would be phosphorylation and dephosphorylation. And iv. It should be possible to design a wide range of polypeptide elastomeric biomaterials that could function in thermomechanical, chemomechanical and electromechanical transduction.

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Synthesis of two component models of elastin

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Morphologically, elastic fibers can be described as a fine fibrillar coating of a large amorphous core referred to as elastin. Elastin is an insoluble, highly cross-linked and very hydrophobic protein with about 90% nonpolar amino acids and about 5% lysines. The insolubility of elastin is due to the presence of cross-links, primarily desmosine and isodesmosine (Fig. 1), which are formed from four lysine residues, two each from two different peptide chains. The cross-linking sequences KAAAK and KAAK were observed to repeat at least six times in the soluble precursor protein, tropoelastin, which is comprised of 800–850 amino acids. Determination of the amino acid sequence of porcine tropoelastin using tryptic peptides is 80% complete [1]. The largest porcine tryptic peptide is 81 residues; the dominant feature of this peptide is the repeating pentapeptide sequence $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ with $n = 11+$ in pig. Conformational studies on oligo- and polypentapeptides of the above repeating sequence, carried out in this laboratory, resulted in the development of a new class of conformations called β-spirals in which a β-turn occurs with regularity along the helical axis. A new mechanism of elasticity, 'a librational entropy mechanism', has been put forward to explain the elastic behavior of the polypentapeptide [2–4]. This contrasts with the 'random chain-network theory' previously proposed for elastin [5]. Demonstration of the librational entropy mechanism and the nonrandom nature of elastin has been achieved by numerous physical characterizations: light and electron microscopy; circular dichroism; Raman spectro-

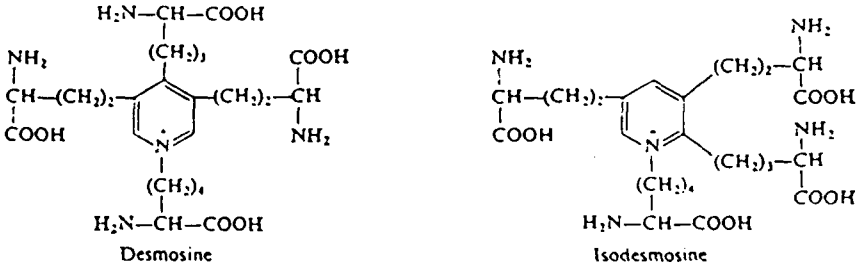


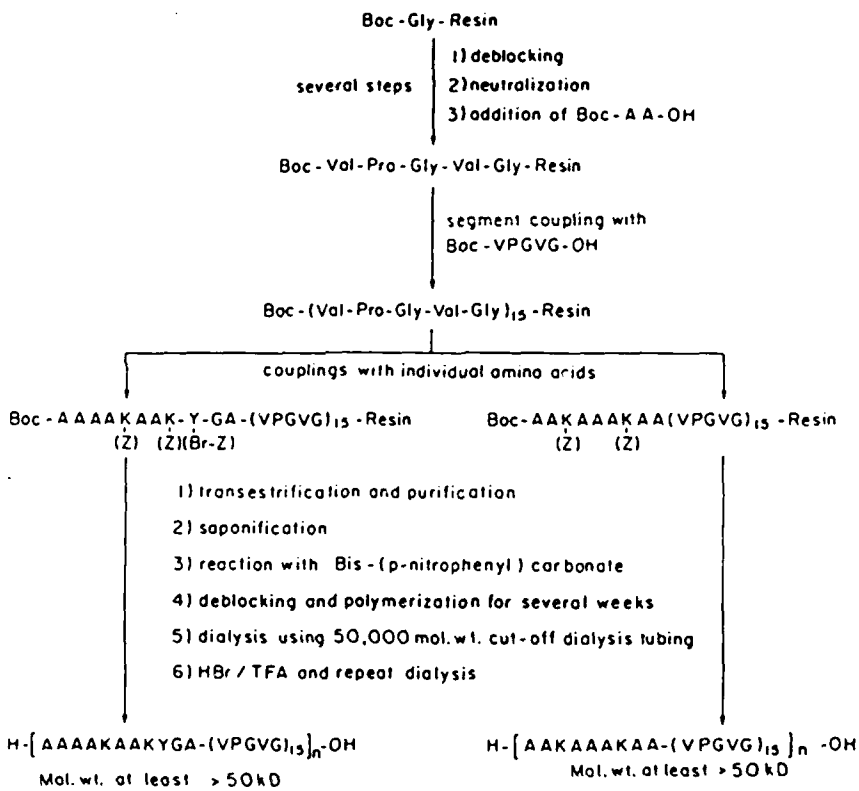
Fig. 1. Structures of desmosine and isodesmosine.

scopy; dielectric relaxation; nuclear magnetic resonance (NMR); temperature dependence of length, of force development, and of elastic modulus; and composition studies [6].

In order to develop polypeptides as models for the natural insoluble elastin, it is useful to synthesize a polypentapeptide molecule with cross-linking sequences and then polymerize them to yield very high molecular weight polymers, which on enzymatic cross-linking by lysyl oxidase could result in biomaterials with physical properties similar to elastin.

Here we report the synthesis of the two polymers $[XL\text{-}1\text{-}(VPGVG)_{15}]_n$ and $[XL\text{-}2\text{-}(VPGVG)_{15}]_n$ where XL-1 is the cross-linking sequence, AAAAKAAK-YGA, and XL-2 is the second cross-linking sequence, AAKAAAKAA.

Synthesis: The synthesis of the two monomeric peptides, AAAAKAAKYGA-



Scheme 1. Synthesis of elastin peptide models with cross-linking sequences.

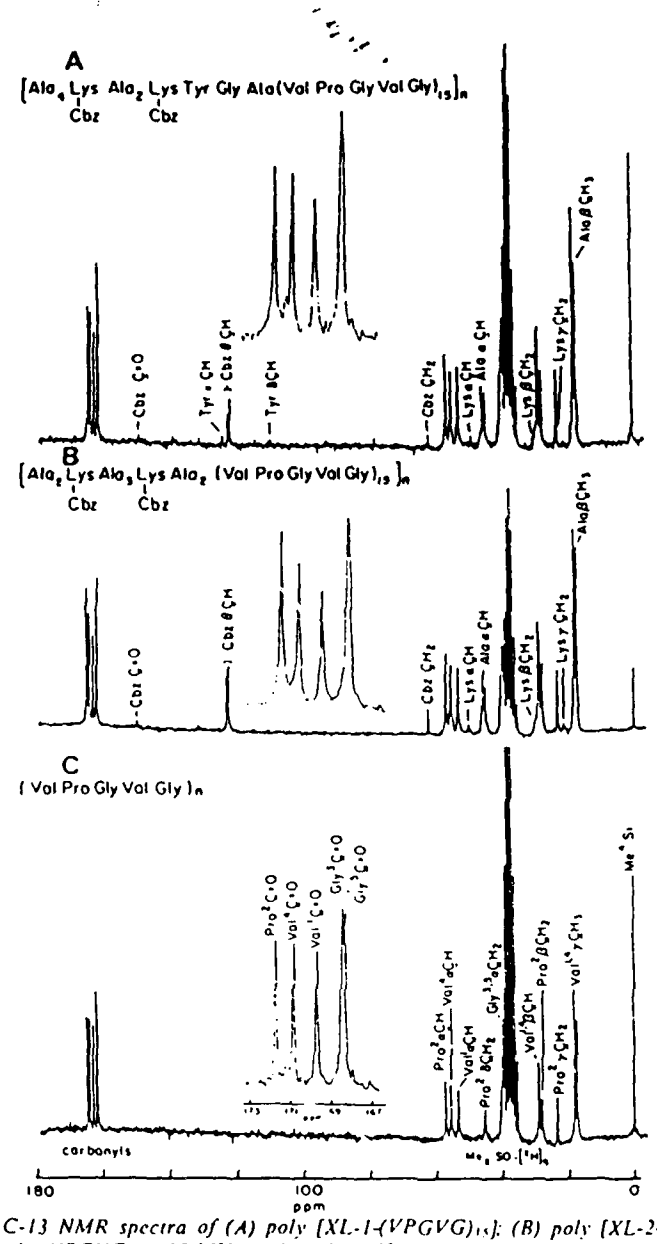


Fig. 2. C-13 NMR spectra of (A) poly [XL-1-(VPGVG)15]; (B) poly [XL-2-(VPGVG)15] and (C) poly (VPGVG) at 25 MHz in dimethylsulfoxide.

