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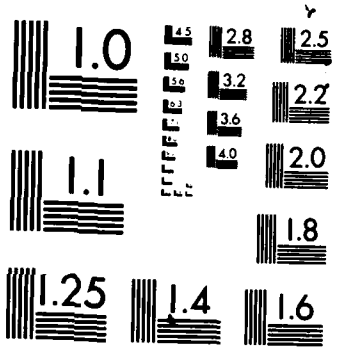

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Life Sciences Conference "From Enzymology To Cellular Biology"

Claire E. Zomzely-Neurath

September 15, 1986

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This report covers presentations on the topics of enzymology, enzyme engineering and biotechnology, metabolic regulation, gene expression, and transcriptional signals, and replication. It includes a table showing the scientific program of the conference.

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LIFE SCIENCES CONFERENCE "FROM ENZYMOLOGY TO CELLULAR BIOLOGY"

1 INTRODUCTION

This conference, entitled "From Enzymology To Cellular Biology," was organized by J. Sjulmajster and other members of the Laboratory of Enzymology of the Centre Nationale de la Recherche Scientifique (CNRS), Gif-sur-Yvette, France, to celebrate the 25th anniversary of its creation. There were 150 participants at this small, focused meeting, including invited speakers and scientists presenting posters. The largest number of participants were from France although five other European countries were represented, as well as the UK, the US, Pakistan, and India.

The topics of the papers at this conference ranged from biochemical and enzymological studies and metabolic regulation to molecular biological approaches including gene regulation and expression, enzyme engineering, and biotechnology. The format of the scientific program is shown in Table 1. In addition there were 43 posters dealing with the topics presented by the speakers at the conference. The major impact of molecular biological techniques, including recombinant DNA (rDNA) methods and site-directed mutagenesis on the progress in the area of enzymology, in terms of structure/function relations was evident from the presentations at this conference. However, the importance of combining information from classical kinetic and equilibrium studies as well as x-ray crystallography with the more recent molecular biological approaches was also emphasized.

A summary of some of the many interesting topics covered at this conference is presented in the following sections. Since the conference was quite extensive, it is not possible to cover all aspects. If additional information is desired, contact Dr. Claire E. Zomzely-Neurath at ONRL.

2 ENZYMOLOGY, YESTERDAY, TODAY, AND TOMORROW

W.N. Lipsomb (Harvard University, Cambridge, Massachussetts) discussed what he considered the important aspects of the present and future research in enzymology. An essential component is the determination of the three-dimensional structures of enzymes and their complexes. Lipscomb stated that x-ray diffraction studies do not establish mechanisms of protein folding, activity, or regulation. He stressed that it is only when the structural results are combined with those of other methods that these mechanisms can be addressed. Studies using the recent method of site-specific mutagenesis are now playing and will play even more in the future a very important role in structure/function studies. Correlated nuclear magnetic resonance (NMR), especially on site-modified residues is developing greatly in effectiveness. Other spectroscopic methods are being used in addition to NMR to identify the presence of intermediates, and low-temperature studies are beginning to be used widely. However, Lipscomb pointed out that the more classical kinetic and equilibrium studies are still required. The research objectives include protein folding, the role of domain structures and structural dynamics, the role of solvent, and the design of new enzymes for catalysts of chosen reactions. They also include mechanisms at the most elementary atomic level of activity, regulation, and assembly.

Lipscomb stated that for the future of x-ray diffraction, he expects that the combination of area detectors, synchotron radiation, low-temperature techniques, and use of large computers will make structures and their complexes available at an increasing rate. New enzymes and other proteins are also becoming available at an increasing rate, owing to genetic engineering and the fact that sequences can be varied at will. Lipscomb also said that the almost untouched area of membrane proteins is opening up as reagents are being developed to crystalize them. Methods for obtaining large amounts of x-ray diffraction data are improving, as are combinations of heavy atom derivatives, anomalous scattering, and molecular (fragment) replacement. Lipscomb thinks that the recent

Table 1

Scientific Program: "From Enzymology to Cellular Biology"

Enzymology, Yesterday, Today, and Tomorrow

Chair: B.L. Horecker, Cornell University Medical School, New York.

"Structural Studies of Activity and Regulation in Enzymes," W.M. Lipscomb, Harvard University, Cambridge, Massachusetts.

"Recent Advances in X-ray Crystallography: Time-Resolved Studies on Catalysis in the Crystal with Glycozen Phosphorylase b.," J. Hajdu, Laboratory of Molecular Biophysics, The Rex Richards Building, Oxford, UK.

"Flux-ratio Studies of Rat Muscle Hexokinase," A. Cornish-Bowden, Department of Biochemistry, University of Birmingham, UK.

"Dynamics of Multienzyme Reactions at the Surface of Plant Cells," J. Ricard, Centre de Biochimie et de Biologie Moleculaire du CNRS, Marseille, France.

"The Structural Organization of Aminoacyl-tRNA Synthetases in Eukaryotes," Laboratoire de Biochimie, École Polytechnique, Palaiseau, France.

Enzyme Engineering and Biotechnology

Chair: J. Yon, Laboratoire d'Enzymologie, Orsay, France.

"From the Renin Gene to Renin Inhibitors," P. Corval, INSERM U36, Vascular Pathology and Renal Endocrinology, Paris, France.

"A Model of Synthetase/tRNA Interaction as Deduced by Protein Engineering," H. Bedonelle, MRC Laboratory of Molecular Biology, Cambridge, UK.

