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REPORT DOCUMENTATI

AD-A254 771

Approved
lo 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	Jul 92	Final 1 Feb 92 - 31 Jul 92

4. TITLE AND SUBTITLE

Development of Rules for Folding of Biotechnology Produced Protein

5. FUNDING NUMBERS

DAAL03-92-C-0004

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

ARO 29480.1-LS-SBI

11. SUPPLEMENTARY NOTES

The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Two fundamental contributions have been made in Phase I. The target of a Glu pK_a shift of up to 3 pH units was exceeded and a series of solutes within the new ΔT_t paradigm can be readily defined as to whether and with what efficacy they enhance or disrupt hydrophobic folding and assembly in proteins.

92-24434

92 9 02 110

424116

32 pg

14. SUBJECT TERMS

Hydrophobicity, Proteins, Polytricosapeptides, Biotechnology Produced Protein, Hydrophobic Interactions

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

ARO PHASE I SBIR CONTRACT NO. DAAL03-92-C-0004

"Development of Rules for Folding of Biotechnology Produced Protein"

RESULTS OF PHASE I WORK

OUTLINE

ARO PHASE I SBIR CONTRACT NO. DAAL03-92-C-0004

"Development of Rules for Folding of Biotechnology Produced Protein"

RESULTS OF PHASE I WORK

1. Scientific Basis for Phase I

From the known crystal structures of globular proteins, it is apparent that proteins fold with their hydrophobic residues in the interior of the protein. So apparent is this that hydrophobicity scales have been developed based on the frequency at which specific amino acid residues are found buried within the globular protein or on the surface of the protein. Such a scale, however, does not discern interactions between protein moieties that may alter expression of hydrophobicity, nor of course could it evaluate the nature and magnitude of the interactions responsible for the distribution and the protein folding principles that give rise to it.

In order to provide an experimental basis for developing rules governing the folding resulting from intramolecular hydrophobic interactions and hydrophobicity-enhanced charge-charge interactions, a polypeptide system is required with the correct balance of hydrophobic (apolar) and polar moieties resulting in a readily perturbable transition temperature for folding, with a readily measurable and near optimized response when compared to other polypeptides and proteins, and with low energy barriers for conformational interconversion such that reversibility can be maintained for the many applied perturbations.

The Model Molecular System

Nature has provided such a polypeptide system within the second most abundant extracellular protein, elastin. The polypeptide system to be used is the most striking repeating peptide sequence known in mammalian elastic fibers; it is well-represented in bovine and porcine elastins where in the former the sequence, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, occurs without a single substitution for a sequence of n = 11 (1-5). Poly(VPGVG) has the correct balance of hydrophobic and polar residues such that it is miscible with water in all proportions below 25°C but, as the temperature is raised through the transitional range to

37°C, it aggregates into a condensed, more-dense state, that is, it exhibits a phase transition from a state I (the solution state) to a state II (the condensed phase called a coacervate) which is in equilibrium with the overlying solution (6).

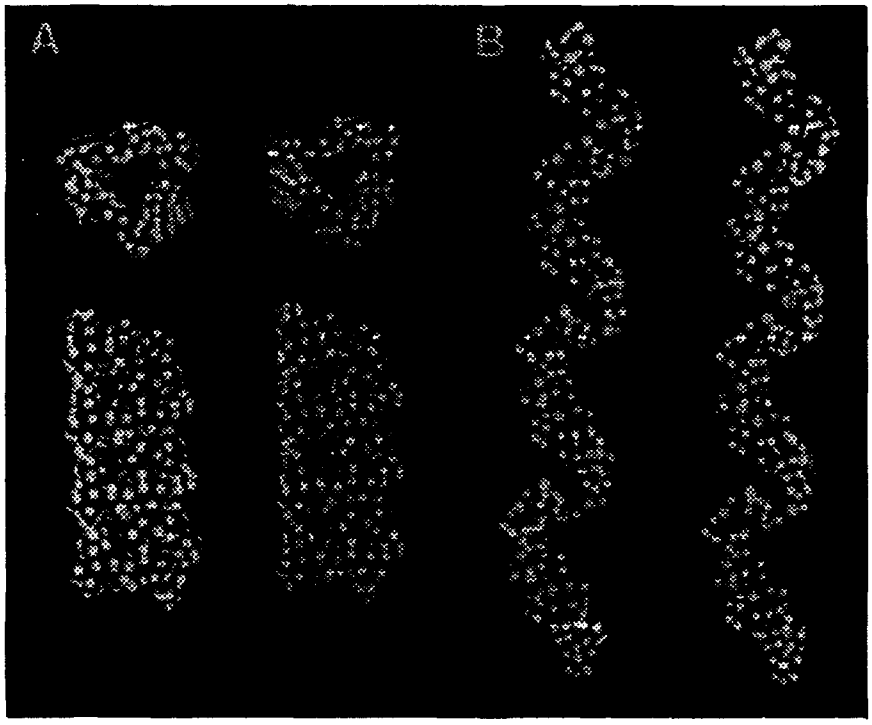
The Inverse Temperature Transition

When high molecular weight poly(VPGVG) is cross-linked as for example by 20 Mrads of γ-irradiation, i.e., X^{20}-poly(VPGVG), on lowering the temperature below 37°C, it swells to extents allowed by the cross-linking and chain length, and it contracts with expulsion of water on raising the temperature from 20°C to 40°C with changes in length of greater than a factor of 2 (6,7). Generally molecular systems (a common example would be molecular crystals in equilibrium with a mother liquor) become more soluble and less-ordered on raising the temperature, whereas poly(VPGVG) becomes less soluble and more-ordered on raising the temperature through the range of the transition. Because of this, the phenomenon exhibited by poly(VPGVG) is called an inverse temperature transition. A naturally occurring analog of VPGVG, with an alanine between the valine and proline, when repeated and cyclized, i.e., cyclo$(VAPGVG)_2$, provides the striking example of a molecular system capable of exhibiting an inverse temperature transition; when having been dissolved in water at low temperature, it reversibly crystallizes on raising the temperature and dissolves on lowering the temperature (8). An understanding of the molecular basis for such inverse temperature transitions necessarily resides in the nature of the hydration shell that surrounds hydrophobic (apolar) moieties.

Hydrophobic Hydration as Basis for the Inverse Temperature Transition

Dissolution of hydrophobic moieties in water is an exothermic process, that is, $\Delta H = \Delta E + P\Delta V$ is negative and heat is released, but the solubility, which depends on a negative Gibbs free energy of hydration ($\Delta G[\text{hydration}] = \Delta H - T\Delta S$), of hydrophobic moieties is generally low because of a significant negative entropy change (ΔS) as water molecules in bulk solution become more-ordered in the shell of water surrounding the hydrophobic moieties. Examples of the nature of the ordered water surrounding hydrophobic groups can be found in the crystal structures of alkane gas hydrates (9). Therefore, even though raising the temperature of poly(VPGVG) dissolved in water can cause it to fold and assemble into a

lower entropy condensed phase, the more-ordered waters of hydrophobic hydration surrounding the hydrophobic moieties in its dissolved state become less-ordered bulk water as the polypeptide aggregates. The net result on raising the temperature through the transition is an increase in disorder, i.e., an increase in entropy, for the system as a whole in keeping with the second law of thermodynamics (10-12), but importantly there has occurred a very useful increase in order, decrease in entropy, for the polypeptide part of the system as has been clearly demonstrated by studies on poly(VPGVG) and its analogs (13).



State II **State I**

Figure 1

Space filling models in stereo perspective (for cross-eye viewing) of the folded β-spiral structure of poly(VPGVG) and of an extended structure where all of the hydrophobic groups would be exposed to solvent and surrounded by waters of hydrophobic hydration.

The proposed molecular structure for poly(VPGVG) is shown in Figure 1A where, in the helically folded state II, there are hydrophobic contacts between turns of the particular helical structure called a β-spiral. It is the optimization of interturn hydrophobic contacts coupled as it is with the change of waters of hydrophobic hydration to bulk water that is the driving force for folding (6,7). Figure 1B is the extended state for which there are no interturn hydrophobic contacts and in which the hydrophobic groups would be surrounded by waters of hydrophobic hydration.

ΔT_t Mechanism of Protein Folding and Free Energy Transduction

Thermomechanical Transduction: In the unfolded, low temperature state (state I), cross-linked poly(VPGVG) is a hydrogel or a superabsorbent which, when the temperature is raised, expels substantial amounts of the water to become the folded, the more-dense, condensed phase (state II). Principals in this SBIR application have demonstrated a number of ways whereby the temperature of the folding transition can be shifted. This means that it becomes possible, without a change in temperature, to shift from state I to state II or vice versa. Since the state I to state II folding transition can be used to carry out work, the cross-linked matrix contracts on raising the temperature and can lift weights that are a thousand times greater than the dry weight of the cross-linked matrix. Thus, for a cross-linked matrix the inverse temperature transition itself is seen as a thermally driven contraction, that is, X^{20}-poly(VPGVG) exhibits thermomechanical transduction (14-16).

Chemomechanical Transduction: Now, if an analog of X^{20}-poly(VPGVG) should be unfolded at physiological temperatures, for example, and a chemical concentration change causes a lowering of the transition temperature to below 37°C, then the chemical concentration change will drive a contraction as the system shifts to the folded state. This is chemomechanical transduction, and it has been achieved by two different general chemical processes, a polymer-based process (16) and a solvent-based process (17).