$(VPGVG)_{15}$ and AAKAAAKAA-$(VPGVG)_{15}$, was carried out by the solid phase methodology [7] (Scheme I). The first pentamer sequence VPGVG was built on the 1% cross-linked Merrifield resin by adding a single amino acid at a time. The next 14 pentamer units were attached by the segment condensation approach using Boc-VPGVG-OH, synthesized by the classical solution methods as reported earlier [8]. The amino acids in the cross-linking sequences were once again coupled by stepwise condensation. The side-chain functional groups of Lys and Tyr were protected by Cbz and O-Br-Cbz groups, respectively. The segment condensations were carried out in DMF, TFE-CH_2Cl_2 or TFE alone in the presence of HOBt. Occasionally preformed symmetrical anhydrides of individual amino acids had to be used to ensure the completeness of the reaction. In order to check the progress of the coupling reactions, the peptides were removed from the resin as methyl esters at various stages of peptide synthesis when there were 6, 9, 12, and 15 pentamer units attached to the growing peptide chain. An approximate determination of the peptide chain length during the course of the synthesis could be obtained from a plot of ln (molecular weight) versus the midpoint of the temperature profile of turbidity (T_{P_T}) as well as from NMR end group analysis [9]. After the synthesis was completed on the resin, the Boc-protected peptides were removed as methyl esters by transesterification and purified by repeated precipitations from different solvent systems. The purity of the peptide was checked by C-13 magnetic resonance (C-13 NMR) spectra and amino acid analysis. After saponification, the peptides were converted to p-nitrophenyl esters by reacting with excess of bis (*p*-nitrophenyl) carbonate [10] for several days.

The Boc group was removed and the peptides were polymerized for 8–12 weeks in dimethylsulfoxide in the presence of *N*-methylmorpholine. After diluting with water, the polymers were dialyzed against water using a 50-kDa cutoff dialysis tubing for 15 days and the retentates were lyophilized. The purity of the polymers was again checked by C-13 NMR (Fig. 2) and amino acid analysis.

In conclusion, the feasibility of synthesizing large peptides of 86 and 84 amino acids in length and polymerizing them into polymers having molecular weights of greater than 50 kDa is demonstrated. The next step will be to remove the Z groups on lysines; submit the polymers separately, and mixed, to lysyl oxidase treatment; study the various intermediate oxidation products and also the formation of final desmosine and isodesmosine structures; and compare the mechanical properties of the insoluble matrix to those of natural elastin.

Acknowledgements

This work was supported in part by NIH Grant HL-29578 and Department of the Navy, Office of Naval Research Grant N00014-K-86-0402.

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ABSTRACT FORM

SYNTHESIS OF TWO COMPONENT MODELS OF ELASTIN; Kari U. Prasad, M. Iqbal and D. W. Urry; Lab. of Molecular Biophys., The University of Alabama at Birmingham, P. O. Box 311/Univ. Sta., Birmingham, AL 35294

The sequence data between the cross-linking regions of tropoelastin, obtained by tryptic digestion, is almost completely determined. The largest tryptic peptide is about 80 AA long and contains 11 (in pig) or 13 (in chick) pentamer (VPGVG) repeats in a continuous sequence. The polypentapeptide, $(VPGVG)_n$, on γ-irradiation cross-linking has been shown to be an entropic elastomer. Two natural cross-linking sequences frequently observed are AAAAKAAKY(F)GA (XL-1) and AAKAAAKAA (XL-2). Two chains of tropoelastin having such sequences cross-link enzymatically by lysyloxidase to form the native elastin. The cross-links utilize four lysines in the formation of desmosine and isodesmosine structures which are substituted pyridiniums. One of the four lysines, the one preceding Y or F, contributes to the nitrogen atom of the heterocyclic ring having been protected from oxidization by lysyloxidase. To test the cross-linking hypothesis and possibly to obtain a biomaterial suitable as a substitute for native elastin, we have prepared polypentapeptide attached to each of the cross-linking sequences (XL-1 and XL-2). Here we present the synthesis of two chains of 86 and 84 AA long, XL-1-$(VPGVG)_{15}$ and XL-2-$(VPGVG)_{15}$, by the solid phase methodology. After characterization by amino acid analysis and NMR, they are activated as p-nitrophenyl esters and polymerized to yield Poly [XL-1-$(VPGVG)_{15}$]-OH and Poly [XL-2-$(VPGVG)_{15}$]-OH with molecular weights demonstrated by dialysis to be greater than 50 kD. This is the first demonstration that monomeric chains of such length (86 or 84 AA) could be polymerized to such a degree. These two polymers are then subjected to lysyloxidase individually and as a mixture to investigate the formation of desmosine and isodesmosine cross-links.

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DEADLINE FOR RECEIPT OF ABSTRACT: FEBRUARY 1, 1987

Entropic Elastomeric Force in Protein Structure/Function

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Abstract

Briefly noting earlier studies on the polypentapeptide of elastin, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, and on elastin, it is emphasized that entropic elastomeric force can be exhibited by nonrandom, anisotropic polypeptide systems and therefore that entropic elastomeric force does not necessarily result from isotropic random chain networks as required by the classical theory of rubber elasticity, nor does it result from solvent entropy effects as deduced from the slow loss of elastomeric force on thermal denaturation. Instead entropic protein elasticity can be the result of internal chain dynamics, specifically of librational processes that become damped on chain extension. This new mechanism of entropic protein elasticity allows for an understanding not only of elastin but also of the passive tension of striated muscle, of the voltage-dependent interconversion between open and closed conductance states in the sodium channel of squid nerve, and of protein elastic forces producing strain in a substrate bond during enzyme catalysis. Because entropic elastomeric force develops as a result of an inverse temperature transition, it becomes possible to shift the temperature of the transition to higher or lower temperatures by decreasing or increasing, respectively, the hydrophobicity of the elastomeric polypeptide chain. In warm-blooded animals this allows for biochemical modulation of the relaxation or development of entropic elastomeric force by an enzymatically modulated decrease or increase of the hydrophobicity, as for example, by phosphorylation or dephosphorylation of the elastomeric polypeptide chain. This understanding provides a mechanism for modulating protein function, whether for example enzymatic or channel, a mechanism for the remarkable reversible structural processes that attend parturition, and a mechanism for the connective tissue anomalies of wound repair and environmentally induced lung disease.

Introduction

Presently recognized as the primary elastomeric protein of warm-blooded animals, elastin is the second most prevalent protein in the extracellular matrix, only collagen is more common [1]. The nature of the elastomeric force was demonstrated by Hoeve and Flory in 1958 to be dominantly entropic in origin [2]. This is an important statement as it provides an understanding of the durability of elastin where single elastin fibers can last the lifetime of an individual, which when used in the human vascular system means undergoing more than one billion stress/strain cycles. That elastin is a dominantly entropic elastomer was reaffirmed by Hoeve and Flory in 1974 when they continued to insist that "A network of random chains within elastin fibers, like that in a typical rubber, is clearly indicated" [3]. This perspective has dominated thinking with respect to protein elasticity for nearly three decades and remains a staunchly held perspective [4–11]. Accordingly, the insistence that entropic elastomeric force

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requires a random network of chains has precluded application to protein systems known to be *nonrandom* chain networks.

Studying the molecular structure and function of the polypentapeptide of elastin, this laboratory has demonstrated a new mechanism of entropic elasticity for this most striking primary structural feature of elastin [12], occurring within the longest sequence between cross-links, a sequence twice as long as any other possible elastomeric sequence between cross-links [12, 13], and has demonstrated its applicability to the elastin fiber as a whole [14, 15]. The mechanism derives from internal chain dynamics and is called the librational entropy mechanism of elasticity. In this report the new mechanism of entropic elasticity is considered relative to other protein systems where elastomeric force is implicated but where the proteins cannot be described as random chain networks.

In particular, the identification and possible origins of entropic elastomeric force are considered briefly. The applicability of internal chain dynamics, that is, librational processes, to protein elasticity as newly understood in elastin is extended to an understanding of the passive tension in muscle, of changing conductance states of channels, and of enzyme mechanisms. Furthermore, the relevance of structural transitions to and from the elastomeric state is considered in regard to elastogenesis, wound repair, fibrotic lung disease, and to processes attending parturition and their reversal, that is, cervical ripening and pubic ligament formation.