"Alteration of the Inhibition Specificity and Oxidation Sensitivity of L1-antitrypsin by Site Specific Mutagenesis," M.G. Courtney, Transgése S.A., Strasbury, France.

"Enzymes as Catalysts for Synthesis Reactions," P. Monsan, BioEurope - C.T. Bio-INSA, Toulouse, France.

Metabolic Regulation

Chair: N. Glansdorff, CERIA, Brussels, Belgium.

"Principles of Metabolic Regulation," E. Stadtman, Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland.

"Regulation of Enzyme Activity at Physiological Concentrations," A. Sols, University of Madrid, Spain.

"Microbial Control of Carbonylphosphate Synthesis," A. Piérard, Laboratoire de Microbiologic, Université Libre de Bruxelles and Institut de Rescherches du CERIA, Brussels, Belgium.

"Virus Multiplication and Polyamine Synthesis in Protoplasts of Chinese Cabbage," S. Cohen, Department of Pharmacological Sciences, State University of New York at Stony Brook, New York.

"Protein Phosphatases and Kinases Cellular Recognition," E.H. Fischer, Department of Biochemistry, University of Washington, Seattle, Washington.

Gene Expression and Transcriptional Signals

Chair: M. Grunberg-Monago, Institut de Biologie Physicochemique, Paris, France.

"Control of Gene Transcription in Lukaryotes and Prokaroytes: a Common Mechanism," M. Ptashne, Harvard University, Cambridge, Massachusetts.

"Enhancers and Cell-type Specificity of Transcription," W. Schaffner, Institut für Molekularbiologie II der Universität Zürich, Switzerland.

"Gene Expression of Aminoacyl-tRNA Synthetases," S. Blanquet, Laboratoire de Biochemie, École Polytechnique, Palaiseau, France.

DNA Replication

Chair: M. Kamen, University of California, San Diego, California.

"DNA Supercoiling as a Regulator of Bacterial Gene Expression: The role of DNA Gyrase," M. Gellert, Laboratory of Molecule Biology, National Institutes of Health, Bethesda, Maryland.

"Single-Stranded Circular Intermediates in Plasmid DNA Replication," S.D. Ehrlich, Institut Jacques Monod, Paris, France.

"DNA Replication and the Regulatory Function of *E. coli* Rec A Protein," J. Roberts, Section of Biochemistry, Molecular and Cell Biology, Cornell Univeristy, Ithaca, New York.

"Error Correction in DNA Replication," M. Radman, Laboratoire de Mutagène, Institut Jacques Monod, France.

Molecular Basis of Cell Motility

Chair: M.W. Kirschner, Department of Biochemistry and Biophysics, University of California, Medical School, San Francisco, California.

"The Molecular Basis of Actinomycin-Dependent Cell Motility," E.D. Korn, Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland.

"Microtubule Dynamics and Cellular Morphogenesis," M.W. Kinschner, Department of Biochemistry and Biophysics, University of California, Medical School, San Francisco, California.

"The Inhibition of Microtuble Assembly by Colchicine: A Review," Y. Engelborghs, Laboratory of Chemical and Biological Dynamics, University of Leuvan, Belgium.

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improvements in refining structures will continue and make it worthwhile to restudy at high resolution structures which have been solved earlier. He also stated that we can only say that we really know how enzymes work when it will be possible to synthesize organic molecules that catalyze reactions as efficiently as enzymes do.

Lipscomb also presented some of his work on proteolytic enzymes, including some "transition state" inhibitors for carboxypeptidase A, and on regulatory enzymes including the allosteric transition and binding of PALA (N-phosphonacetyl L-aspartate) to aspartate transcarbamylase.

J. Hajdu (Laboratory of Molecular Biophysics, Oxford, UK) presented some of his recent studies on glycogen phosphorylase b. Glycogen phosphorylase is the key control enzyme of glycogen metabolism both in muscle and in liver. Detailed x-ray crystallographic studies in progress by Hajdu and colleagues in Oxford are aimed at elucidating the stereochemical basis of the enzyme mechanism and its control.

In resting muscle, phosphorylase is inactive, and this form is termed phosphorylase b. In response to nervous or hormonal stimulation, phosphorylase b is converted to phosphorylase a by the reversible phosphorylation of a single serine residue. Phosphorylase a is active and can catalyze the first step in glycogen breakdown, leading eventually to the formation of ATP to supply energy to the muscle. Alternatively, when ATP levels are low and AMP levels are high, phosphorylase b can be activated by a different mechanism, i.e., noncovalent allosteric control. Thus, phosphorylase exemplifies two different mechanisms of control designed to meet the energy requirements of the cell. The crystal studies of phosphorylase b have resulted in a detailed understanding of the enzyme structure and the metabolite binding sites.

Hajdu et al. are now exploring the mechanism of catalysis of phosphorylase b. Fast crystallographic data-collection

techniques and the intense x-radiation generated on the Wiggler magnet of SRS Daresbury were used to monitor events in three dimensions on the catalytic pathway as the substrate is converted to product. Each experiment required a new crystal. Hajdu stated that recent developments have enabled him to return to the earliest method of data recording, the Lane method (stationary crystal, white radiation: 0.45 Å<λ<2.5 Å). With this method data can be recorded in 250 milliseconds. This has made possible a series of dynamic experiments in which the conversion of substrate to product can be monitored in the same crystal. Hajdu thinks this method shows great promise for the future.