The polymer-based process utilizes functional side chains in, for example, poly[4(VPGVG),(VPGEG)] where E = Glu. A protonation (due to a decrease in pH), causes a shift from the less hydrophobic state (i.e., from the more polar COO^- moieties) to the more hydrophobic state (i.e., to the less polar COOH moieties). This is because the temperature of the transition is lowered as the polypeptide becomes more hydrophobic, and it is raised as the polypeptide becomes less hydrophobic (16). The molecular interpretation is that the pull of the COO^- moiety to achieve its waters of hydration destructures the waters of hydrophobic hydration, thereby removing the thermodynamic driving force for folding (18). This interpretation derives from the observed hydrophobicity induced increase in pK_a of

constituent Glu residues on increasing hydrophobicity by conversion of Val^1 to Ile^1 as in poly[4(IPGVG),(IPGEG)] or in the increase in pK_a of Glu residues on stretching to increase exposure of hydrophobic groups (19,20). Even though in the latter case there is an increase in the amount of water in the cross-linked matrix on stretching, the free energy of COO^- moieties is increased causing a delay in ionization on lowering the proton concentration. This defines the mechanism as $(\partial\mu/\partial f)_{n=\alpha} < 0$ for the use of the $COOH/COO^-$ chemical couple to achieve chemomechanical transduction by modulation of the temperature of an inverse temperature transition. In the partial derivative, f is force; μ is chemical potential, and n is constant composition which in this case is a constant degree of ionization, α (20). Significantly, the sign is reversed for the same chemical couple for the charge-charge repulsion mechanism where $(\partial\mu/\partial f)_n > 0$ as shown by Katchalsky and colleagues (21). Importantly for directing protein folding the expression of hydrophobicity depends on whether a proximal Glu residue has its side chain as COOH or as COO^-.

In the solvent-based process, changes in composition of the solvent can change the temperature of the transition. For example, increasing the concentration of salt (NaCl) lowers the transition temperature (17); adding urea raises the transition temperature; adding ethylene glycol (22) or dimethyl sulfoxide lowers the transition temperature, etc. A quantitative understanding of how solvent composition can shift the temperature of an inverse temperature transition would improve the understanding as to how particular solutes may be used to direct proper folding of proteins.

Simplicity of the Assay System and efficiency of the ΔT_t Mechanism for Chemomechanical Transduction: For both thermomechanical transduction and chemomechanical transduction, the demonstration of forces developed and work performed can be by means of the simple assay of watching a strip of the cross-linked matrix pick up a weight when the energy source is thermal for thermomechanical transduction (thermally driven folding), or pick up a weight when the energy source is chemical, for example, as when the salt concentration is raised or when the concentration of proton is raised to achieve chemically driven folding (16,17). The forces are obviously real, and they are dramatic with an efficiency, for example, in the conversion of chemical energy into mechanical work that is more than an order of

magnitude greater than previously demonstrated for the charge-charge repulsion mechanism of chemomechanical transduction (13). At the polypeptide level, the increase in temperature or concentration, as described, drives the conversion from the unfolded (hydrophobically hydrated) state of Figure 1A to the contracted (folded) state of Figure 1B.

Baromechanical Transduction: It has recently been shown that a change in pressure can shift the transition temperature (23). An increase in pressure raises the temperature of the transition by very significant amounts when there are aromatic residues in the polymer. The molecular interpretation is that waters of hydrophobic hydration surrounding an aromatic residue occupy less volume than bulk water. In terms of baromechanical transduction in a water-filled pressurizable cylinder with a weight attached to a suspended elastomeric band containing aromatic residues, e.g., Phe residues, application of pressure would cause the band to swell and lower the weight whereas releasing the pressure would cause the band to contract and lift the weight. This has now been observed. Thus pressure could be used to unfold a protein containing aromatic residues and then the slow release of pressure could be used to allow correct refolding of the protein and this could be done simultaneous with changing solute concentration by dialysis.

Electromechanical Transduction: Demonstration of the feasibility of electromechanical transduction has been achieved with the electrochemical reduction of poly[3(GVGVP),(GK{NMeN}GVP)], where {NMeN} indicates the N-methyl nicotinate moiety attached by amide linkage to the ε-amino of lysine (K). At pH 9, –1.6 volts and a concentration of 3.5 mg/ml, the electrochemical reduction of this protein-based polymer causes a decrease in the value of T_t from 47°C to 36°C. This protein-based polymer has also been cross-linked such that now the reduction of the elastic matrix, with the use of a mediator, will be attempted to drive contraction (folding), and thereby to have demonstrated electromechanical transduction.

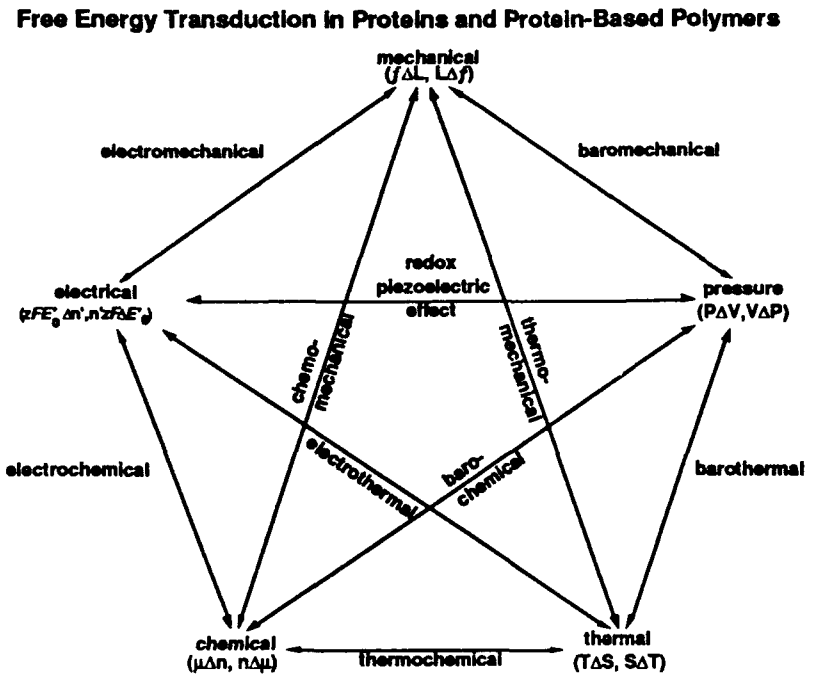


Figure 2

Thus there is the potential for all of the free energy transduction of Figure 2 to be achieved by controlling folding and assembly. There is then demonstrated potential for the four transduction coupled to mechanical force. It is then our proposal that any free energy input – pressure, thermal, chemical or electrical – that can drive folding to give the mechanical component, can utilize the driving force for folding to convert energy among the other four free energy driving forces. This is controlling higher order structure to achieve function.

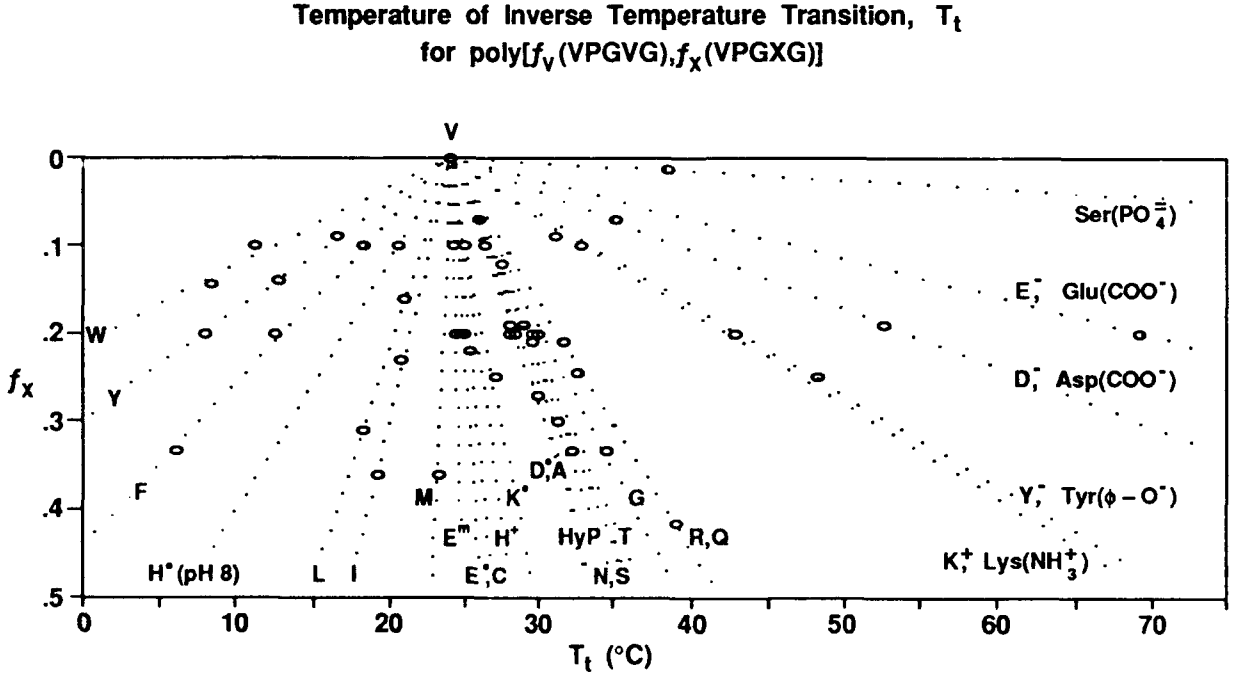


Figure 3: Plot of the temperature of the inverse temperature transition, T_t, as a function of the mole fraction, f_X, of guest residue X. The order of the residues is given in single letter code for the closely grouped residues. The order and values of T_t, for the residues provides an experimental hydrophobicity scale. (See Table I, page 8.)