Possible Origins of Entropic Elastomeric Force in Proteins

Elasticity, of course, is the property whereby a material resists and recovers from deformation. The elastomeric force, f, can be considered to be comprised of two components: an internal energy component, f_e, and an entropy component, f_s, or

$$f = f_e + f_s \tag{1}$$

Following Flory and colleagues, the relative magnitudes of the internal energy and entropy components can be determined by means of thermoelasticity studies [16]. In these studies the elastomer is extended to a fixed length and the elastomeric force is measured as a function of temperature. A plot of $\ln[f/T(°K)]$ versus temperature allows evaluation of the f_e/f ratio, or

$$\frac{f_e}{f} = -\bar{T}\frac{\partial \ln(f/T)}{\partial T}_{P,L,eq} - \frac{\beta_{eq}T}{\alpha^3(V_i/V) - 1} \tag{5}$$

where the experiment is carried out at constant pressure, P, with the elastomer at fixed length, L, and with the elastomeric matrix in equilibrium, eq, with the solvent. The second term in Eq. (2) is a correction term allowing the analysis to proceed at constant pressure rather than constant volume, and in equilibrium with solvent rather than at constant composition [17]. In this term $\beta_{eq} = (\partial \ln V/\partial T)_{P,L,eq}$ is the thermal expansion coefficient; α is the fractional increase in length; and V_i and V are the elastomer volumes before and after elongation. This correction term is of the order of 0.1 for elastin [18] as well as for the polypentapeptide of elastin [19]. Figure 1 shows thermoelasticity studies for elastin and for the polypentapeptide of elastin, where particularly for the latter, the near zero slope argues for a dominantly entropic elas-

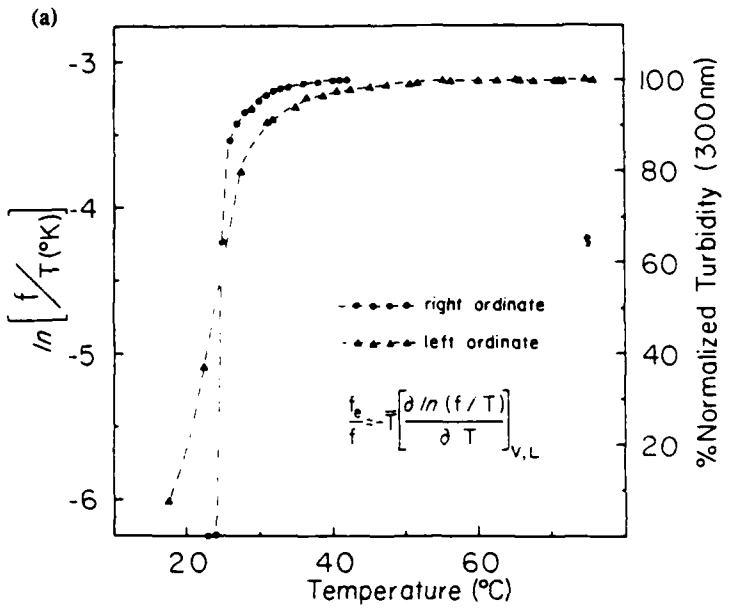

Figure 1. Thermoelasticity studies: Temperature dependence of elastomeric force at fixed extension. (A) Polypentapeptide of elastin cross-linked by 20 Mrads of γ-irradiation while in the coacervate state which is obtained by raising the temperature of solutions of polypentapeptide plus water from 20°C to 40°C to form a dense viscoelastic phase that is 62% water, 38% peptide by weight. The sample is extended to 60% at 40°C and then the force is measured as a function of temperature. In going from 20 to 40°C there is an abrupt development of elastomeric force, but above 40°C the plot of ln[force/T(K)] versus temperature exhibits a near zero slope. Since the slope is proportional to the f_e/f ratio and since this is near zero, it can be argued that the polypentapeptide of elastin exhibits dominantly entropic elastomeric force in the temperature range above 40°C. The development of elastomeric force in the 20–40°C range correlates with an inverse temperature observed by numerous physical methods and seen to be a process of self-assembly into fibers. Therefore the polypentapeptide of elastin is an ansiotropic, entropic elastomer. (B) Ligamentum nuchae elastin exhibits a similar development of elastomeric force on raising the temperature over a somewhat broader temperature range, but at higher temperatures the slope approaches zero and a dominantly entropic elastomer has been concluded. This conclusion is assisted by carrying out the study in 30% ethylene glycol in water which shifts the transition to lower temperature giving a wider temperature range where the slope is near zero. In both cases there is plotted on the right-hand side the temperature profile for aggregation, actually for fiber formation as observed by microscopy, for the constituent peptide on protein. (●—●) Right ordinate; (▲—▲) left ordinate. Reproduced with permission from Ref. 20.

tomeric force [20]. On changing the solvent to ethylene glycol-water, 3:7 by volume, the rapid rise in elastomeric force is shifted to lower temperature and the near zero slope becomes more apparent for elastin [unpublished data, 2, 3]. Furthermore, a near zero slope for elastin has been found in triethylene glycol [10]. Thus elastin and the polypentapeptide of elastin are considered to be dominantly entropic elastomers.

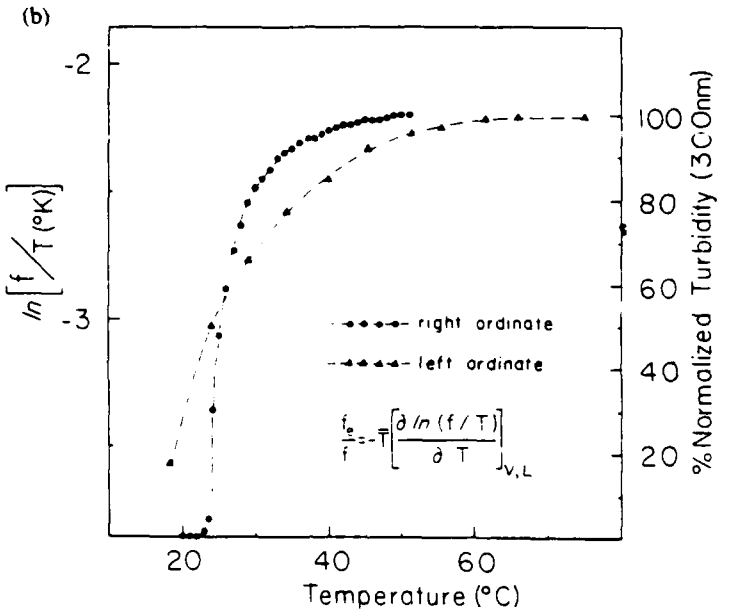

Figure 1. (*Continued.*)

The Classical Theory of Rubber Elasticity for Random Chain Networks

The classical or statistical theory of rubber elasticity holds that entropic elastomeric force derives from random chain networks [21–23]. At rest the network is described as being comprised of a random distribution of chain end-to-end lengths. This is the highest entropy state. On stretching, the distribution of end-to-end lengths is displaced from that of highest entropy. This decrease in entropy provides the resistance to deformation and the driving force for recovery. A representative distribution of chain end-to-end lengths is given in Figure 2 where $W(r)$ is the probability distribution of the end-to-end lengths, r, in nm. In this theory the f_e/f ratio is given by $d \ln\langle r^2\rangle o/dT$ where $\langle r^2\rangle o$ is the mean square end-to-end chain length.

Solvent Entropy

When the elastomer is comprised of hydrophobic groups that become exposed to polar solvents such as water on extension, several workers [24–27] have suggested that the formation of clathrate-like water surrounding these exposed hydrophobic groups constitutes a decrease in entropy that would provide an entropic restoring force.

Internal Chain Dynamics: Librational Process

Another source of decrease in entropy on extension has been derived from studies on the polypentapeptide of elastin [14, 15, 28–31] but it is an entirely general mechanism. It asserts that chain segments within a bulk matrix have freedom to undergo rocking motions. Since the chain segments in the dense, cross-linked bulk matrix will be essentially immobilized at their ends, motion occurs by rotation about one bond

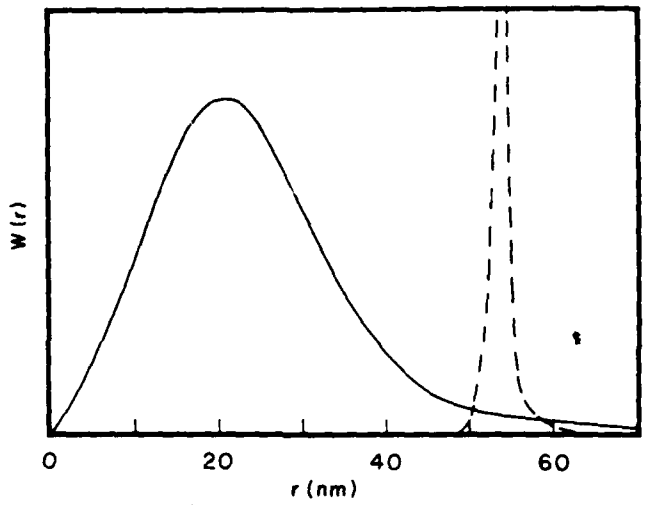

Figure 2. Probability distribution, $W(r)$, of chain end-to-end lengths r in nm. The solid line gives the distribution for a freely jointed chain with 10,000 segments of 0.25 nm each [22]. This is a random distribution of end-to-end lengths representing the highest entropy state. On stretching of a bulk cross-linked matrix of such a collection of chains, the distribution is displaced from that of a random chain network. The decrease in entropy provides a resistance to deformation and a restoring force. This is a description of the classical theory of rubber elasticity. The dashed curve represents a possible distribution of chain end-to-end lengths where the chains are nearly the same length. In this case an entropic restoring force can derive from the damping of internal chain dynamics on extension. This has been referred to as the librational entropy mechanism of elasticity which as represented, can occur with anisotropic fibrillar elastomers.

being paired with compensating rotations about one or more other bonds such that rocking motions or librational processes occur. On stretching these librational motions become damped. This has been termed the librational entropy mechanism of elasticity [29].