Studies of the dynamics of multienzyme reactions at the surface of plant cells were presented by J. Picard (Centre de Biochemie et de Biologie Moleculaire du CNRS, Marseille, France). The enzymes which are located in the plant cell walls are involved in both cell-wall extension and growth as well as in the transport of organic solutes in the cell. Most of these enzymes (for instance, an acid phosphatase) display different types of kinetic behavior depending on whether they are bound to the cell wall or are in free solution. This difference of behavior, which is controlled by ionic strength, is explanable by electrostatic repulsion effects of charged substrates (e.g., phosphate) by the fixed negative charges of the polyanionic cell-wall matrix. Although the acid phosphatase displays classical Michaelis-Menten kinetics in free solution, it shows apparent negative cooperativity when bound to the cell surface. These effects are suppressed upon raising the ionic strength. Calcium, which by itself has no effect on the enzyme in free solution, strongly binds to the cell wall and modulates the extent of negative cooperativity by controlling the Donnan potential of the cell wall.

Microenvironmental effects on plant cell-wall enzyme activity has enabled J. Picard to propose a novel theory of the ionic control of plant cell-wall extension and growth. The model, which is based on various experimental results, involves several assumptions:

1. A cell-wall pectin methyl esterase de-esterifies pectins and thus creates the fixed negative charges of the cell wall.
2. Various enzymes incorporate unchanged carbohydrates in cell-wall material and allow the sliding of cellulose microfibrills.
3. The enzymes responsible for carbohydrate incorporation and cell-wall extension are activated by protons in a pH 4-8 range, whereas methyl esterase is inhibited by protons in the same pH range.

The very basis of the theory is the existence of an electrostatic potential difference, $D\psi$, between the inside and the outside of the cell-wall. When this $D\psi$ value is large, the local proton concentration is high. Thus, the enzymes involved in cell-wall extension and growth are active, but pectin methyl esterase is not. Therefore, the cell wall extends and the charge density decreases. The $D\psi$ value then declines, as well as the local proton concentration. Under these conditions, the pectin methyl esterase becomes activated, whereas the "growth enzymes" do not. This activation of pectin methyl esterase restores the initial, or an even higher, electrostatic potential difference which, in turn, results in a decrease of local pH.

J.P. Waller (Laboratoire de Biochemie, École Polytechnique, Palaiseau, France) presented some of his recent research on the structural organization of aminoacyl-tRNA synthetases (AARS) in eukaryotes. All AARS of lower eukaryotic (yeast) and mammalian origin display the ability to bind to immobilized polyanionic supports (heparin, RNA) through electrostatic interactions in conditions where the corresponding enzymes from prokaryotes do not. Studies on purified lysyl-tRNA from yeast by Waller and coworkers showed that binding is mediated by a structural domain excisable by proteolysis, with full retention of activity. The ensuing truncated enzyme

displays the same subunit size as the corresponding prokaryotic enzyme. The evolutionary acquisition of this binding property by all eukaryotic AARS suggests to Waller that this acquisition fulfills an important function *in vivo* unrelated to catalysis which may ensure the compartmentalization of AARS within the cytoplasm through electrostatic interactions with negatively charged components.

Unlike lower eukaryotic AARS, those of mammalian origin additionally display the property of forming multienzyme complexes. Waller et al. found that analysis by gel filtration of the size distribution of each of the twenty AARS from a crude extract of cultured Chinese hamster ovary cells showed that nine AARS (specific for the amino acids Ile, Leu, Met, Lys, Arg, Glu, Gln, Asp and Pro) co-elute at a volume corresponding to an apparent molecular weight of 1.2×10^6; the remainder displayed values characteristic of "free" enzymes. The high-molecular-weight entities were co-purified to yield a heterotypic multienzyme complex comprising each of the nine AARS and composed of 11 polypeptides, as revealed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Multienzyme complexes containing the same nine AARS and displaying very similar polypeptide compositions were also purified from rabbit, rat, and sheep liver. Waller et al. obtained evidence that complex formation is mediated by hydrophobic interactions. The isoleucyl-lysyl and leucyl-tRNA synthetase components dissociated from the complex with full retention of activities and purified to homogeneity, bind strongly to hydrophobic support in conditions where the corresponding enzyme from yeast to *E. coli* do not. Moreover, mild proteolysis of the complex generates fully active, truncated, free forms of the lysyl- and methionyl-tRNA sythetase components which have lost the ability to bind to hydrophobic supports. This implies the existence of a hydrophobic domain, distinct from the catalytic domain and responsible for complex formation. In accordance with this interpretation, Waller et al. found that the mammalian AARS specific for Trp, His and Thr, representative of those enzymes which are not encountered as complexes, do not display hydrophobic properties.

The reason why only certain AARS have acquired hydrophobic properties allowing their assembly as a multienzyme complex remains obscure. Waller suggests that complex formation may contribute to the stabilization of the "loose" network of AARS electrostatically bound to cellular components at or near the site of protein synthesis; also that this is established by interconnections between these enzymes through hydrophobic interaction. Waller et al. have obtained immunocytological evidence supporting the association of mammalian AARS with cellular components.