TABLE I

Temperature of Inverse Temperature Transition, T_t for poly[f_V(VPGVG),f_X(VPGXG)]

(A New Hydrophobicity Scale)

Residue X	T_t at f_X = 1	Correlation Coefficient
Trp (W)	-90°C	0.993
Tyr (Y)	-55°C	0.999
Phe (F)	-30°C	0.999
His (pH 8) (H^o)	-10°C	1.000
Pro (P)	(-8°C)	
Leu (L)	5°C	0.999
Ile (I)	10°C	0.999
Met (M)	20°C	0.996
Val (V)	24°C	
Glu($COOCH_3$) (E^m)	25°C	1.000
Glu(COOH) (E^o)	30°C	1.000
Cys (C)	30°C	1.000
His (pH 4) (H^+)	30°C	1.000
Lys(NH_2) (K^o)	35°C	0.936
Asp(COOH) (D^o)	45°C	0.994
Ala (A)	45°C	0.997
HyP	50°C	0.998
Asn (N)	50°C	0.997
Ser (S)	50°C	0.997
Thr (T)	50°C	0.999
Gly (G)	55°C	0.999
Arg (R)	60°C	1.000
Gln (Q)	60°C	0.999
Lys(NH_3^+) (K^+)	120°C	0.999
Tyr(ϕ-O^-) (Y^-)	120°C	0.996
Asp(COO^-) (D^-)	170°C	0.999
Glu(COO^-) (E^-)	250°C	1.000
Ser($PO_4^=$)	1000°C	1.000

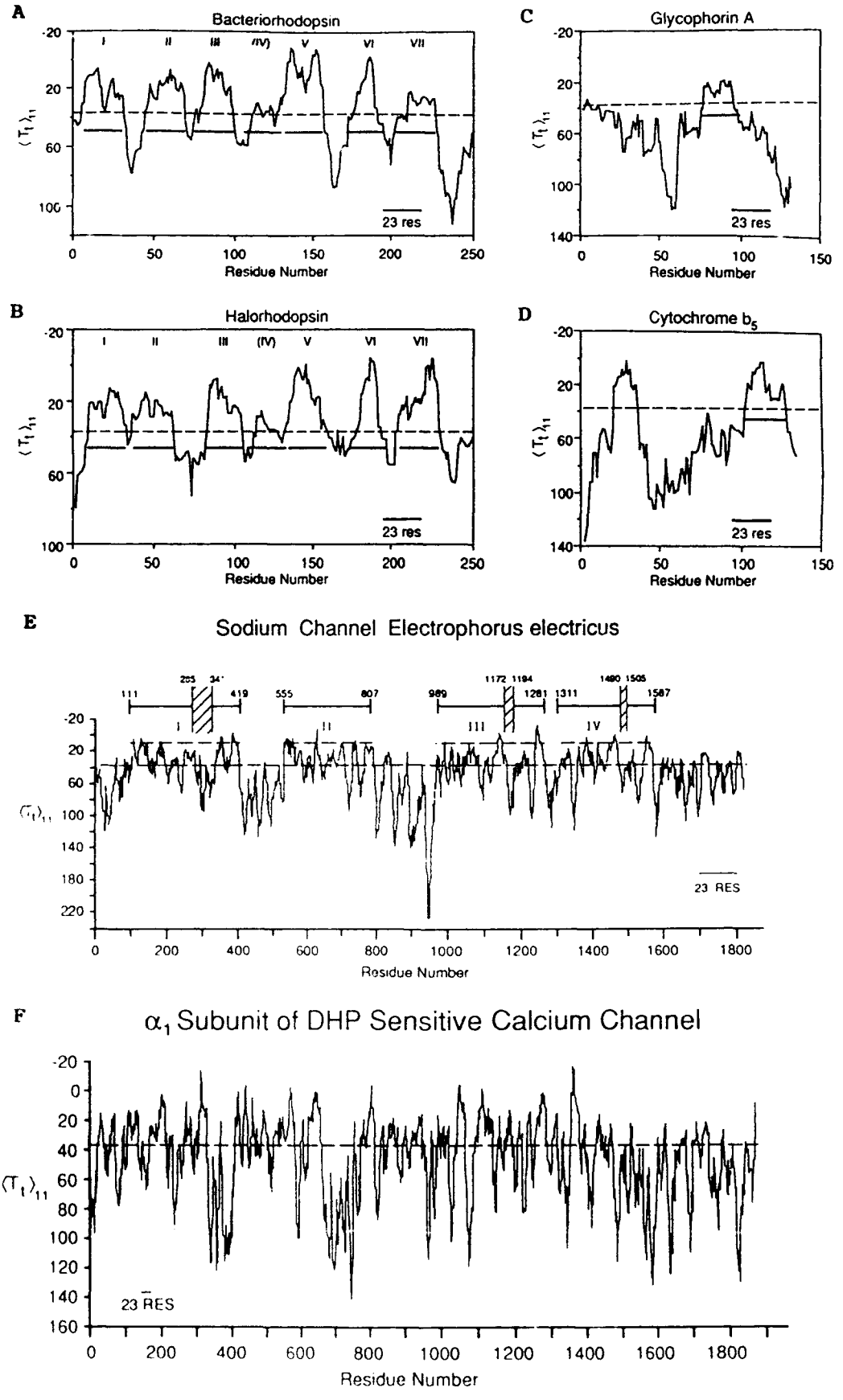


Figure 4: Mean transition temperature, $(T_t)_{11}$, plots averaged over an eleven residue sliding window for bacteriorhodopsin (A), halorhodopsin (B), glycophorin (C), cytochrome b_5 (D). Na Channel Protein (Electrophorus electricus) (25) (E) and Ca Channel Protein (α_1 subunit of DHP sensitive) (26) (F). Values less than 37°C indicate sequences that would be hydrophobically folded or interacted as in the case of a transmembrane helix. In each case the bar indicates a length of 23 residues, the approximate number of residues required for an α-helix to span a lipid bilayer. Note for each membrane protein that the transmembrane helices are observed.

Hydrophobicity Scale

Understanding the contribution of each chemical moiety of a protein and solute to the temperature of the transition make it possible to introduce energy in one form to shift protein folding from one state to another while directing proper folding. As a major first step in this process, a new type of hydrophobicity scale has been experimentally derived (24) which is the relative contribution of each amino acid residue to the transition temperature of an inverse temperature transition (see Figure 3, page 7 and Table I, page 8). With this set of values of T_t for each amino acid residue, a plot of a mean value of T_t, i.e. $\langle T_t \rangle$ averaged over a sliding window of a given number of residues for a given protein sequence can evaluate the relative hydrophobicity of sequences. In particular in the now classical test, a plot of $\langle T_t \rangle$ versus residue number for bacteriorhodopsin, halorhodopsin glycophorin A, cytochrome b_5 and the sodium and calcium channels is capable of identifying the transmembrane helices (see Figure 4, page 9). A sequence for which $\langle T_t \rangle$ is less than 37°C would be hydrophobically folded, or in the case of a membrane protein, becomes a potential transmembrane helix (24).

Enzymatic Control of Folding by Phosphorylation

The most dramatic means so far identified for shifting the temperature of the inverse temperature for protein folding is phosphorylation/dephosphorylation (27). Because of the central role that phosphorylation plays in modulating protein structure and function (28), an elastomeric polypentapeptide was prepared with a site for phosphorylation. When a cyclic AMP-dependent protein kinase site (29), RGYSLG, is introduced into the polypentapeptide, e.g., poly[30(IPGVG),(RGYSLG)], phosphorylation of one-half of the serine residues in the polypeptide by the α catalytic subunit of the protein kinase causes a 15°C shift in the transition temperature from 18°C to 33°C. For an intermediate temperature of 25°C, for example, the phosphorylation induced shift in the temperature of the inverse temperature transition would be sufficient to be essentially fully folded before phosphorylation and to become fully unfolded after phosphorylation of one-half of the serine residues in th RGYSLG-containing poly(IPGVG). One phosphate in a mean molecular weight of 20,000 Da is sufficient to produce such a dramatic effect. The interpretation here is the same as for the COO^- moiety. The phosphate moieties destructure the waters of

hydrophobic hydration that would otherwise provide the driving force for folding at 25°C. Therefore, the polypeptide unfolds on phosphorylation. Accordingly should a phosphorylation site be present in a microbially produced protein, it would be a reasonable consideration to phosphorylate it to achieve the proper folding for the phosphorylated state, with care taken to prevent aggregational potential, and then carefully to remove the phosphate by a phosphatase thereby lowering the transition temperature to complete the refolding.