Elastomeric Processes in Protein Systems

The Polypentapeptide of Elastin

As shown in Figure 1(A), when the polypentapeptide of elastin is γ-irradiation cross-linked at a concentration of about 40% peptide, 60% water by weight, the resulting elastomer exhibits dominantly entropic elastomeric force above 40°C. On raising the temperature from 20° to 40°C, however, there is a dramatic development of elastomeric force. This development has been demonstrated by five independent physical methods — nuclear magnetic resonance structural and relaxation studies, dielectric relaxation studies, circular dichroism studies, microscopic characterization, and composition studies — to correlate with development of molecular order, that is, to correlate with an inverse temperature transition [14, 19, 32]. In the 20–40°C temperature range, development of molecular order correlates with development of elastomeric force. That the entropically elastomeric state above 40°C is an ordered state is further demonstrated by thermal denaturation followed by circular dichroism [19],

by extrusion of water [15, 19] and most directly by the slow loss of elastomeric force and of elastic modulus [15, 33], all demonstrated by heating at 80°C. As the elastomeric state is not a random chain network and since at 80°C destructuring of clathrate-like water would occur with time constant of the order of nanoseconds or less whereas the loss of elastic modulus at 80°C occurs with a half-life of days, the entropic elastomeric force must be due to internal chain dynamics.

The proposed elastomeric structure of the polypentapeptide of elastin is given in Figure 3 [31, 34, 35, 28] and the effect of stretching on the damping of the librational

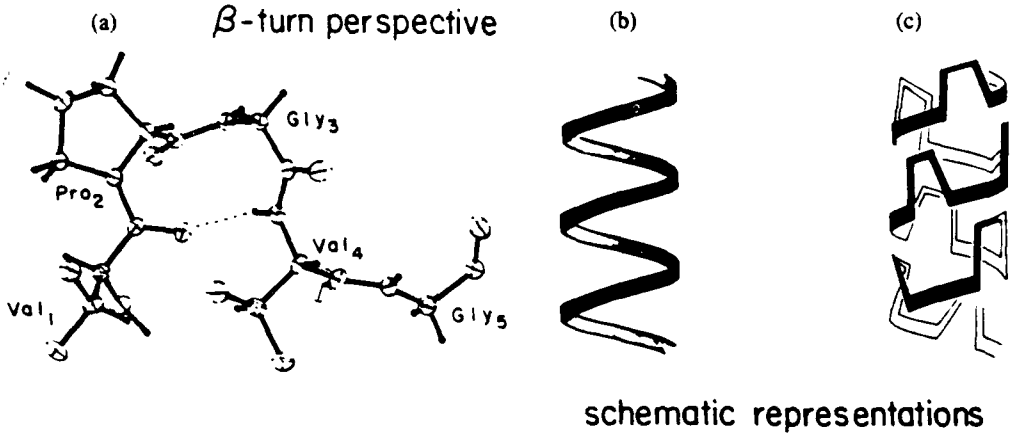

Figure 3. Proposed conformation of the elastomeric state of the polypentapeptide of elastin: (A) β-turn perspective showing the 10-atom hydrogen-bonded ring which utilizes the $Val^1C—O\cdots HN\ Val^4$ hydrogen bond. This conformation was first developed in solution using NMR methods and then demonstrated in the crystal for the cyclopentadecapeptide which was shown to be the cyclic conformational correlate of the polypentapeptide of elastin. Reproduced with permission from Ref. 34. (B) and (C) Schematic representations of the helical state (β spiral) of the polypentapeptide of elastin which is the elastomeric state. In (C) the β turns are included showing them to function as spacers with hydrophobic contacts between the turns of the spiral. Reproduced with permission from Ref. 31. (D) Detailed stereo pair of the spiral axis view showing space for water within the β spiral and showing suspended segments between the β turn. The suspended segment runs from the α-carbon of Val^4 to the α-carbon of Val^1 and is referred to as the Val^4-Gly^5-Val^1 suspended segment. It is within the segment where the large amplitude, low frequency librational motions are most pronounced (see Figs. 4 and 5). Reproduced with permission from Ref. 31. (E) Stereo pair of the side view of the β spiral of the polypentapeptide of elastin. This is one of a family of closely related β spirals. Seen here are gaps in the surface of the β spiral on each side of the suspended segments. The contacts between turns of the spiral utilize the Val and Pro hydrophobic side chains. The structure in (E) is displayed the same as in the schematic representation in (C). It is the optimization of intramolecular hydrophobic interactions that is responsible for β-spiral formation. Reproduced with permission from Ref. 35. (F) and (G) Supercoiling of β spirals to form twisted filaments of dimensions similar to those observed in transmission electron micrographs of negatively stained polypentapeptide, α-elastin and tropoelastin coacervates [14, 30, 40] and of negatively stained elastin. The structure is given in (F) in α-carbon to α-carbon virtual bond representation and in (G) in terms of spheres of different sizes centered at the α-carbon locations. Reproduced with permission from Ref. 28.

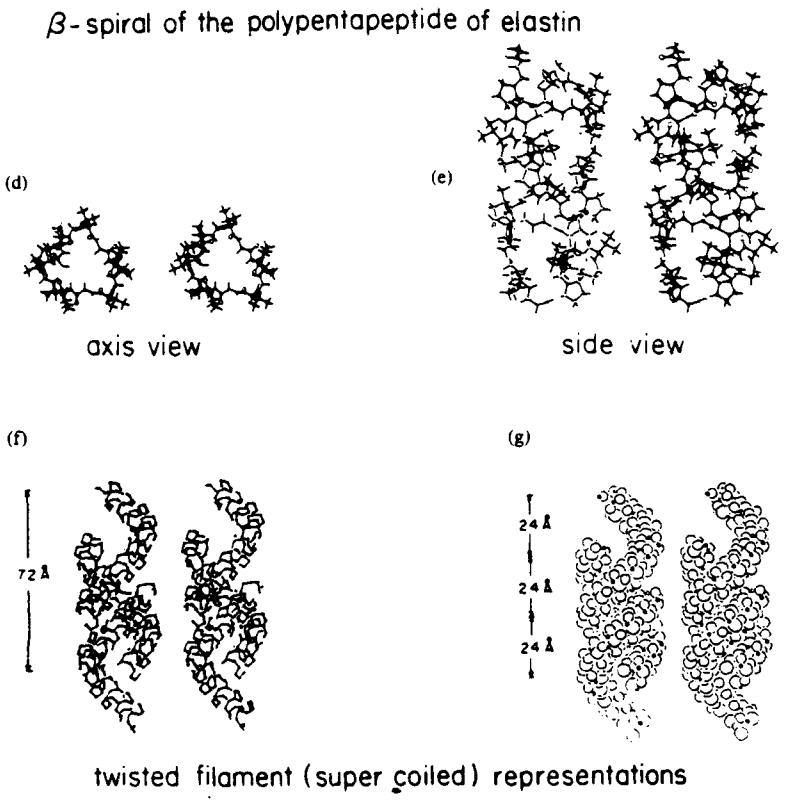

Figure 3. (*Continued.*)

motions is shown in Figure 4 [29]. The regularly repeating structure of the polypentapeptide provided the opportunity to demonstrate unequivocally that entropic elastomeric force occurs on formation of a regular nonrandom structure. One of the particularly interesting demonstrations is provided by dielectric relaxation studies [36]. At 20°C where there is minimal elastomeric force, the real part of the dielectric permittivity in the 1 GHz to 1 MHz frequency range exhibits a monotonically increasing curve. This is shown in Figure 5. As the temperature is raised and elastomeric force develops, there develops a localized Debye-type relaxation centered near 20 MHz. This has been assigned to a peptide librational mode [14, 36]. The intensity at 40°C, $\Delta\varepsilon \simeq 70$, and the localized nature of the relaxation require a regular nonrandom elastomeric state and the relaxation identifies a backbone (peptide) librational mode that is directly contributing to the high entropy of the relaxed state. While the phenomenology enumerated above require setting aside the random chain network analysis and the elimination of solvent entropy as a consideration, this experiment allows direct observation of the responsible internal chain dynamics. This is the remarkable contribution of the polypentapeptide of elastin.