3 ENZYME ENGINEERING AND BIOTECHNOLOGY

P. Corvol (INSERM Unit 36, Vascular Pathology and Renal Endocrinology, Paris, France) reviewed the research by his group as well as that of other laboratories on the structure of renin and its precursor; he then presented some of the recent work on the search for inhibitors of renin. More than 80 years elapsed between the discovery of the pressor effect of a kidney extract and that of the structure of renin and its precursor. In 1982, the first reports were published concerning the primary structure of mouse submaxillary renin and the nucleotide sequence of this renin's structural gene. The techniques of molecular biology enabled the structure of the gene for human kidney renin to be elucidated 2 years later, and from this data it was possible to deduce the primary structure of renin and its precursor. The site of renin catalysis is now known and three-dimensional models for renin have been proposed, thus providing a rational basis for attempts to identify its inhibitors.

There is a considerable degree of homology (68 percent) between the primary structures of human renin and mouse submaxillary renin. However, the latter is not glycosylated, whereas human renin, whose fate is plasmatic, possesses two potential N-glycosylation sites. Renin precursor maturation was resolved using

the mouse submaxillary renin. The initial precursor was a preprorenin form which is converted to prorenin. Upon release of a signal peptide, the prorenin is cleaved in two sites to produce mature, active renin containing two chains linked by a disulfide bridge. The precise maturation of human kidney renin is not known. However, it is highly probable that the maturation of the human renin precursor is superimposable on that of mouse renin because of the similarity of its primary structure to that of mouse renin at the primary cleavage sites.

It has been found that renins belong to the acid protease family. This class of proteins comprises pepsin, chymosin, penicillopsin, and cathepsin D. These proteins all contain two aspartic acids which have a key role in the hydrolysis of the peptide bond. Recent studies of the organization of the submaxillary renin gene and the human renin gene showed that this gene essentially comprised four exons arranged symetrically in two blocks, each exon coding for one lobe of the protein. According to Corval, the fact that renin belongs to the acid protease family has three important consequences as regards the search for its inhibitors:

1. The exact tertiary structure of renin is not known as it has not as yet been possible to crystalize human renin and therefore to obtain its atomic coordinates. However, on the basis of the coordinates of other acid proteases, it is possible to establish models for the three-dimensional structure of mouse submaxillary and human kidney renin.

2. Given the similarity of the active sites of renin and those of the other acid proteases, specific inhibition of renin or any other acid protease is likely to be difficult because the mechanisms by which these proteases are inhibited as regards the attack on the peptide bond is unambiguous, and the specificity of any inhibitor therefore resides in the substrates of inhibiton and substrate recognition by the enzyme.

3. Acid proteases are inhibited by the peptides derived from their prosegment. Thus the 1-16 NH_2 terminal segment of pig pepsinogen inhibits pepsin activity. This property has been applied to renin, which Corvol showed was inhibited by peptides derived from this prosegment.

Renin has only one known substrate--angiotensinogen. Contrary to the other acid proteases which have a large variety of substrates, renin only hydrolyzes angiotensin I from angiotensinogen. Human renin has very little affinity for rat or dog angiotensinogen which it hydrolyzes only very slowly. One of the reasons hyman renin is specific for human angiotensinogen is due to the N-terminal sequence of human angiotensinogen which differs markedly from that of rat and pig angiotensinogen at the Leu_{10}-Val_{11} cleavage site and also because of the residues that follow on the C-terminal side. The other characteristic of human renin is its immunological specificity. The polyclonal and monoclonal antibodies (Mabs) obtained against human renin recognize neither the other acid proteases nor the renin of other species than man except primates. All these observations indicate the difficulty of identifying renin inhibitors. These inhibitors will have to be large enough to occupy the many sites of interaction with the enzyme. At the outset, their structure will have to be modeled on that of human angiotensinogen in order to obtain specific inhibitors of human renins. Lastly, renin inhibitors will have to be tested in *in vitro* systems of human or primate renin or human angiotensinogen. *In vivo* experimentation will mainly have to be done in the primate.

Corvol et al. have found in studies with human renin antibodies using primates on a normal sodium diet that renin regulates the level of arterial pressure. These results are important as they constitute a strong incentive to discover inhibitors of the renin-angiotensinogen reaction. Corvol and his group have also been studying the inhibitor effect of prorenin segments (natural inhibitors). They hypothesized that renin might be inhibited by peptide segments derived from its own prosegment. Indeed, Corvol

et al. found that the synthetic peptide corresponding to residues 15-19 of the mouse and human prorenin sequence proved to be an inhibitor of submaxillary renin with a Ki the order of one micromole. It is therefore likely, according to Corvol, that these peptides located near the N-terminal part of the prosegment interact with the rest of the molecule near the active site by blocking access to the catalytic site.

The present inhibitors of renin include an analog of the transition state of peptide bond hydrolysis by renin. Corvol et al. have prepared renin inhibitors homologous with pepstation which is a natural pepsin inhibitor originally isolated from actinomyces culture. The synthesis of more specific inhibitors with a greater affinity for human renin was worked out by Corvol et al. by retaining the central statine (of pepstatin) as an inhibitor of the site of acid protease catalysis and by replacing the amino acids surrounding it by amino acids found in angiotensinogen or those displaying an affinity for the corresponding renin subsites. They have synthesized peptides with 1-10,000 times more affinity for human renin than pepstatin and with markedly reduced affinity for pepsin. Intravenous administration of these peptides to the monkey lowered arterial blood pressure in a dose-dependent manner, blocked plasma renin activity, and caused a fall in the level of angiotensin II. A parallel can be drawn between the hypotensive effect of these compounds and the reduction or abolition of the enzymatic activity of renin. Like renin antibodies, they act on the normotensive primate on a normal or sodium-depleted diet, exerting their maximal effect in cases of sodium depletion. The duration of the action of statine-containing compounds varies, depending on their structure and the doses administered. For example, Corvol et al. found that administration to the macque of 3mg/kg of the pentapeptide Iva-Phe-Nle-Sta-Ala-Stn (SR 4218) lowered arterial pressure for 3 hours and completely blocked renin activity during that time.