It is now becoming apparent that if biotechnology is to more fully reach its potential for the production of proteins by their expression from unnatural cells and by unnatural processes, it will be necessary to develop rules that will allow the protein folding required for the desired property.

2. Technical Objectives of Phase I

Elements of three technical objectives are being addressed in Phase I which are central to development of protein folding and purification strategies and to the design of protein for free energy transduction:

(1) to assess the magnitude and distance dependence of the repulsive free energy of interaction that exists between carboxylate moieties and hydrophobic moieties that can lead to large shifts in the pK_a (up to several pH units, i.e., a target of 3 pH units) of the carboxyl moiety and the raising of T_t, the temperature for the hydrophobic folding transition.

(2) to evaluate hydrophobic domain sizes, both as to intensity of the hydrophobicity and the number of residues in the domain, that can give rise to independent folding of the domains.

and, (3) to quantitate the effects of changes in solvent composition on shifting the value of T_t, for poly(VPGVG) and for this host polypentapeptide with guest residues i.e. for poly[f_V(VPGVG),f_X(VPGXG)] where f_V and f_X are mole fractions with $f_V + f_X = 1$ and where X is the guest residue.

3. Progress in Phase I

The progress achieved in Phase I can be discussed in terms of the technical objectives, but first the syntheses of the model protein-based polymers will be enumerated.

Peptide Syntheses: The necessary first step in reaching any of the technical objectives is the syntheses of those protein-based polymers which were designed to provide the information sought in the technical objectives. Extraordinary progress has been made in the synthesis component. Instead of the proposed ten polypentapeptides with the highest complexity being polypentadecapeptides, poly(15 mers), 22 polypentapeptides have been synthesized and another 6 are under polymerization with the greatest complexity being the much more difficult polytricosapeptides, poly(30 mers). These protein-based polymers are listed in Table II, page 13.

a. Hydrophobicity-Induced pK_a Shifts

(1) Successful Design at Nanometric Dimensions to Enhance Hydrophobicity-Induced pK_a Shifts.

At the writing of this Phase I proposal, a one pH unit hydrophobicity-induced pK_a had been observed. The target was to achieve as much as a 3 pH unit shift. Our objective has been exceeded. What has actually been achieved is a 3.8 pH unit shift. This is described with a succinct focus in what follows.

With poly[4(GVGVP),(GEGVP)] (I) as a reference state, the polytricosapeptides, poly[3(GVGVP),2(GFGFP),(GEGFP)] (II); poly[2(GVGVP),2(GVGFP),(GFGFP), (GEGFP)] (III); poly(GEGFP GVGVP GVGVP GVGVP GFGFP GFGFP) (IV); and poly(GEGFP GVGVP GVGFP-GFGFP GVGVP GVGFP) (V), all having the same theoretical and essentially the same analytical amino acid compositions, were synthesized and designed for the specific purpose of testing the limits of hydrophobicity-induced pK_a shifts in this system. Polymers I, II, III, IV and V exhibited pK_a values for the Glu(E) residues of 4.3, 4.7, 6.3, 7.7 and 8.1, respectively. Due to the limited number of Glu

TABLE II

Polymers Synthesized

Polymers	pK_a 37°C	pK_a 20°C
CG56PF poly(GVGVP)		
CG58PF poly[(GEGFP)2(GVGVP)2(GVGFP)GFGFP)]	5.38	6.30
CG59PF poly[(GEGFP)3(GVGVP)2(GFGFP)]	4.90	4.80
CG61PF poly(GEGFP GVGVP GVGFP GFGFP GVGVP GVGFP)	5.18	8.00
CG62PF poly(GEGFP GVGVP GVGVP GVGVP GFGFP GFGFP)	5.40	7.60
CG70PF poly(GDGIP)	3.84	
CG71PF poly[0.8(GDGIP)0.2(GVGIP)]	3.86	
CG72PF poly[0.6(GDGIP)0.4(GVGIP)]	4.07	
CG73PF poly[0.4(GDGIP)0.6(GVGIP)]	4.12	
CG74PF poly[0.3(GDGIP)0.7(GVGIP)]	4.30	
CG75PF poly[0.2(GDGIP)0.8(GVGIP)]	4.74	
CG76PF poly[0.1(GDGIP)0.9(GVGIP)]	5.04	
CG77PF poly[0.05(GDGIP)0.95(GVGIP)]	6.82	
CG78PF poly(GVGIP)		
CG79PF poly(GKGIP)		
CG80PF poly(0.8(GKGIP)0.2(GVGIP)]		
CG81PF poly(0.6(GKGIP)0.4(GVGIP)]		
CG82PF poly(0.4(GKGIP)0.6(GVGIP)]		
CG83PF poly(0.3(GKGIP)0.7(GVGIP)]		
CG84PF poly(0.2(GKGIP)0.8(GVGIP)]		
CG85PF poly(0.1(GKGIP)0.9(GVGIP)]		
CG86PF poly(0.05(GKGIP)0.95(GVGIP)]		
CG87PF poly[(GEGIP)2(GVGVP)2GVGIP)(GIGIP)]		
CG88PF poly[(GEGIP)3(GVGVP)2(GIGIP)]		
CG89PF poly[(GDGIP)2(GVGVP)2GVGIP)(GIGIP)]		
CG90PF poly[(GDGIP)3(GVGVP)2(GIGIP)]		
CG91PF poly[(GKGIP)2(GVGVP)2GVGIP)(GIGIP)]		
CG92PF poly[(GKGIP)3(GVGVP)2(GIGIP)]		

residues, the structures themselves exclude the often invoked charge-charge interaction mechanism for pK_a shifts. The differences in pK_a values arise from the differences in proximity of the Glu(E) and Phe(F) residues within and between pentamers with optimal arranging of five nearest-neighbor Phe residues at nanometer distances from the Glu residue in IV and V. This remarkable increase of 3.4 pH units within polymers II through V of essentially the same amino acid composition and of 3.8 pH units between polymers I and V within a system which is approximately 50% water by weight in its folded state demonstrates a repulsive free energy of interaction between hydration processes of the Phe and Glu side chains of 4.6 kcal/mole within the polytricosapeptide group and of 5.1 kcal/mole overall. To our knowledge these are the largest pK_a shifts documented for the Glu residue, and the recently identified interactions responsible are considered to be important free energies of interaction in modulating protein structure and function (13).

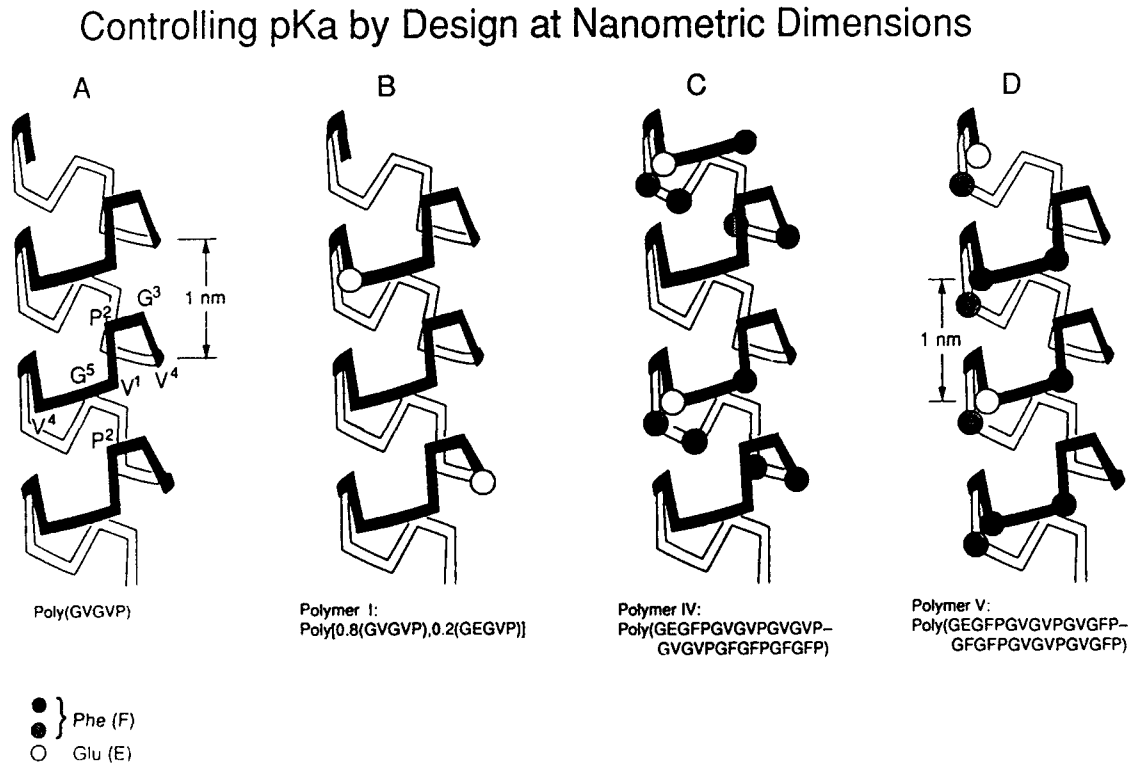


Figure 5 Schematic representation of the β–spiral structure proposed for the polypentapeptide, poly(Val[1]-Pro[2]-Gly[3]-Val[4]-Gly[5]), which is equivalent to poly(GVGVP). The Pro[2]-Gly[3] β-turn is seen to function as a spacer between the turns of the β-spiral in A. and the hydrophobic folding arises principally from interturn Val[1]γCH3 ´ Pro[2] β-CH2 ⋮nteractives. In B. there are occasional Glu(O) residues randomly dispersed. In C. the Phe residues are placed in proximity to the Glu residues based on primary structure. In D. the Phe residues are placed in proximity to the Glu residues based on a β-spiral with approximately 3 pentamers per turn, i.e., based on tertiary structure.