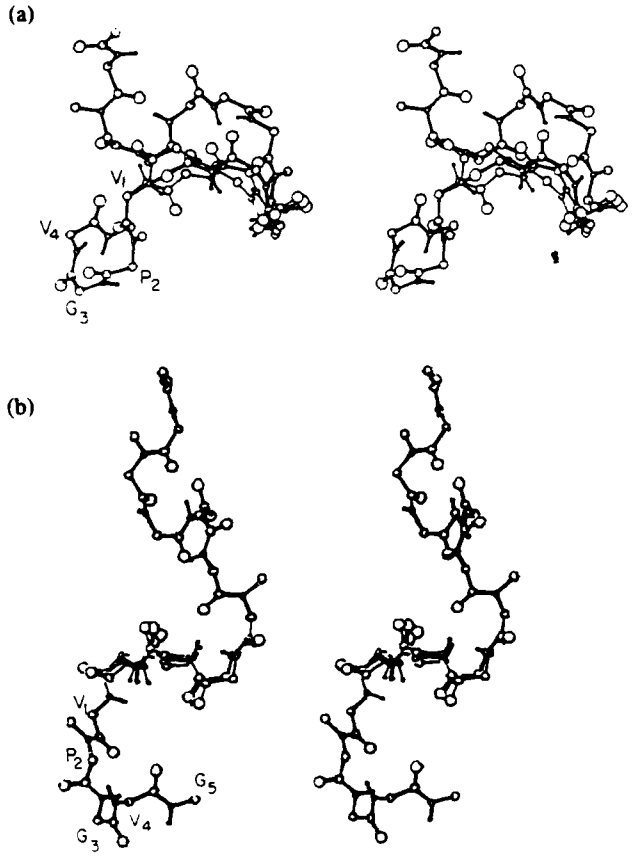

Figure 4. Stereo pair view of a pentadecapeptide segment in the β-spiral conformation of Fig. 3(E) in which the central Val^1 α-carbon to Val^1 α-carbon pentamer has been allowed to assume conformations within a 2 kcal/mole residue cut-off energy. What is observed is a rocking motion of peptide moieties. In the relaxed state in (A), large librational motions are observed whereas in an extended state, in (B) at 130% extension, the librational amplitudes are markedly damped. This decrease in amplitude of the librations and possibly an associated increase in the frequency of the librational motions on extension is the decrease in entropy that resists elongation and that provides the restoring force. This is called the librational entropy mechanism of elasticity and this mechanism for developing entropic elastomeric force can occur in any polypeptide segment wherein the structure favors librational processes. Reproduced with permission from Ref. 29.

The Elastin Fiber

In the case of the elastin fiber, 3 of the 5 physical methods, utilized to demonstrate that increase in elastomeric force in the below 40°C temperature range correlates with increase in molecular order in the polypentapeptide, have been applied to elastin, to the precursor protein, or to a chemical fragmentation product of elastin. Those physical methods are microscopy [37–40], dielectric relaxation [41], and circular dichroism [42]. Furthermore, thermal denaturation has been directly observed on elastin, as on the polypentapeptide of elastin, by following the slow loss of elastomeric force in

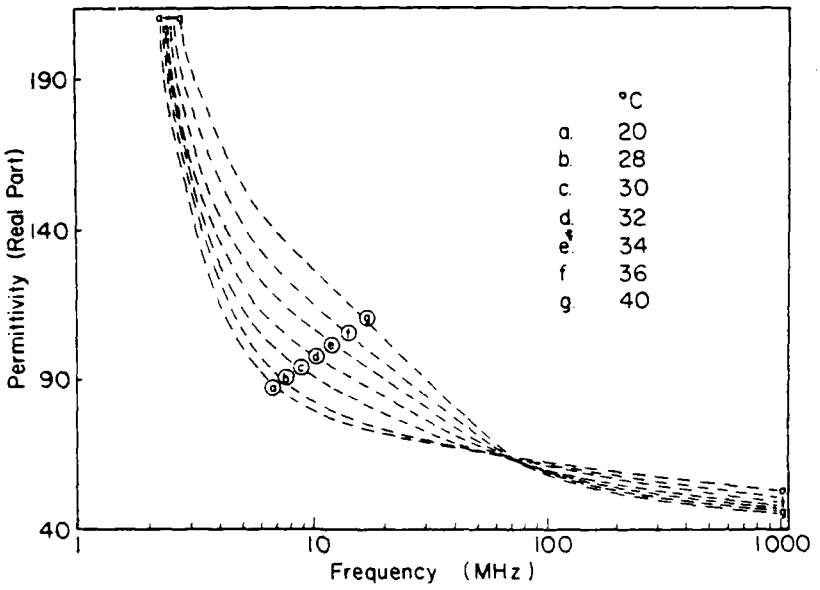

Figure 5. Dielectric permittivity (real part) of the polypentapeptide of elastin coacervate which is 38% peptide and 62% water by weight. On raising the temperature from 20 to 40°C there develops an intense, localized, Debye-type relaxation near 20 MHz. As the only dipolar entities are water and peptide moieties and because the intensity of the relaxation is so large and the frequency relatively low with a low temperature dependence, the relaxation is assigned to a peptide librational motion. Because the relaxation is at a localized frequency the polypentapeptides must be developing a regular structure as the temperature is raised from 20 to 40°C. The development of this relaxation correlates with the development of elastomeric force observed in Figure 1(A). The relaxation is taken to be due to the librational motions shown in Figure 4(A). Reproduced with permission from Ref. 36.

a thermoelasticity study and the slow loss of elastic modulus monitored by stress/strain curves at 37°C which resulted from heating at 80°C [15, 33]. Therefore, the entropic elastomeric force exhibited by this protein is not due to a random chain network nor is it due to the formation of clathrate-like water structures, rather it too must derive from internal chain dynamics. It may be noted that the slow thermal denaturation is in the practical sense irreversible in water. Here again the internal chain dynamics can, with the insight of the studies on the polypentapeptide of elastin and with awareness that the most prominent sequence between cross-links is where the polypentapeptide resides, be directly observed by dielectric relaxation studies on α-elastin (the chemical fragmentation product of elastin) in the 1 GHz to 1 MHz frequency range as shown in Figure 6 [41]. While the intensity of the relaxation is less, as expected with the polypentapeptide being a fractional component of α-elastin, a relaxation is again observed near 20 MHz.

Elastomeric Filaments of Muscle

Studies of Maruyama et al. [43, 44] and of Wang et al. [45, 46] have resulted in the