H. Bedquelle (MRC Laboratory of Molecular Biology, Cambridge, UK) presented a model of synthetase/tRNA interaction as deduced by protein engineering. The recognition of transfer RNAs(tRNA) by their cognate aminoacyl-tRNA synthetases is the crucial step in translation of the genetic code. In order to construct a structural model of the complex between tyrosyl-tRNA synthetase (Tyr Ts) from *B. Stearothermophilus* and tRNA Tyr, 40 basic residues at the surface of the Tyr Ts dimer were mutated by oligonucleotide site-directed mutagenesis. Heterodimers were created *in vitro* by recombining subunits derived from different mutants. A cluster of basic residues (Arg 207-Lys 208) in the N-terminal domain of one Tyr Ts subunit interacts with the acceptor stem of tRNA Tyr, and two separated clusters of basic residues (Arg 368-Arg 371; Arg 407-Arg 408; Lys 410-Lys 411) in the C-terminal domain of the other subunit interact with the anticodon arm. The Tyr TS would thus change the tRNA in a fixed orientation. The precise alignment of the flexible ACCA3' end of the tRNA for attack on the tyrosyl adenylate is made by closer contacts to the catalytic groups of the enzyme, such as with Lys 151.

Studies of protein engineering using site-specific mutagenesis were also reported by M.G. Courtney (Transgene, S.A., Strasbourg, France). He carried out research on the alteration of the inhibition specificity and oxidation sensitivity of α_1-antitrypsin (α-AT) by site-specific mutagenesis. This enzyme is a human serine protease inhibitor whose major physiological role is to protect the lower lung from damage mediated by the elastolytic neutrophil proteases, elastases, and cathepsin G. Hereditary deficiency of α_1-AT leads to a protein burden in the lung and is associated with a high incidence of severe pulmonary emphysema. However, the major cause of emphysema is cigarette smoking, and in these cases there is also evidence of an imbalance between α_1-AT and proteases, partly due to oxidative inactivation of the α_1-AT. With a view to its application in

replacement therapy, Courtney and his group have produced recombinant α-AT in an *E. coli* host/vector system. Using site-specific mutagenesis techniques they have isolated a series of active center mutants of α_1-AT. These were analyzed both for their ability to inhibit various serine proteases and for their sensitivity to oxidative inactivation. Courtney et al. have identified oxidation-resistant variants that inhibit neutrophil elastase, cathepsin G, or both. α_1-AT (Met^{358}→Leu) does not react with neutrophil proteases but is an excellent inhibitor of thrombin and the contact phase proteases kallikrein and factor XII.

Another variant of these studies showed that the specificity of inhibition of α_1-AT can be altered by creating a potential cleavage sequence for a particular serine protease at the active center. Because of its oxidation-resistant inhibition of both neutrophil elastase and cathepsin G, the variant α_1-AT (Met^{358}→Arg) is considered by Courtney et al. to be particularly suitable for the treatment of pulmonary emphysema. α_1-AT (Met^{358}→Arg) as an efficient cofactor-independent thrombin inhibitor has potential according to Courtney, as a clinical anticoagulant as, for example, in the treatment of various thromboembolisms or disseminated intravascular coagulation (DIC). The additional ability of α_1-AT (Met^{358}→Arg) to inhibit kallikrein and factor XII allows the possibility of controlling disease states associated with activition of the kinin-forming system; e.g., in hereditary angioedema and septic shock. These studies indicate that it has been possible in the case of α-AT, to perform protein engineering without access to pertinent three-dimensional structural analysis. The raw data for this work comes from a comparison of the amino acid sequences of the serine proteases family and from a detailed knowledge of the substrate specificities of the proteases themselves. In this way, Courtney et al. have created new molecules with definite potential as therapeutic agents.

P. Monsan (BioEurope, Toulouse, France) discussed biotechnological applications of enzyme catalysts. These applications involve mainly hydrolytic reactions. Starch and protein hydrolysis are today's most important industrial applications of enzymes. Although about 3000 different enzyme activities have been characterized up to now, less than 30 are currently in use at the industrial level. The increase of the application of enzymes on technological levels thus involves the development of new reactions. Monsan thinks that, more particularly, there is a lack of the use of enzymes in the field of synthesis reactions.

Monsan reviewed some recent developments in synthesis reactions catalyzed by enzymes. Three main approaches are followed to achieve synthesis reactions:

1. Use of cofactor enzymes. As in metabolic pathways, the coupling of an exerogenic reaction involving the stoichiometric consumption of a cofactor molecule (ATP, NADH,...) makes possible an enderogenic synthesis reaction. The use of such reactions at a preparative scale involves the development of an efficient cofactor regeneration system. An example is the case of nicotinamide cofactors and their application to amino acid synthesis.

2. Reversion of hydrolysis equilibria. Hydrolytic enzymes can also catalyze the reverse reaction. The use of appropriate thermodynamic(al) reaction conditions and/or the continuous removal of reaction product thus allows a shift of the reaction equilibrium toward the direction of synthesis. For example, quite interesting developments have been obtained in the application of proteolytic enzymes to peptidic bond synthesis. Concentrated media or immiscible organic solvents can be used to allow a reaction equilibrium shift.