Polymers IV and V were designed on the basis of the working β-spiral structure of poly(GVGVP) shown schematically in Figure 5A (13,16). For the β-spiral structure, the distance between turns is approximately one nanometer (30,31). This results in the nanometer proximities of the Glu and Phe residues shown in Figure 5C and D. The polytricosapeptides were constructed by the sequential addition of pentamers. The composite pentamers were also mixed in the appropriate ratios and polymerized to obtain polymers II and III with a more random ordering of pentamers. A complete description of the syntheses will be presented elsewhere. The structures were verified by carbon-13 nuclear magnetic resonance and amino acid analyses. The amino acid analyses for polymers I through V are given in Table III (page 16) for the average values and standard deviations of four runs on each sample.

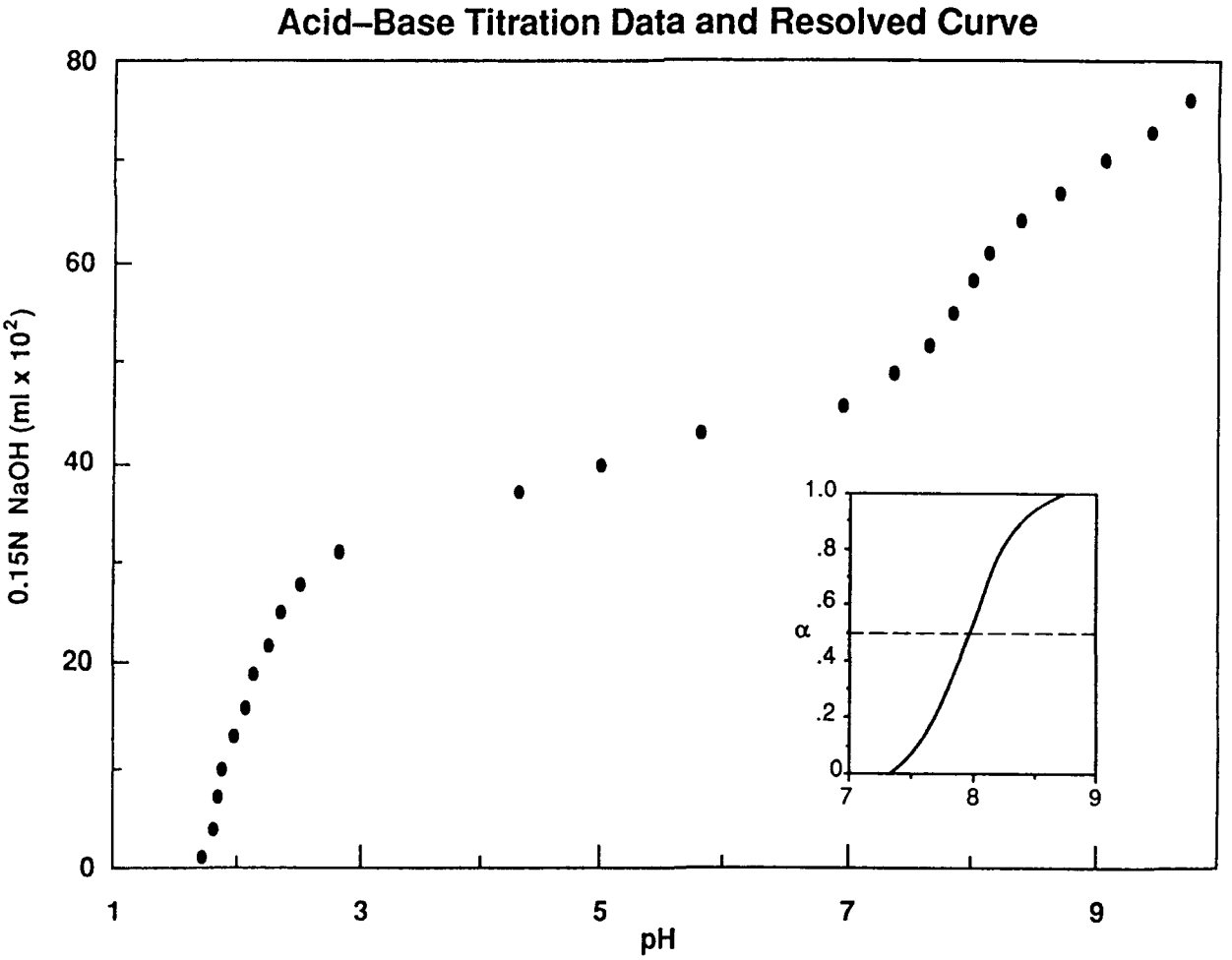


Figure 6 An acid-base titration curve for polymer V and the resolved curve included as an inset. The starting concentration was 40 mg/ml and the temperature was 20°C.

The raw acid-base titration data and the resolved curve for one of the three titrations for polymer V are given in Figure 6 (page 15) and the averaged value and standard deviations for three runs each for polymers II through V are included in Table III below. Curve resolution to obtain α, the degree of ionization, and the pK_a value utilized the second derivative of the experimental titration curve. The negative extremum for the second derivative signals the start of the ionization process, and the positive extremum signals the completion of the ionization process. The midpoint between the two extrema is the pK_a value (32,33). Resolved acid-base titration curves for polymers I, II, III, IV and V are given in Figure 7 (page 17).

Table III

Amino acid compositon, experimental pKa values and calculated mean hydrophobicities, $\langle T_t \rangle$, for polymers I through V

Amino Acid or Property	Polymer						
	I Found	I Theoretical	II Found	III Found	IV Found	V Found	II – V Theoretical
Glutamic Acid (E)	0.20	0.20	0.17 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.17
Glycine (G)	1.99	2.00	2.13 ± 0.03	1.89 ± 0.03	2.05 ± 0.01	1.98 ± 0.02	2.00
Proline (P)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Valine (V)	1.79	1.80	1.03 ± 0.16	1.10 ± 0.10	0.90 ± 0.01	0.99 ± 0.04	1.00
Phenylalanine (F)	- - - -	- - - -	0.74 ± 0.06	0.90 ± 0.01	0.79 ± 0.06	0.84 ± 0.01	0.83
Experimental pKa	4.3 ± 0.02		4.7 ± 0.05	6.3 ± 0.05	7.7 ± 0.1	8.1 ± 0.1	
Calculated $\langle T_t \rangle$ E°	30°		23°	20°	22°	21°	
E^-	39°		30°	26°	29°	27°	

In the amino acid analyses, Pro is taken as 1.0.

E° stands for Glu (COOH) and E^- for Glu(COO^-).