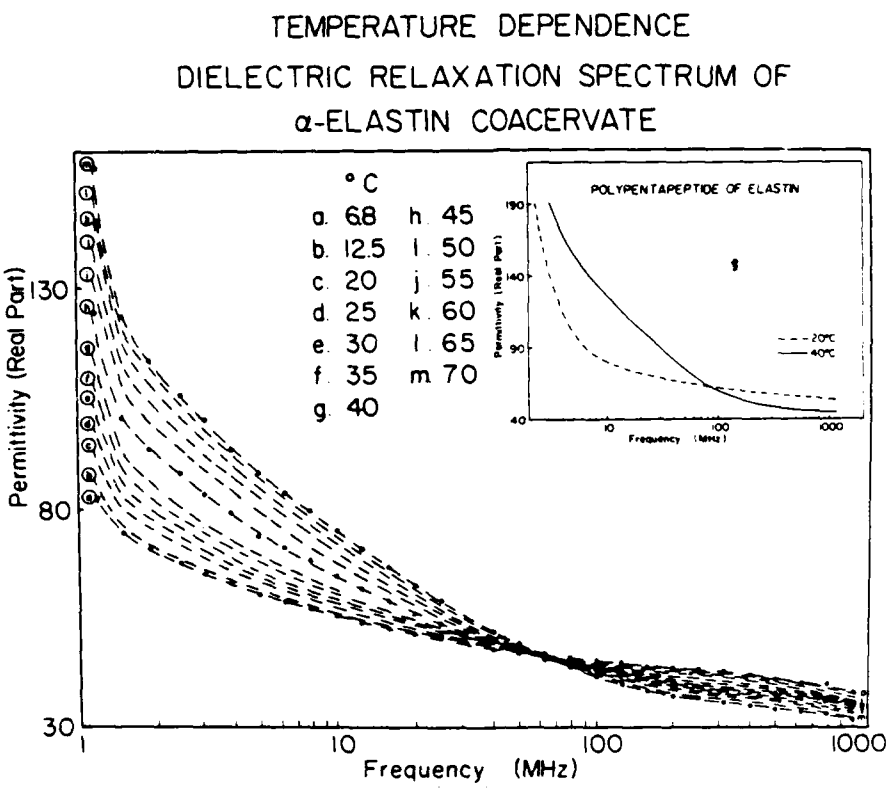

Figure 6. Dielectric permittivity (real part) of the coacervate state of α-elastin which is a 70,000 molecular weight chemical fragmentation product of elastin. Below 15°C there is a monotonically increasing permittivity from several hundred MHz to 1 MHz. But as the temperature is raised there develops a relaxation near 20 MHz. A α-elastin contains the polypentapeptide of elastin which exhibits a similar relaxation (see inset and Fig. 5) this relaxation in α-elastin has been assigned to the same or similar peptide librational processes. The development of the relaxation with temperature in the 15° to 45°C temperature range correlates with the development of elastomeric force over the same temperature range as seen in Figure 1(B). Thus this along with considerable other data on elastin, α-elastin and tropoelastin including thermal denaturation of elastomeric force and elastic modulus of elastin at 80°C [33] allows the conclusion that elastin too is a nonrandom entropic elastomer. Reproduced with permission from Ref. 41.

isolation of a several million molecular weight elastic protein from muscle. Efforts to characterize this protein microscopically have demonstrated the protein to be filamentous [43]. This protein becomes a possible explanation for the passive tension of muscle and for the residual passive force exhibited when the sarcomere length has been extended beyond the point where the thick and thin filaments no longer overlap. Microscopic studies on pulled fibers have led to the identification of long narrow filaments either connecting the thick filaments to the Z lines or directly running from Z line to Z line [47, 48]. Consistent with an effort to understand elastomeric force in terms of random networks, it has been suggested that the stretching itself causes the filaments to form from a gel state (see discussion following Ref. 47). Consistent with the random chain network theory of entropic elasticity, efforts are made to understand

elasticity in terms of an isotropic gel state rather than in terms of the anisotropic filaments observed microscopically both on the pulled fibers and for the isolated elastic protein of muscle. Having demonstrated internal chain dynamics to be the source of entropic elastomeric force, it now becomes possible to understand durable nonrandom, anisotropic elastomeric filaments, and thereby to accept the microscopic observations of the isolated elastic protein of muscle and of the pulled muscle fibers.

Interconversion of Sodium Channel Conductance States

To a physical chemist, one of the very challenging aspects of biology is understanding the molecular structure and mechanisms of ion-selective, voltage-dependent transmembrane channels. The conductance state — open, closed, refractory — depends on the transmembrane potential. It is of fundamental interest, for example, to understand what structural changes and processes result in changing the conductance state. This issue has been addressed in an interesting way by Rubinson [49] who modelled the sodium channel opening/closing equilibrium of squid nerve "as a charged region of a macromolecule moving under the influence of the applied field and confined elastically by interconnection with other masses." The result was the characterization of the mechanical properties of the polypeptide chain segment which controlled the gating process as rubber-like with an elastic modulus in the range of that of elastin. Taking the elastic modulus to be 5×10^6 dynes/cm^2 as for elastin, the ratio of the cross-sectional area to length (~400Å) of the connecting chain segment would not be unlike that of the polypentapeptide β-spiral in Figure 3. This is not to imply in any way that a β-spiral like that of the polypentapeptide of elastin actually exists in the sodium channel but rather to emphasize that internal chain dynamics and specifically librational processes rather than random chain networks would be required to understand this elastomeric process.

Enzyme Mechanisms

Several aspects of enzyme mechanisms may involve entropic elastomeric forces within the protein, for example, the structural rearrangements resulting from the binding of an allosteric effector [50], induced fit elements of substrate binding [51], and the catalytic process itself. In the former two processes it is apparent that binding to the surface of a viscoelastic protein could result in stretch-damping of librational motions within proximal regions of the active site. In addition the catalytic process itself has been considered in terms of elastic forces. Recalled, for example, is the elastomeric "rack" of Eyring et al. [52]. A recent elegant description of this element of enzyme catalysis has been presented by Gavish [53] in an exposition of "molecular dynamics and the transient strain model of enzyme catalysis." With emphasis on the viscoelastic properties of proteins [54], Gavish described a detailed model for stress and strain in the enzyme-substrate complex. The protein exerts an elastic force on the scissile bond of the substrate resulting in a strain that contributes to the potential energy required for bond cleavage. An effective means of increasing the rate of the catalytic process would seem to be to employ an entropic elastomeric force to induce strain in a substrate. Gavish states [53] "factors that dominate structural mobility in proteins should affect enzyme catalysis." On the basis of the new

understanding of entropic protein elasticity it might be said that *factors that modulate entropic elastomeric force should modulate enzyme catalysis*. For entropic elastomeric force as demonstrated by the polypentapeptide of elastin, it is not mobility *per se*, but rather it is mobility arising from a regularity of structure that gives rise to force capable of inducing significant strain. As shown by the nuclear magnetic resonance (NMR)-derived rotational correlation times [15], the mean mobility of the peptide moieties is greater at 25°C before the inverse temperature transition than at 37°C after the inverse temperature transition, yet the entropic elastomeric force is minimal at 25°C and dramatically increases until 37°C [15]. Thus it is not motion *per se* but the nature of the motion. In the dielectric relaxation studies at 25°C there is no localized relaxation in the 1 GHz to 1 MHz frequency range, but as the temperature is raised to 40°C there develops in concert with the development of elastomeric force an intense, Debye-type relaxation near 20 MHz, indicating motion within a regular structure [36]. Thus it is coherent motion (e.g., a librational mode) within a regular structure that gives rise to entropic elastomeric force. This provides for an anisotropic structure capable of producing a strain in an enzyme substrate by means of an entropic elastomeric force.

Modulation of Transitions in the Elastomeric State: Turning Entropic Elastomeric Force On and Off

In the preceding discussion of elastomeric processes in protein systems it was generally the elastomeric state itself that was considered, but the modulation of the transition to and from the elastomeric state can be an effective means of turning on and off an entropic elastomeric force. The modulation can be biochemical and it can be involved in such disparate processes as the modulation of enzyme catalysis, wound repair, the destruction of elastic tissue in environmentally induced lung disease, and relaxin-induced cervical ripening and pubic ligament formation attending parturition and their reversal.

Elastogenesis

Before addressing the more biomedical issues, it is necessary to consider the implications arising from the fact that, for elastin and the polypentapeptide of elastin, elastogenesis arises out of an inverse temperature transition and is therefore dependent on the hydrophobicity of the chains which are to constitute the elastomer. Generally, elastogenesis of elastin has been considered to be the physical process of fiber formation but as will be seen below it is simultaneously fiber formation *and* the development of elastomeric force. This is not possible within the constraints of the classical theory of rubber elasticity requiring, as it does, random chain networks, because the formation of an isotropic random chain network could not result in the formation of anisotropic fibers. *Once the random chain network perspective is set aside, it becomes apparent that modulation of elastomeric force in homoiothermic animals can be achieved by shifting the temperature range in which the inverse temperature transition occurs.*

Effect of Changing the Hydrophobicity. Using the polypentapeptide of elastin as the model elastomer, analogs can be prepared in which the hydrophobicity of

the repeating unit is changed. Three physical characterizations can be compared: (1) the temperature profile for aggregation, which is actually the temperature profile for fiber formation, (2) the temperature dependence of conformational change followed by circular dichroism, and (3) the temperature dependence of elastomeric force of the γ-irradiation cross-linked analog which has been stretched to a fixed length at 40°C. As shown in Figure 7, these transitions occur near 30°C for (Val^1-Pro^2-Gly^3-Val^4-Gly^5)n, the polypentapeptide of elastin. When the hydrophobicity of the repeating unit is increased as in (Ile^1-Pro^2-Gly^3-Val^4-Gly^5)n, the Ile^1 polypentapeptide, the temperature of the transition, as followed by all three means, shifts to lower temperature by some 20°C to near 10°C [55]. When the hydrophobicity of the repeating unit is decreased as in (Val^1-Pro^2-Gly^3-Gly^4)n where the Val^4 residue has been deleted, the temperature of the transition shifts some 20°C higher to a temperature near 50°C [56]. These shifts are proportional to the hydrophobicity of the repeating unit as estimated by the Nozaki and Tanford [57] and the Bull and Breese [58] scales. This reaffirms the transition to be an inverse temperature transition, with a temperature inversely proportional to the hydrophobicity of the repeating unit. It is to be emphasized that the transition for the development of elastomeric force follows the hydrophobicity shifts; this further reaffirms development of elastomeric force to be the result of an inverse temperature transition leading to increased order for the elastomeric state [55, 56].