3. Transferase enzymes. This class of enzymes is used to catalyze the synthesis of a wide range of molecules through the transfer of a chemical moiety from a donor to an acceptor. Glycosyl transferases, for example, have been applied to oligo- and polysaccharides synthesis (cyclodextrans, dextrans, etc.).

4 METABOLIC REGULATION

E.R. Stadtman (Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland) presented an extensive and excellent review of the principles of metabolic regulation. In general, the regulation of highly independent metabolic processes is achieved by controlling the concentrations and activities of key enzymes. The activity of enzymes is regulated by allosteric effectors, mass action effects, covalent modification of enzyme functional groups, compartmental separation of enzyme function, and the organization of functionally related enzymes into macromolecular complexes. Feedback inhibition of the first enzyme in a pathway by the ultimate product of the pathway regulates most unifunctional enzymes, but more complex patterns of allosteric control are used to regulate branched biosynthetic pathways, catabolic pathways, and amphibolic pathways. Balance between different metabolic functions is achieved by compensatory control systems that include feed-forward activation and feedback inhibition of rate-limiting steps in one pathway by the accumulation of metabolites in another.

Stadtman used studies of glutamine synthetase (GS) from *E. coli* to illustrate fundamental mechanisms implicated in the control of many key enzyme activities, enzyme level, and enzyme turnover. GS as well as other protein components involved in its enzymatic function have been cloned, thus greatly facilitating the studies. Furthermore, it has been shown that the structural gene (glnA) for GS is controlled by several gene products. Stadtman also reported that the accumulation of altered inactive or less active forms of enzymes during aging may reflect changes in the levels of one or more of the enzymatic regulating factors or in the levels of proteases that degrade altered enzymes. This possibility is supported by studies showing that the levels of oxidized proteins in cultured human fibroblasts increase with the age of the donor and that the levels in fibroblasts of young individuals with premature aging disease (progenia, Werner's syndrome) are about the same as those in fibroblasts from a 75-year-old normal individual.

A. Sols (University of Madrid, Spain) presented some of his recent studies on the regulation of enzyme activity at physiological concentrations. He had found in previous work that at the macromolecular environment prevailing in animal cells some regulatory enzymes exhibit markedly different kinetic properties from the *in vitro* system. Notable among such enzymes is animal phosphofructokinase. In an attempt to account for the behavior of certain enzymes *in situ*, Sols and colleagues have recently developed two alternative or complementary approaches for kinetic studies of enzymes at or near their physiological concentrations. They are the "slow motion" approach, in which enzymes are studied in conditions that greatly lower the actual molecular activity without affecting the conformation of the enzyme, and the "crowding" approach based on the presence of high concentrations of polyethylene glycol. Sols et al. found that the main reason for the large differences in kinetic behavior of animal phosphofructokinases between *in situ* and *in vitro* were homologous interactions at the higher degree of aggregation that corresponds to the concentration of the enzyme prevailing *in vivo*. Similar, although less marked effects, were found for pyrurate kinase but not for several other enzymes examined. Sols et al. have also explored the possibility of heterologous interactions in the case of the highly multimodulated muscle phosphofructokinases. Applying the "slow-motion" and "crowding" approaches to mixtures of phosphofructokinase and metabolically neighboring enzymes, Sols et al. found kinetically significant heterologous interactions between phosphofructokinase and fructose phosphate, with activation of the former and inhibition of the latter. Sols considers that these approaches are of value for a better approximation of the accurate knowledge of enzyme regulation *in vivo*.

Studies on the microbial control of carbamoyl phosphate synthesis utilizing molecular biological techniques were presented by A. Piérard (Laboratoire de Microbiologie, Université Libre de Bruxelles, Belgium). Microorganisms exhibit considerable diversity regarding the organization and control of the biosynthesis of phosphate, a key precursor of both arginine and pyrimidine biosynthesis. Most prokaryotes use a single carbamoyl phosphate synthetase (CPSase) regulated according to its dual metabolic function. This is illustrated by *E. coli*. CPSase, which is subject to allosteric control of activity by intermediates of both pathways and cumulative repression of synthesis by arginine and pyrimidines. The mechanism of the cumulative expression of the *Car Ab* operon encoding the nonidentical subunits of CPSase has been elucidated by Piérard and coworkers.

A significantly different situation prevails in fungi and in particular in *S. Cerevisiae* where they have found that carbamoyl phosphate synthesis is carried out by two independently regulated synthetases. CPSase P, the pyrimidine pathway synthetase, is part of a multifunctional protein confined to the nucleus; it is repressed and feedback inhibited by the pyrimidine. The arginine pathway synthetase, CPSase A, encoded by the unlinked *CPA1* and *CPA2* genes is subject to both the general control of amino acid biosynthesis and specific repression by arginine. In a detailed study of the expression of gene *CPA1*, Piérard et al. found that the repression of this gene by arginine operates at the level of translation. The *CPA1* messenger RNA (mRNA) has a 250-nucleotide-long leader sequence which, moreover, contains a small open reading frame (ORF) potentially coding for a 25-amino-acid peptide. Investigation of the AUG (initiation codon) of this ORF by oligonuclentide-directed mutagenesis suggests a control consisting in regulated selection of the translational initiation code. Piérard et al. are also working on the elucidation of the complete sequence of various carbamoyl phosphate synthetases and in a study of the evolution of carbamoyl phosphate metabolism.