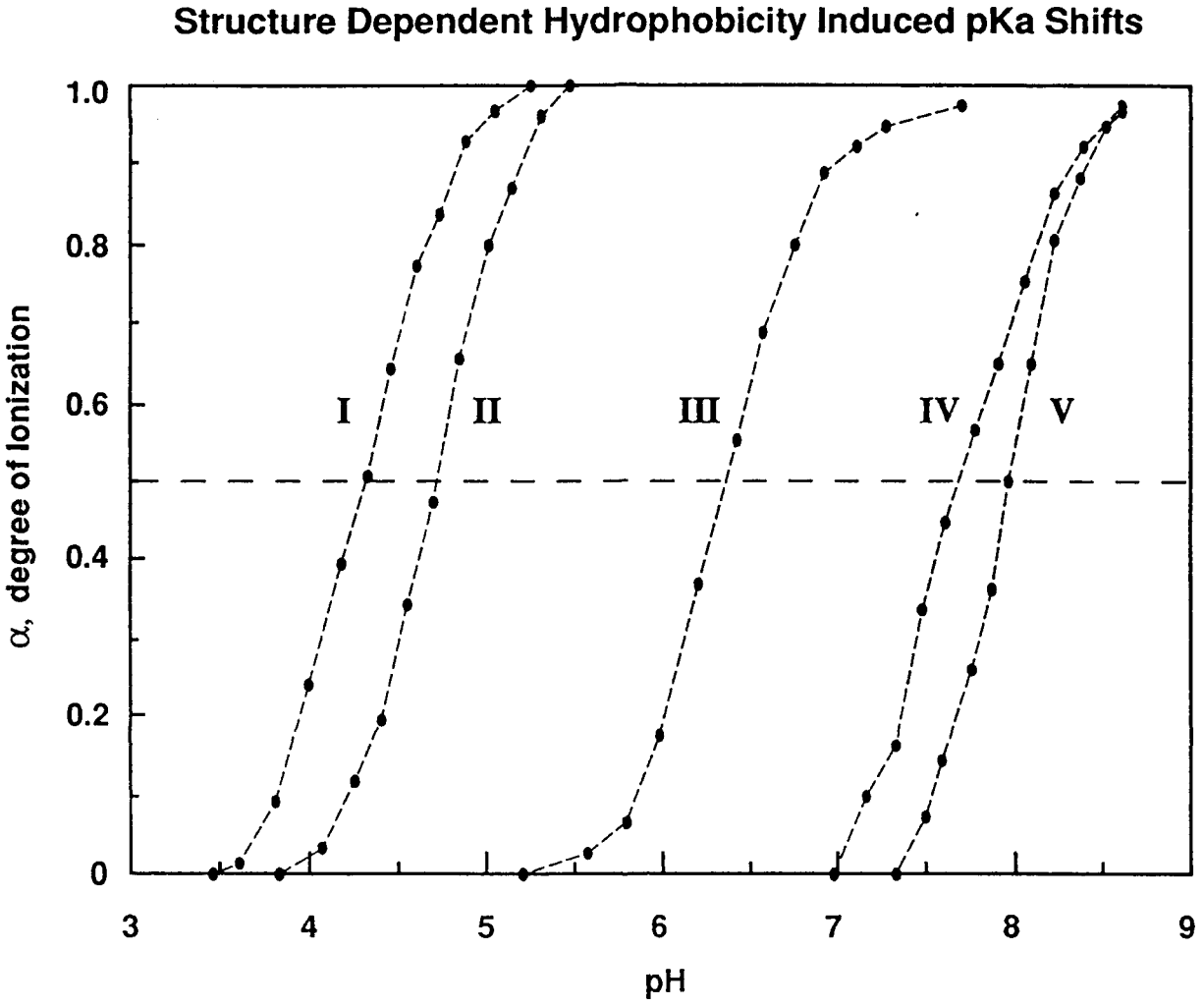


Figure 7 Resolved acid-base titration curves for polymers I through V. A remarkable overall pK_a shift of 3.8 pH units is observed. For all curves the starting concentration was 40 mg/ml and the temperature was 20°C.

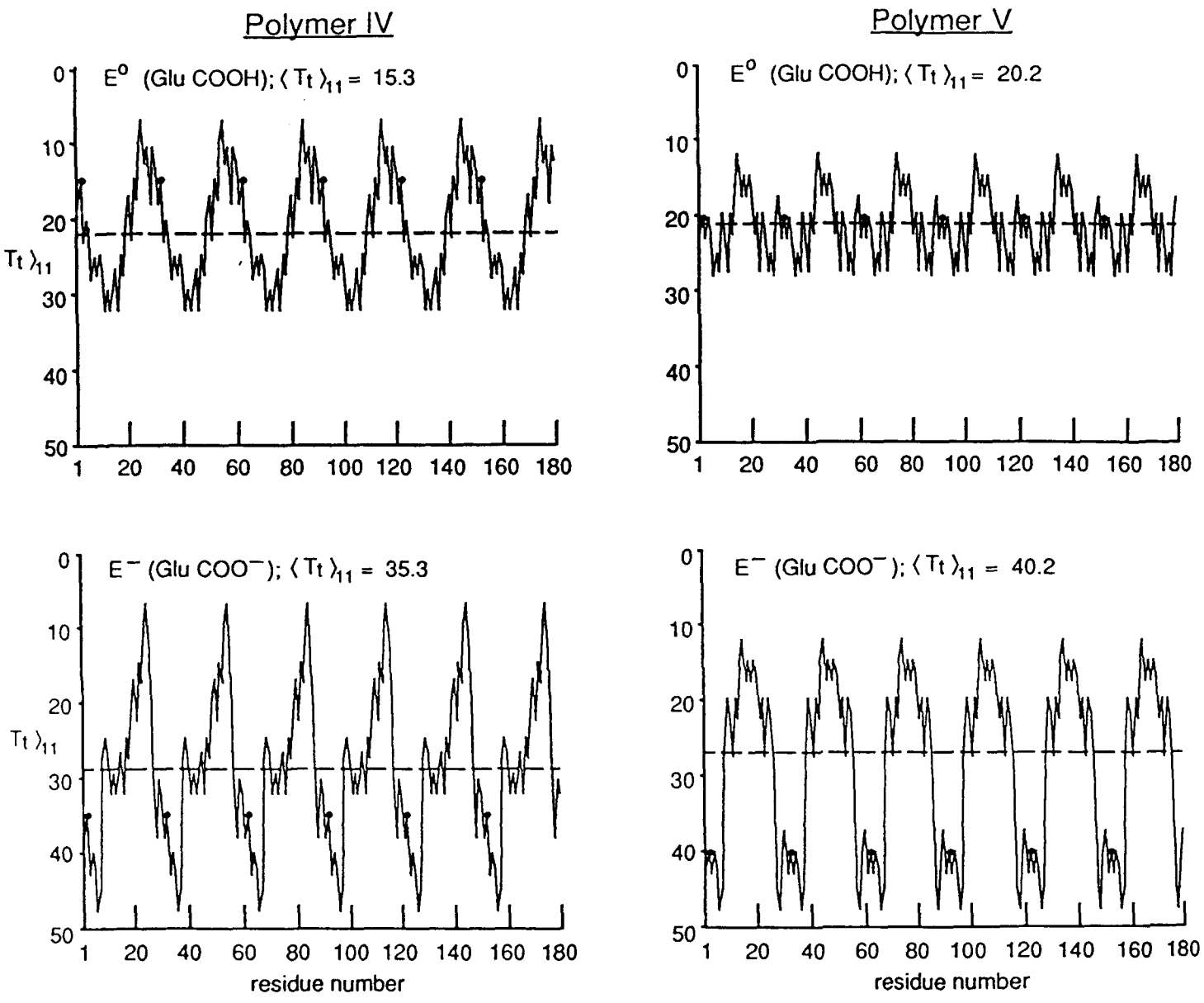
Poly(GVGVP) and analogs thereof undergo phase transitions, the temperatures for which vary systematically with composition (13). The general compositions can be written poly[f_V(GVGVP),f_X(GXGVP)] where f_V and f_X are mole fractions with $f_V + f_X = 1$ and where X can be any amino acid residue or chemical modification thereof. These polypentapeptides are soluble in water

at low temperatures but on raising the temperature aggregation and settling occurs to form a more dense viscoelastic phase, called a coacervate, which is approximately 50% water by weight. The onset temperature for this transition is indicated as T_t. Changes in composition change the value of T_t in a systematic way such that plots of T_t vs. f_X are linear (See Figure 3, page 7) and such that more hydrophobic residues lower the value of T_t and less hydrophobic residues than Val and more polar residues increase the value of T_t. This property has been used to develop a hydrophobicity scale (24) (See Table I) in which the reference values for T_t are obtained by extrapolating to $f_X = 1$. The values of T_t range from -90°C for poly(GWGVP) to 250°C for poly(GEGVP) with Glu(COO^-).

The mean residue hydrophobicities, $\langle T_t \rangle$, calculated using this hydrophobicity scale and the amino acid analysis data are included in Table III, page 16, for polymers I through V where the values of $\langle T_t \rangle$ are seen to be nearly the same for polymers II through V yet the pK_a values differ remarkably. Previous studies have shown that increased hydrophobicity increases pK_a (13,20,34). The present study was designed to determine the effect of differing proximity of Glu and Phe residues on pK_a. Significant differences in pK_a values were also observed between polymers IV and V where the sequences are fixed and where variable incorporation of different pentamers during polymerization would not be a factor.

The dominance in this molecular system of the tertiary structure over the primary structure in achieving the largest pK_a shift can be exemplified by mean residue hydrophobicity plots for an internal six repeats (180 residues) using a sliding window of eleven residues for the average value, i.e., $\langle T_t \rangle_{11}$ for polymers IV and V (24). These plots are given in Figure 8 where the smaller number indicates the greater hydrophobicity. The value of $\langle T_t \rangle_{11}$ for the Glu COOH state is 15 for polymer IV and 20 for V, and the value for the Glu COO^- state is 35 for IV and 40 for V. Thus, based on primary structures, the Glu residues in Polymer IV would experience the greater hydrophobicity and would be expected to give the larger pK_a shift. The pK_a shift is greater, however, for polymer V. Only when the proper folding is taken into account does the spatial proximity become apparent, and this Glu-Phe proximity resulting from the β-spiral folding, as shown in Figure 5C and D, provides the understanding for the larger pK_a shift being exhibited by Polymer V.

Mean Residue Hydrophobicity Plots for the Poly(tricosapeptides), IV and V
Sliding Window of 11 Residue Average, $\langle T_t \rangle_{11}$



- Value of $\langle T_t \rangle_{11}$ for the Glu residue for each polymer for both the Glu COOH and Glu COO^- states

---- Mean value of $\langle T_t \rangle$ based on the amino acid analysis data

Figure 8 Mean residue hydrophobicity plots with an eleven residue sliding window plotted at the central residue of count for polymers IV and V. The small dot positions the recurring Glu residues and the dashed lines are the mean values of T_t calculated from the amino acid analysis data. The upward deflections (smaller numbers) signify greater hydrophobicity. Note that the Glu residues for polymer IV based on primary structure are in the greater hydrophobic environment than for polymer V, yet polymer V exhibits the greater pK_a shift. This is considered to be due to the tertiary structure as seen in Figure 1D.