Effect of the Transition on the Length of the Elastomer. The steepness of the curve for the development of elastomeric force of the Ile^1-polypentapeptide near 10°C [see Fig. 7(C)] is the result of matrix shortening and the fact that this sample had been stretched to 40% elongation at 40°C whereas the other samples had been stretched to 60% elongation at 40°C. As reflected in the temperature profiles of aggregation, the noncross-linked polypeptide is soluble in all proportions at a temperature below the onset of the inverse temperature transition [19]. This means that the cross-linked elastomers would dissolve on lowering the temperature below the transition if it were not for the cross-links. Instead of dissolving, the cross-linked polypeptides simply swell to the limit allowed by the cross-links and by the structural transition. This results in remarkable changes in the length of the cross-linked matrix as shown in Figure 8 where the length is measured as a function of temperature under zero load [59]. For 20 Mrad cross-linked polypentapeptide, the length of a strip of matrix increases 2.2 fold as the temperature is decreased from 40 to 20°C. Elastin shows analogous but less dramatic lengthening; a classical rubber such as latex, of course, shortens on lowering the temperature under zero load.

Biochemical Modulation of Hydrophobicity (i.e., of Transition Temperature). Rather than decreasing the temperature to relax the elastomeric force, it is possible to modify enzymatically the hydrophobicity of the elastomeric polypeptides and thereby to shift the temperature of the inverse temperature transition. This shift in temperature of the inverse temperature transition has been demonstrated with the enzyme prolyl hydroxylase. As shown in Figure 9, when the polypentapeptide is exposed to prolyl hydroxylase with the resulting hydroxylation of some of the Pro residues, this decrease in hydrophobicity causes the temperature profile for aggregation [60], equivalently for fiber formation and for elastomeric force development, to shift to higher temperature. This shift occurs with only about one Pro in 10 hydroxylated; this is only 1 hydroxylation in 50 residues. Thus enzymatic prolyl hydroxylation with a

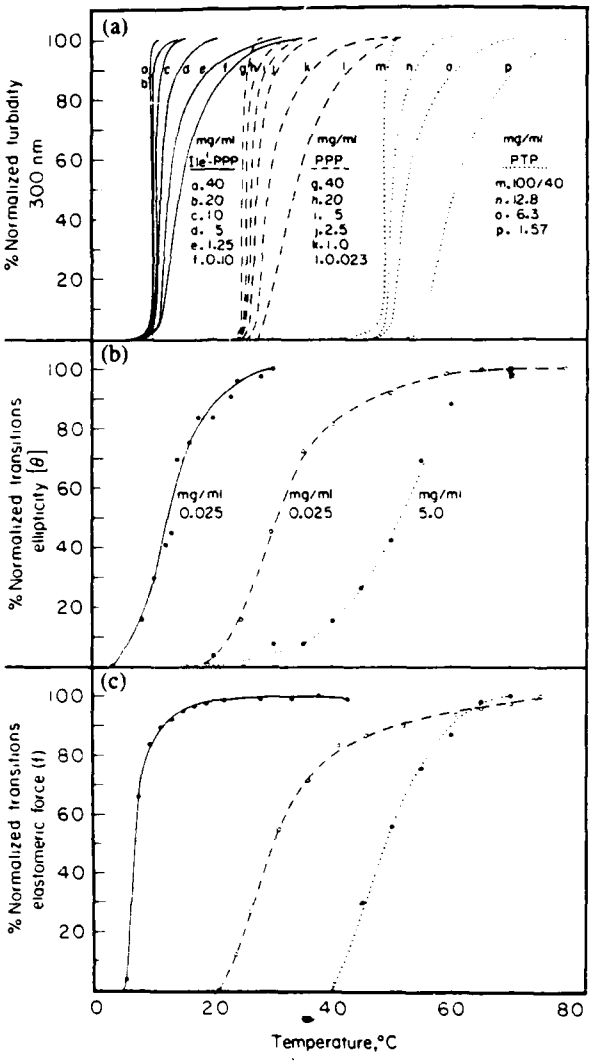

Figure 7. Comparison of a series of studies on a related series of elastomeric sequential polypeptides: Ile[1]-PPP is $(Ile^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; PPP is the polypentapeptide of elastin, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$; and PTP is $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$. These are all high polymers with molecular weights greater than 50,000. (A) Temperature profiles for aggregation which have been shown to be temperature profiles for fiber formation, that is, fiber formation occurs by an inverse temperature transition utilizing intermolecular hydrophobic interactions. Increasing the hydrophobicity of the repeating unit as in Ile[1]-PPP causes the transition, i.e., fiber formation, to occur at lower temperature than for PPP; Ile is more hydrophobic than Val. Decreasing the hydrophobicity of the repeating unit as in PTP causes the aggregations, i.e., fiber formations, to occur at higher temperature. (B) The conformation of each of the sequential polypeptides is followed by circular dichroism of suspensions wherein the concentration was kept low enough so that the particulate distortions due to the small suspended aggregates were not significant. Observed in each case is an increase in intramolecular order as the temperature is raised through the transition. (C) Temperature dependence of elastomeric force, when the γ-irradiation cross-linked coacervates are set at a fixed extension, is followed. The development of elastomeric force is found to have shifted to the temperature range of the inverse temperature transition. This is a clear demonstration that elastomeric force develops as the result of an inverse temperature transition dependent on the hydrophobicity of the polypeptide. The elastomeric state is the more-ordered state and loss of elastomeric force can be achieved by decreasing order. The temperature range of the inverse temperature transition can be shifted by changing hydrophobicity of the polypeptide. If the temperature range of the transition could be reversibly shifted at body temperature then elastomeric force could be turned on and off. Adapted with permission from Refs. 55 and 56.

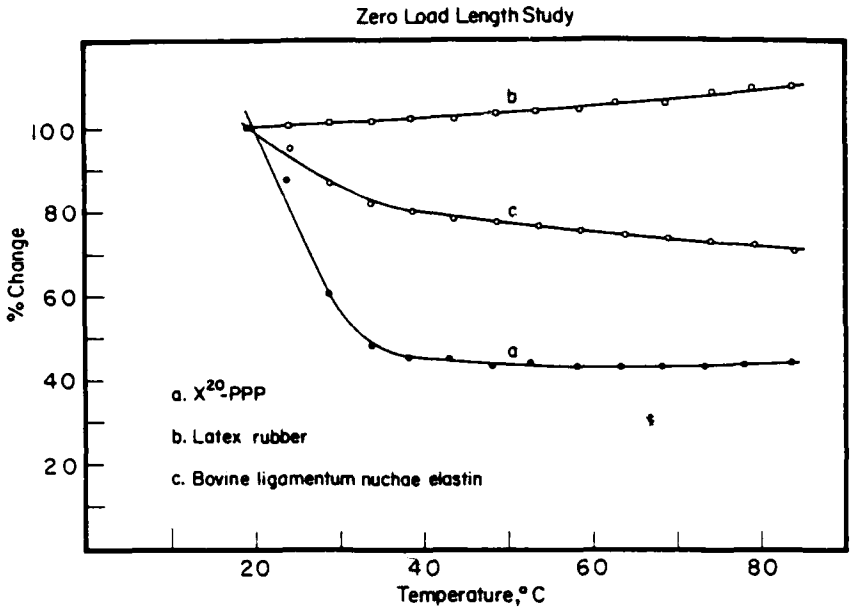

Figure 8. Effect of inverse temperature transition on the length of the elastomer. On raising the temperature from 20 to 40°C the 20 MRad γ-irradiation cross-linked polypentapeptide of elastin, X^{20}-PPP (●—●), undergoes a dramatic shortening to 45% of its 20°C length. This study is carried out at zero load (zero force). The structuring that occurs during the inverse temperature transition to form the β-spiral type of structure results in a shortening of the strip of X^{20}-PPP. A similar but less dramatic and more gradual shortening is observed for bovine ligamentum nuchae elastin (□—□). Typical of rubbers, latex (○—○) expands on raising the temperature. Thus elastomeric force is lost in part due to the structural transition. If by making the polypeptide less hydrophobic, the transition temperature range should shift to higher temperature and the elastomer would at body temperature lengthen and release or relax the force between two contact points. Reproduced with permission from Ref. 59.

sample of X^{20}-PPP held extended at 37°C should result in a decrease in elastomeric force when held at constant length and an elongation of the sample when maintained at a constant force.