E.H. Fischer (Department of Biochemistry, University of Washington, Seattle) discussed his studies on a heat stable inhibitor of cyclic AMP-(cAMP) dependent protein kinase (PKI) and the structure and regulation of phosphorylase phosphatase, an enzyme that may be under insulin control. Current modification of enzymes and proteins by phosphorylation-dephosphorylation has been recognized as a major mechanism by which cellular processes can be regulated. In carbohydrate metabolism, catabolic reactions seem to be triggered by the phosphorylation of regulatory enzymes while anabolic enzymes are active in their dephosphorylated forms. Therefore, according to Fischer, insulin, an anabolic hormone, should exert its metabolic function either by bringing about the inhibition of protein kinases or the activation of protein phosphatases.

Fischer and coworkers have determined the complete amino acid sequence of PKI and identified its inhibitory site. It displays all the determinants required for recognition by the enzyme except that instead of the seryl residue that is normally phosphorylated, there is an alanine. That this region represents the inhibitory site was confirmed by the synthesis of a number of inhibitory peptides; it shows homology with the "hinge region" of the regulatory subunit of cAMP-dependent protein kinase, thought to interact with, and inhibit, the catalytic site.

Fischer et al. found phosphorylase phosphatase exists as an inactive 70 kDa (kilodalton) complex made up of a catalytic subunit (38 kDa) and a regulatory subunit (inhibitor 2, 31 kDa). The enzyme can be activated by the protein kinase FA which phosphorylates inhibitor 2 and converts the catalytic subunits to an active conformation. While the activated enzyme undergoes autodephosphorylation, it remains active for a while until the regulatory subunit regains its inhibitory state. The catalytic subunit finally returns to its original inactive state

but only in the presence of inhibitor 2, indicating that the regulating subunit is required for both the activation and inactivation processes. The enzyme is also strongly inhibited by PKI in a competitive manner. As of now, no direct effect of insulin or epidermal growth factor (EGF) in the presence of their receptors has been demonstrated on the purified enzyme even though it appears to be under the control of these growth factors in 3T3 or A431 cells in culture.

5 GENE EXPRESSION AND TRANSCRIPTIONAL SIGNALS

M. Ptashne (Harvard University) presented some of his recent studies on the control of transcription in eukaryotes and prokaryotes which indicate a common mechanism. Several examples are known of genes in eukaryotes that differ from those in lambda in the following stiking characteristic. For each of these genes, transcription is activated by a protein bound to a DNA site hundreds of base pairs away from where transcription begins. These examples include genes of humans, rodents, and yeast. Ptashne stated that two principal molecular mechanisms governing the action of transcriptional activator proteins emerge from his studies of lambda (λ). The first concerns the interaction of regulatory proteins with DNA and the second, the mechanism of gene activation by a DNA-bound regulatory protein. He presented evidence that both of these descriptions apply to the regulation of gene expression in other organisms, including eukaryotes.

His recent experiment with the yeast gene *GAL 1* (eukaryote) show that, as with lambda repressor, DNA-binding and positive control are two distinct functions of *GAL 4* protein (a positive regulatory protein) and that these functions are separable. His experiments with *GAL 4* indicate that, like lambda repressor, DNA-bound *GAL 4* stimulates transcription by touching some other molecule, presumably another protein. In other words, the *GAL 4* bound at the upstream activating sequence (μAS_G) directly contacts another protein bound at the transcription start site and thereby helps polymerase bind and begin transcription. According to this view, the DNA between μAS_G and the start of the gene is looped out, perhaps covered with other proteins. This would require that DNA be sufficiently flexible so that "touching at a distance" can occur at low energy cost. Ptashne's experiments with lambda repressor suggest that as a dimers bind cooperatively to two separated sites, the DNA bends, allowing the carboxyl domains to touch. As the repressors bind, the DNA between the two sites acquires a pattern of sensitivity to nuclease digestion, indicating that it is bent smoothly. The repressors cannot cooperate if they are on opposite sides of the DNA helix because, according to Ptashne, the energy required to twist the DNA so that they could touch is too great. Interactions that depend upon DNA flexibility might be facilitated, especially over long distances, in DNA that is supercoiled and hence folded upon itself.

Ptashne is continuing his studies on a common mechanism for the control of transcription in prokaryotes and eukaryotes. His theory is novel and important for research on control of transcription, especially in eukaryotes.

W. Schaffner (Institute for Molecular Biology II, University of Zurich, Switzerland) spoke about enchancers and cell-type specificity of transcription. Enhancers were originally identified as long-range activities of gene transcription in higher eukaryotes. In addition, they were the first DNA sequences found to confer tissue specificity. These properties had set them apart from previously described promoter elements of eukaryotic genes. Although the enhancer mechanism and the phenomena of inducibility are not as yet understood, recent research is beginning to provide answers. Schaffner reviewed much of the recent work in this area from his laboratory as well as from others. Schaffner described his recent work and that of others particularly on tissue-specific immunoglobulin (Ig) gene enhancers. He found that the enhancer is located 3' to the promoter and within the Ig transcription unit. The Ig heavy chain

enhancer has the same characteristics as a typical viral enhancer: it works in both orientations and acts over long distances on heterologous promoters. In addition, it shows a pronounced tissue specificity functioning only in lymphoid cells.