From the primary structures themselves, it is clear that the differences in pK_a do not arise from the commonly considered electrostatic interactions (35,36). The pK_a shifts instead are an expression of a repulsive free energy of interaction which exists between the hydration shells of hydrophobic (apolar) and polar(COO^-) moieties as discussed in more detail elsewhere (13). The change in Gibbs free energy per mole obtained from the change in chemical potential, $\Delta\mu$, required to maintain 50% ionization of the side chain is given by $\Delta\mu = -2.3\ RT\ \Delta pK_a$. At 20°C with a ΔpK_a of 3.8 pH units, $\Delta\mu$ is 5 kcal/mole, that is, there is observed a repulsive Gibbs free energy of interaction arising out of the proximity of Phe and Glu side chains in polymer V.

It is not the Glu side chain becoming folded in the hydrophobic, water-free interior of a globular protein that provides an adequate description of the pK_a shift; rather it is the competition between Glu and Phe side chains for appropriately structured hydration shells in a filamentous folded state having approximately 50% water by weight that is responsible for the unfavorable free energy for carboxylate anion formation (13). This is referred to as an apolar-polar repulsive free energy of hydration. This mechanism can be used to drive chemomechanical transduction as the polypeptide folding into a β-spiral can be used to lift a weight in elastomeric bands obtained on γ-irradiation of the coacervate state (13,16,17). This mechanism for driving folding and unfolding is an order of magnitude more efficient in the amount of chemical energy (due to an increase in proton concentration) required to lift a given weight than is the charge-charge repulsion mechanism using the same $COOH/COO^-$ chemical couple (13,21), and $(\partial\mu/\partial f)_\alpha$, where f is force, is negative for the new mechanism (13,35) and positive for the latter (21).

In this case the formation of a charged species is viewed as destroying the structured water surrounding hydrophobic moieties when exposed to water such that the thermodynamic driving force for hydrophobic folding is lost and unfolding can occur. The phosphate moiety attached to a serine residue has been shown to be the most potent chemical moiety in destroying structured hydrophobic hydration, that is, phosphorylation is the most effective moiety on a molar basis for driving unfolding. One phosphate moiety in 300 residues in poly[30(IPGVG),(RGYSLG)] (VI) can raise the temperature of

the folding transition by 14°C from 18°C to 32°C (13,27). Thus phosphorylation of the Ser residue of polymer VI to the extent of one phosphate per 300 residues, at 25°C, can result in the unfolding of the polymer. This gives for the hydrophobicity scale a value of T_t for Ser-phosphate of over 1,000°C. Because of the ubiquitous use of phosphorylation to drive protein function, because of the dramatic effect of phosphorylation in the mechanism demonstrated by this system and because of the efficiency of this mechanism in driving polypeptide folding to perform chemomechanical transduction, it is believed that the mechanism demonstrated here in terms of dramatic pK_a shifts would be a dominant mechanism in protein structure and function.

(2) The Relative Sensitivity of Amino Acid Side Chains to Hydrophobicity-Induced pK_a Shifts.

Comparison of the relative sensitivity of ionizable amino acid side chains to hydrophobicity-induced pK_a shifts should be achieved under conditions wherein the charge-induced pK_a shifts can be shown to be negligible.

As the first comparison Glu(E) and Asp(D), both of which have the carboxyl side chains, are studied in the protein-based polymers, poly[f_V(GVGIP),f_E(GEGIP)] and poly[f_V(GVGIP),f_D(GDGIP)] where the mole fractions, f_E and f_D are varied between one and 0.05, and the acid-base titrations are carried out in normal saline at 37°C. Plots of f_E and f_D versus pK_a are given in Figure 9. In both cases there is no significant pK_a change in the 1.0 to 0.8 mole fraction range. At lower mole fractions, that is, as the carboxyl containing side chain is further replaced by an isopropyl side chain, the pK_a systematically increases up to values approaching 7.0.

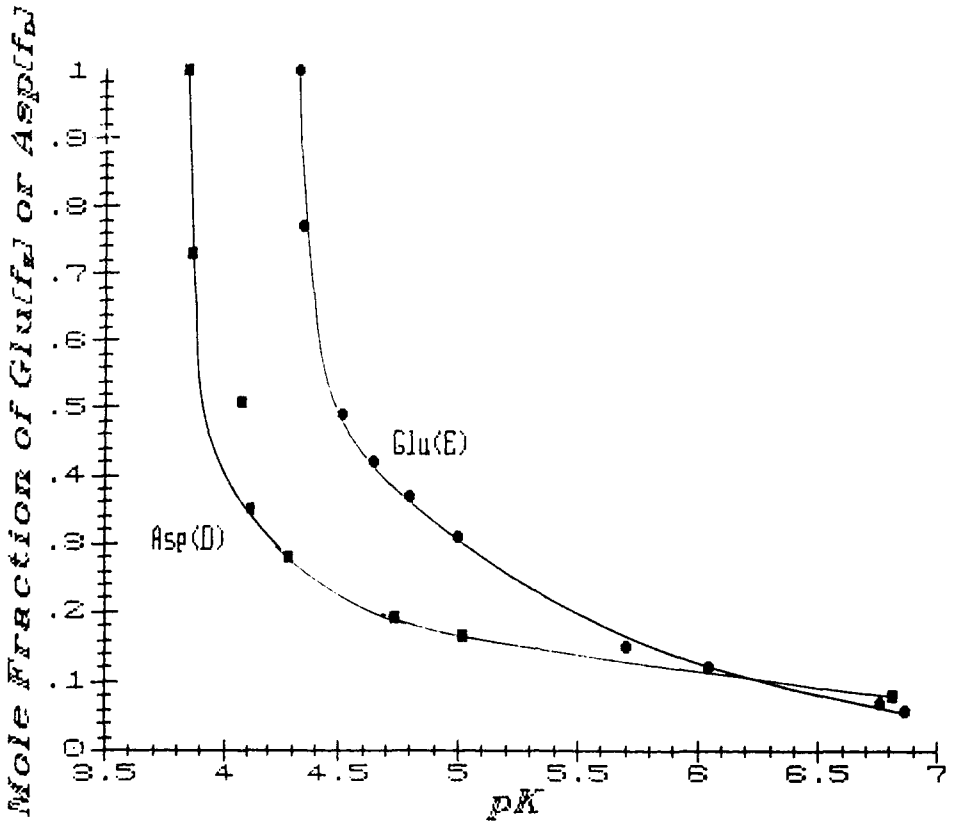


Figure 9

It is clear from the data in Figure 9 that the observed pK_a shifts are due exclusively to hydrophobicity increases. This is because charge-induced pK_a shifts would be seen as increases in pK_a as the mole fractions f_E and f_D increased rather than decreased. A situation where both charge-induced and hydrophobicity-induced shifts were observed would have curves which were parabolic with regard to the mole fraction axis. Interestingly, both the pK_a for Glu and that for Asp approach 7 as the mole fraction drops below 0.1. As the pK_a for Asp begins at about 3.8 for $f_D = 1$ whereas that for Glu is near 4.3, the greater pK_a shift appears to be exhibited by Asp. This will be tested by additional data points for Asp in the 0.14 to 0.08 mole fraction range and in the polytricosamer system of Figure 5D where Glu is replaced by Asp. This is planned in Phase II.

b. Hydrophobic Domain Size and Intensity Studies

When poly(GVGVP) and poly(GVGIP) are mixed at low temperatures, below 10°C, and the temperature of the mixture is raised, the latter begins separating out (coacervating) at 11°C, whereas the former does so at 28°C. The molecules self separate with two transitions based on their hydrophobicities.

When the random copolypentapeptide, poly[0.5(GVGVP),0.5(GVGIP)], is studied, it separates out (coacervates) at an intermediate temperature of 19°C as a single transition. The objective is to find, for a set of protein-based polymers $(GVGVP)_l$-$(GVGIP)_m$, the lower limit of l and m, for which the two domains will exhibit separate transitions. To date, low enough molecular weights have yet to be obtained. Presently underway are polymerizations for 4, 8 and 16 hours which are to be fractionated by dialysis and coupled.

In addressing the issue of hierarchical hydrophobic folding in proteins, the pentamers required to synthesize the polymers, that is, Boc-GVGVP-OH, Boc-GVGIP-OH and Boc-GFGVP-OH, have been synthesized. A screening effort was undertaken to obtain polymers of lower molecular weight, i.e., less than 50 kDa. The efforts are:

Boc-GVGVP-ONp was deblocked with TFA and polymerized for 48 hours instead of our usual 14 days (to obtain greater than 50 kDa) and dialyzed against 50,000 molecular weight cut-off dialysis tubing and lyophilized. Surprisingly, the yield in terms of the quantity of polymer retained in 50,000 molecular weight cut-off tubing after extensive dialysis against water was in the 75 - 80% range with a T_t of 25°C. This means that high quality high molecular weight $(GVGVP)_l$ was obtained with $l >$ 120 in just 48 hours of polymerization.

Boc-GVGVP-OH was deblocked and polymerized for 24 hours using EDCI as the polymerizing agent in the presence of HOBt. The polymer was dissolved in water and dialyzed against 50,000 molecular weight cut-off dialysis tubing and lyophilized. It was found that the yield of the polymer having greater than 50,000 molecular weight was about 75%. It has also been found in our previous studies that EDCI alone does not produce polymer having molecular weight greater than 3500.