While hydroxylation is an irreversible process, it becomes a trivial conceptual step to consider an elastomer with occasional serine or threonine residues that could be phosphorylated by a kinase causing the elastomer to extend (i.e., to relax) and that could be dephosphorylated by a phosphatase causing the elastomer to shorten and elastomeric force to again develop. It is suggested that such processes could be involved in the relaxin-induced cervical ripening and interpubic ligament formation and their reversal after parturition. Phosphorylation of enzymes and other proteins such as channels could be expected to have analogous effects on polypeptide segments capable of exerting entropic elastomeric force.

Biomedical Relevance

Wound Repair. In scar tissue there is a preponderance of collagen fibers with few or no elastin fibers [61]. In optimizing wound repair which involves sewing the breach together with collagen fibers, high levels of prolyl hydroxylase occur. Hydroxylation of proline residues in collagen is necessary for release of collagen from the cell; it is required to stabilize the collagen triple-stranded helix, and it protects collagen from nonspecific proteolysis (see references within Ref. 62). The same en-

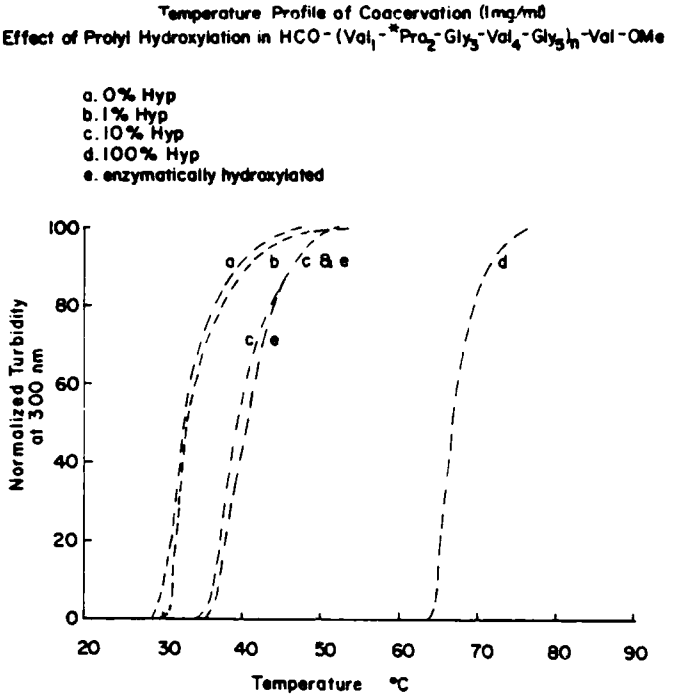

Figure 9. Prolyl hydroxylation of the polypentapeptide of elastin by the enzyme prolyl hydroxylase decreases the hydrophobicity of the polypeptide and shifts the temperature range for the inverse temperature transition 10°C to higher temperatures. Using synthetic polypentapeptide in which 10% of the pentamers contained hydroxyproline instead of proline causes a similar shift. Of the order of one hydroxyl introduced in 50 residues causes a substantial shift in the transition, as much as 10°C. Considered in terms of Figure 7(C), this would shift the development of elastomeric force to a higher temperature. Considered in terms of Figure 8, this prolyl hydroxylation would at 37°C result in a lengthening of the elastomer. Thus an enzymatic modification is expected to cause a relaxation of elastomeric force at body temperature. If the enzymatic modification were phosphorylation and dephosphorylation then entropic elastomeric force could be turned off and on as desired for changing structural states in connective tissue and elastomeric components of muscle or for changing the functional state of an enzyme or channel, for example.

zyme hydroxylates proline residues in tropoelastin, the single precursor protein of elastin fibers. Based on the shift to higher temperatures of the temperature profile for fiber formation of the polypentapeptide of elastin (see Fig. 9) that results from prolyl hydroxylation, this decrease in hydrophobicity of tropoelastin would be expected to have a similar effect. The result would be less elastic fiber formation and the fiber formed would be in a more nearly relaxed state and unable to provide an appropriate entropic elastomeric restoring force. This has been demonstrated in cell cultures of aortic smooth muscle cells induced to high levels of hydroxylation by the addition of ascorbic acid required by prolyl hydroxylase [63].

Environmentally Induced Lung Disease. In environmentally induced lung disease, such as pulmonary emphysema, the elastin fibers are fragmented and dysfunctional. When the lung is challenged by toxic substances, it is proposed that the

ensuing repair response results in the elaboration of high levels of prolyl hydroxylase. The consequence of overhydroxylation of tropoelastin would limit elastin fiber formation; those fibers that did form would be able to exert a more limited elastomeric function because of the shift to higher temperature of the inverse temperature transition; and it is not unreasonable to expect that the poorly formed fibers would be more susceptible to proteolytic degradation [62]. In general, any process, such as inhalation of cigarette smoke, that resulted in oxidation of the elastomeric chains in elastin would cause a loss of elastic recoil.

Events Attending Parturition and Their Reversal. *Interpubic Ligament Formation*. There are remarkable deformations and restoring forces attending and following parturition. In mice and guinea pigs [64, 65] and in some women there is the development of an interpubic ligament in the days prior to delivery. In mice, for example, the pubic symphysis is normally less than 2 mm in width. In the days before delivery an interpubic ligament develops becoming 5–6 mm in length allowing for enlargement of the birth canal. By the third or fourth day after delivery the gap between the pubic bones is drawn back to 2 mm [65]. What connective tissue processes could allow this elongation, and then within the time period of a few days what restoring forces could result in the shortening? The above mentioned biochemical process of decreasing the hydrophobicity by phosphorylation could lead to lengthening by shifting of the temperature range of the inverse temperature transition for the development of elastomeric force to higher temperature. The result would be a biochemically controlled relaxation of elastomeric force. Subsequent removal of the phosphate moieties by phosphatases would result in a restoration of elastomeric force and a shortening of the elastomer. Interestingly, the shortening from about 5 mm to 2 mm is similar to the shortening of the cross-linked polypentapeptide (seen in Fig. 8) on going from the relaxed state at 20°C to the elastomeric state at 37°C. A 20°C increase in the temperature range of the inverse temperature transition by decreasing hydrophobicity due to phosphorylation could result in the lengthening and then dephosphorylation could return the transition temperature to its normal physiological range being completed as it is just at body temperature.

Cervical Ripening. The relaxing and softening of the cervix is referred to as cervical ripening. This occurs in the hours preceding delivery and is thought to be under the control of the hormone relaxin [66–68]. Here one could employ elastin fibers as considered for the interpubic ligament formation. However, if uterine smooth muscle fibers contained elastomeric filaments as observed in striated muscles, then phosphorylation and dephosphorylation of intracellular elastomeric filaments could readily be considered as a potential mechanism. This is a particularly attractive hypothesis as the mechanism of action of relaxin is considered to involve the activation of kinases and phosphatases in a time-dependent manner [69]. Once such a hypothesis is raised involving uterine smooth muscle cells it is natural to inquire whether such a process could be operative in vascular smooth muscle cells and be relevant to some forms of essential hypertension.

Requiem for the Random Chain Network Theory of Entropic Protein Elasticity

One of the purposes of the above limited enumeration of the possible roles of en-

tropic elastomeric force in protein structure and function is to demonstrate the reasoning that becomes possible once the shackles of the classical theory of rubber elasticity (requiring as it does random chain networks) are removed from consideration of entropic protein elasticity. Useful approaches of three decades ago should give way to more accurate descriptions, made possible by improvements in physical methods and their interpretation. These more correct descriptions can lead to new contributions, to new concepts of mechanism that can be tested by a wide range of experimental approaches. It is pernicious to hold that polypeptide backbone motions of the order of nanoseconds can only be achieved by random chain networks. It is contrary to progress in understanding protein structure and function to assume that the only examples of ordered polypeptide states are α-helix, β-sheet, and triple-standed helix and that all else is random. It is particularly curious to see protein structure deduced on the basis of a theoretical approach that has found it necessary to invoke phantom chains that occupy no space and that can pass through one another [70]. Once the random chain network theory of entropic protein elasticity is set aside, progress in understanding many fundamental processes utilizing entropic protein elasticity can occur more readily.

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