Schaffner and his group are carrying out studies of the murine Ig heavy chain (IgH) enhancer and have found that it is located within a restriction fragment between the J and the C regions. To delineate the units of tissue-specific enhancer function within the IgH enhancer, they are using small restriction fragments and oligomers of these sequences. The data obtained so far indicate that even small segments of the IgA enhancer exhibit cell type-specificity and are inactive in fibroblast cells. Oligomerization of these segments increases their activity in lymphoid cells but not in fibroblasts. To characterize the binding sites of tissue-specific transcription factors, Schaffner et al. are currently analyzing the effect of point mutations within the active sequence elements.

6 DNA REPLICATION

The role of DNA gyrase in DNA super coiling on a regulator of bacterial gene expression was discussed by M. Gellert (Laboratory of Molecular Biology, National Institutes of Health, Bethesda, Maryland). In bacteria, and perhaps in eukaryotes, DNA supercoiling has a powerful influence on gene expression. Transcription from various bacterial promoters responds very differently to changes in the superhelix density of cellular DNA. When supercoiling is decreased by inhibition of DNA gyrases, transcription of some genes is repressed, while transcription of others is induced. The molecular basis for this behavior has been obscure.

Gellert and his colleagues have studied, as a model system, the response of the genes for the two subunits of DNA gyrase (gyr A and gyr B) to changes in DNA. Expression of both genes is strongly induced by DNA relaxation and remains high as long as the template DNA is held in a relaxed state. By recloning the gyr A and gyr B promoter regions, Gellert et al. have shown that DNA relaxation stimulates transcription initiation primarily and that the stimulation is a local and transferable property of short DNA sequence. A deletion analysis has put further limits on the extent of DNA sequence needed to maintain this form of transcription control. Gellert et al. have also used the method of transient electric dichroism to investigate the structure of complexes between the enzyme and defined fragments of DNA. They are also studying the behavior of these complexes with and without added nucleotide cofactor.

S.D. Ehrlich (Institut Jacques Monod, Paris, France) and his group have been studying single-stranded circular intermediates in plasmid DNA replication. Plasmid pc194, originally isolated from *Staphylococcus aureus* and which also replicates in *Bacillus subtilis* and *E. coli*, exists in the three hosts in the form of double-stranded and single-stranded circular DNA. Ehrlich et al. have obtained five other *S. aureus* plasmids and one *B. subtilis* plasmid which generate single-stranded DNA in *E. subtilis* in amounts similar to that measured for pC194. The studies of Ehrlich et al. make it likely that many plasmids from gram-positive bacteria replicate via a single-stranded intermediate, using a mechanism analogous to that of single-stranded DNA phages. They are carrying out further work to ascertain how widespread such a life cycle is among plasmids from other bacterial species.

J. Roberts (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York) spoke about DNA replication and the regulatory function of *E. coli* Rec A protein. This protein has a central role in DNA repair: it provides recombinational repair of DNA by catalyzing DNA strand exchange, and when the cell's DNA is damaged, it induces DNA repair by promoting proteolytic cleavage of the Rec A repressor. However, it is not known how DNA damage leads to activation of the proteolytic cleavage of the Rec A protein in the cell. Biochemical studies have shown that the Rec A

protein-mediated inactivation of repressors requires binding of Rec A protein to single-stranded DNA as well as to small molecules. This result, and a consideration of the cellular role of Rec A protein, suggest to J. Roberts that an important pathway of DNA-repair gene induction may involve attachment of Rec A protein to gaps left when movement of the replication fork uncovers obstructive lesions. To provide evidence for this model, Roberts and coworkers have examined the effect of interrupting replication on activation of Rec A protein as determined by measuring the intracellular concentration of Rec A protein. They found that blocking DNA replication by temperature-sensitive mutations in genes for subunits of the elongation complex, or with drugs that inhibit DNA gyrase, interrupts development of the inducing signal after ultraviolet irradiation, as long as excision repair is prevented. This result, according to J. Roberts, suggests that Rec A protein activation is closely coupled to DNA replication.

M. Radman (Laboratory of Mutagenesis, Institut Jacques Monod, Paris, France) discussed error correction in DNA replication. High fidelity of DNA replication results from enzymatic activities associated with the DNA replication machinery (e.g., nucleotide selection and the proofreading of 3' to 5' exonuclease activity) as well as from the post replicative mismatch correction. Radman and his group studied the characterization of the mismatch correction acivity in *E. coli*. They found that two mechanisms appear to operate: one by excission resynthesis directed on the newly synthesized strands by the transient hemimethylation of GATC sequences, another operating in the absence of DNA methylation, presumably involving recombination repair among the two sister molecules.

Radman stated that the efficiency and the molecular specificity of the *E. coli* mismatch repair system suggests that mismatch repair has two important genetic effects in DNA replication: it eliminates over 99 percent of spontaneous mutations arising as replication errors and, in so doing, equalizes the frequencies of diverse mutations along the DNA at a level of 10^{-10} to 10^{-9} per nucleotide replicated. The specificity of mismatch repair is such that it compensates for the replication errors by repairing most efficiently the G:T, A:C, and frameshift mismatches and by repairing G:C-rich regions more efficiently than the A:T-rich regions.

7 CONCLUSION

The conference entitled "From Enzymology to Cellular Biology" was an intensive meeting with limited attendance. Molecular biological approaches to the study of enzyme structure/function relationships were emphasized at this conference. These approaches included, for example, protein (enzyme) engineering, recombinant DNA methods, and site-directed mutagenesis using a wide range of enzymes from proparyotes and eukaryotes. However, more classical biochemical enzymatic studies were also presented, and the need for a combination of research at both the biochemical and molecular biological levels was stressed.

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