Boc-GVGVP-OH was deblocked and polymerized for 4 hours, 8 hours, and 16 hours separately using EDCI and HOBt. The 50,000 dialysis of these polymers is in progress. By using the

shorter times, it is hoped that lower molecular weights will be obtained which could then be coupled to $(GVGIP)_m$ polymers similarly prepared. An additional approach is included in the Phase II proposal.

c. Effect of Protein Naturation and Purification Solutes on Hydrophobic Higher Order Structuring.

There are many solutes which are routinely used in protein renaturation/denaturation and purification efforts for which a better understanding of function is required. Our model protein-based polymers allow identification and even quantitation of their relative effects on hydrophobic folding and assembly. The solutes studied in Phase I are urea, guanidine HCl, sodium dodecyl sulfate (SDS), Triton X-100, ammonium sulfate, sodium chloride, trifluoroethanol (TFE), trifluoroacetic acid (TFA), and glycerol. All of these solutes have been used in protein purification, renaturation/denaturation and structure studies. The results are as follows: In order of potency in raising the temperature, T_t, of the inverse temperature transition of 40 mg/ml of poly(GVGVP) are SDS (600°C/mole, measured at 0.1 M), guanidine HCl (120°C/mole at 0.3 M), urea (55°C/mole at 0.3M), and Triton X-100 (30°C at 0.3 M). This is, therefore, the relative effectiveness in disrupting hydrophobic folding and assembly. These data are plotted in Figure 10A (Data for triton X100 ends at 0.3 M since at higher concentrations it separates out of solution.). In order of potency in enhancing hydrophobic association, that is, in lowering the value of T_t for 40 mg/ml of poly(GVGVP) are ammonium sulfate (–80°C/mole measured at 0.2 M), sodium chloride (–14°C/mole at 0.4 M), trifluoroethanol (–11°C/mole at 0.4 M), trifluoroacetic acid (–9°C/mole at 0.4 M), and glycerol (–2.2°C/mole at 0.4 M). The data are given in Figure 10B. Also included in Figure 10 are data for dimethylsulfoxide (DMSO) and ethylene glycol which are plotted with respect to the top scale in the figure from 0 M to 5 M. It is now becoming possible, therefore, to plan a strategy based on primary structure to identify regions in a protein and use the above studied solutes to fold and unfold or assemble and disassemble hydrophobic associations. What is required now is a determination of the relative effectiveness of these as a function of degree of hydrophobicity, that is, it is now necessary to carry out these studies on protein-based polymers of differing mean hydrophobicities as measured by the scale

of Table I. Studies to date using sodium chloride indicate that the slope of –14°C/mole is the same for different hydrophobicities. This will need to be determined for the other solutes.

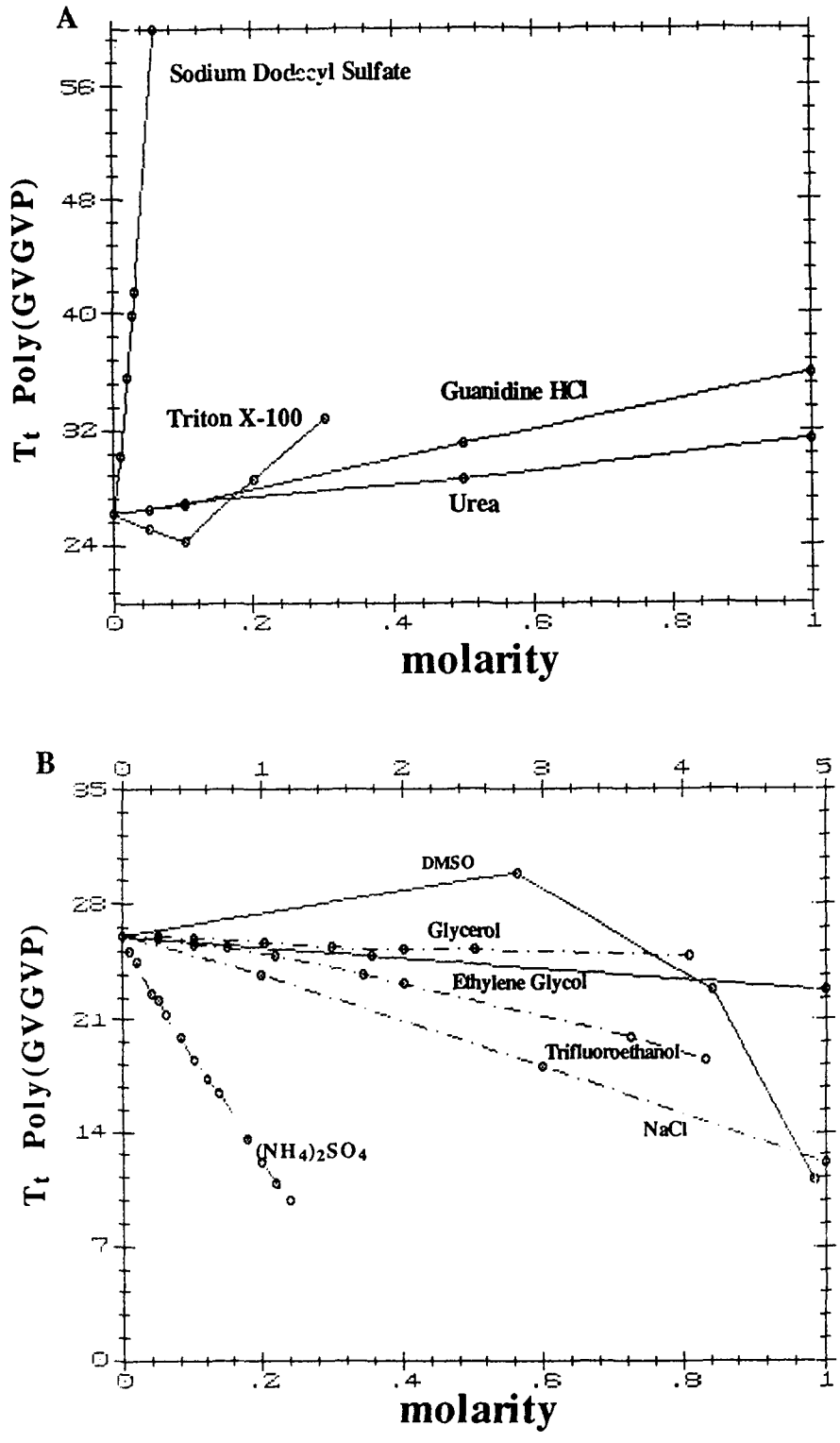


Figure 10

4. Technical Feasibility Demonstrated in Phase I

Two fundamental contributions have been made in Phase I. The target of a Glu pK_a shift of up to 3 pH units was exceeded and a series of solutes within the new ΔT_t paradigm can be readily defined as to whether and with what efficacy they enhance or disrupt hydrophobic folding and assembly in proteins.

The observed pK_a shift for Glu from 4.3 to 8.1, to our knowledge, is the largest shift ever documented for a Glu residue, and it was achieved by design using polytricosapeptides in which 5 apolar Phe(F) side chains were at approximately 1 nm distances from the polar Glu (COOH) side chain (See Figure 5). This pK_a shift represents a free energy of interaction of over 5 kcal/mole, as calculated from the expression for the change in chemical potential, $\Delta\mu$, required to maintain the Glu side chain at 0.5 degree ionization, i.e., $\Delta\mu = 2.3\ RT\ \Delta pK_a$ with R = 1.987 cal/mole degree and T in °K. These are referred to as apolar-polar repulsive free energies of hydration, and they are clearly the magnitudes of interaction energies that control protein folding and drive protein function.

The data of Figures 10 A and B show the relative effects of solutes in disrupting (raising the value of T_t) or in enhancing (lowering the value of T_t) hydrophobic folding and assembly, which is basic to higher order structuring in proteins. Accordingly, the coarse outline of an approach for achieving properly folded and assembled protein begins to emerge. One begins with the $\langle T_t \rangle_{11}$ profile for the protein of interest, examples of which are given in Figure 4. These identify the hydrophobic domains. Next with the data of Figures 10 A and B the hydrophobic associations within and between the domains can be controlled by addition or removal of a particular solute and while favoring or disfavoring secondary structure formation. For example, consider TFE and TFA, both of which enhance hydrophobic association. TFE enhances secondary structure, particularly α-helix formation, while TFA disrupts secondary structure. This means that it is possible, depending on the folding regimen to be tried, to enhance or to disrupt secondary structure while favoring hydrophobic association.

When the variable of solute is combined with effective use of the degree of ionization of particular functional side chains within or proximal to the hydrophobic domains, considerable control over the higher order structuring of protein is obtained. What is now required are the determination of the hydrophobicity-induced pK_a shifts of the rest of the amino acid residues, the determination of hydrophobic domain size and intensity for independence of folding, the determination of combinatorial effects of organic and inorganic solutes with varied hydrophobicity, and the determination of the effect of hydrophobicity on enhancing charge-charge interactions. These are planned in the Phase II effort.

Therefore in Phase I, an extraordinary candidate system has been identified and partially characterized for detailed study and design of appropriate methodology for verification of rules governing higher order (tertiary and quaternary) structure formation.

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