MEETING REPORT

THE MECHANISM OF ENZYME CATALYSIS
Report of a Pre-symposium held in Riga, U.S.S.R., on June 19 and 20, 1970, within the scope of the VII. International Symposium on the Chemistry of Products*

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1. Introduction

The pre-symposium-Mechanism of Enzyme Catalysis was attended by an international audience of about 100. It was devoted to discussion of general aspects of the functioning of enzymes, including the physical and chemical basis of their catalytic activity, and evaluation of the major experimental approaches used. The topics selected for discussion (indicated below as headings of the sections of this Report) were communicated well ahead of the Meeting to all invited participants, so as to focus attention on the specified problems.

Discussion of each topic was initiated by an introductory lecture by an Animator, followed by the contributions of several invited speakers and by free discussion.

In the Chairman's opening speech, A.E.Braunstein pointed out that the time is not yet ripe when a general, universally applicable theory of enzyme catalysis might be construed. However, investigations of the mechanism of action of individual enzymes and the study of congruent model reactions have brought into relief a number of common features and basic principles determining the high efficiency and selectivity of biological catalysts. These include, for example: multipoint interactions between enzyme proteins, cofactors and substrates; conformational flexibility of the enzyme protein and its specific ligands; "induced" or " compulsory" fit between these reactants, entailing stabilization of electronically and conformationally strained states of the enzyme and of enzyme-substrate complexes, etc. The same principles account, to a considerable extent, for the characteristic high susceptibility of enzyme catalysis to regulation under the influence of subtle environmental changes.

2. Fast reaction studies of elementary steps in enzyme mechanisms

The following questions were set forth for discussion by the Animator, G.G.Hammes, USA: What unique information about enzyme mechanisms can be obtained with fast reaction methods? What are current limitations on fast reaction studies? What types of new methods are needed and what types are currently being developed?

Information about the time sequence of all intermediary steps on the way from substrate to product is necessary for complete understanding of mechanisms of enzyme action. Current methods for fast reaction studies (stopped flow, temperature jump, etc) permit one to record processes involving steps with time constants as short as microseconds; the limiting time intervals for ultrasonic relaxation methods are several orders of magnitude shorter. Sequential steps of an enzyme reaction proceed with different velocities which are strongly dependent on the geometry of the substrate. Thus, the rate constant for formation of the complex of aspartate aminotransferase with aspartate exceeds 10^8 M^{-1} sec^{-1}; with α-methylaspartate it is about 10^4 M^{-1} sec^{-1}. The rates of conformational transitions in enzymes and enzyme-substrate complexes

* Communicated by the Organizing Committee

are relatively low (relaxation times ranging from 10^{-4} to 10^{-1} sec), due to the cooperative nature of such transitions. Results relating to ribonuclease and aspartate aminotransferase were presented to illustrate the application of fast reaction studies to the detection and investigation of discrete, transient steps. Thus, no less than eight intermediary steps were revealed in the reaction catalysed by aspartate aminotransferase. Such data, in conjunction with data on the structure of an enzyme, allow one to suggest a complete mechanism for its catalytic function, as illustrated by results of fast reaction studies on ribonuclease. As a general conclusion, Dr Hammes stated that an enzyme is capable of dividing the catalytic process into a series of discrete steps, the structure of the enzyme being optimized for each step by small conformational changes.

One of the promising new approaches to stepwise studies of enzyme reactions is the investigation of enzymes at low temperatures in mixed aqueous organic solvents, developed by P. Douzou, France. It allows one to reduce markedly the velocities of transformation of individual intermediates, and to study by conventional techniques both the kinetics of conversion of a given product into another and the properties of the intermediates.

The method permits one to work with stoichiometric concentrations of the reactants and is particularly promising in combination with simple procedures for rapid mixing of components or with relaxation techniques. Its application was illustrated by results obtained in the study of reactions catalysed by peroxidase. K. Yagi, Japan, reported, as an example of combined use of the methods of stopped flow and of isotopic H-D exchange, on his studies of sequential steps in the reaction catalysed by D-amino acid oxidase.

A novel method for the study of fast reaction kinetics has been proposed by I.V.Berezin, USSR, based on the application of flash photolysis to a light-sensitive pre-substrate or effector precursor. Within an interval of the order of 10^{-5} sec the active substrate is formed, whose reaction with the enzyme is recorded by conventional methods. For example, chymotrypsin reacts with *p*-nitrophenyl *p*-nitro-*cis*-cinnamate one-thousand-fold more slowly than with the *trans*-isomer. The latter is formed on irradiation of the *cis*-isomer with a xenon bulb or a laser. The main advantage of the method is the circumvention of rapid mixing.

3. The physical meaning of the parameters of energy and entropy of activation in enzymic reactions

Thermodynamic activation parameters are often used for characterization of enzymic processes. However, applicability of the theory of the activated state to such processes is not self-evident. The Animator for this topic, L.A.Blumenfeld, USSR, negated the physical meaning of activation parameters for enzyme reactions. As a rule, the true activation barrier ΔE_a, of a given elementary stage in an enzyme-catalysed process may change with temperature mainly because of structural changes in the enzyme macromolecule. In the necessarily narrow temperature interval for measurements, these changes can always be approximated by a straight line, $\Delta E_a = \Delta E_a^0 - bT$.

Then the Arrhenius equation can be transformed to $k = Ae^{b/r} \times e^{E_a/RT}$, i.e. the estimated parameters of energy and entropy of activation will be the apparent values, ΔE_a^0 and $b\Delta S_a^{\ddagger}$, which are linearly related. It also follows that "compensation effects", observed with many enzymes on alteration of external conditions and implying a mutual compensation of changes in the energy and the entropy of activation, are a consequence of incorrect treatment of the data rather than real effects. In order to elucidate the true cause of temperature-dependent changes of ΔE_a^0 it is necessary, in the speaker's opinion, to know the construction of the enzyme and the detailed mechanism of its action. But when this is known, we do not need the "activation parameters".

The Animator's negative attitude induced a lively discussion. G.I.Lichtenstein, USSR, pointed out that the compensation effect is not necessarily an artifact. It can arise as a consequence of peculiarities of the enzyme-catalysed process. At each elementary stage the number of nuclei that must be oriented along the reaction coordinate is substantially decreased in comparison to the overall process. In order to maintain the necessary contact between substrate and enzyme, the latter must change its conformation, all the time adapting it to the structure of reactants which changes in the course of the reaction. A compensation effect is necessary in this case, to obviate the necessity for an increase in free energy of the system; i.e., the change in free energy associated with conformational transitions equals zero.

B. Havsteen, Denmark, thinks that the meaning of energy and entropy of activation in enzyme reactions is the same as in reactions in the gas phase. In the former case, however, it is difficult to assign the estimated values to any concrete stage of the process. Valuable information can be obtained by investigation of the effect of steric factors upon the activation parameters, particularly within a series of substrates of the same type, as well as by analysis of the deviations of Arrhenius plots from linearity. The speaker reported on such deviations in the case of chymotrypsin-catalysed reactions.

M.V.Volkenstein, USSR, stressed that the notion of activation energy becomes meaningless in the case of cooperative transitions occurring in proteins with changes in temperature.

Summarizing most of the comments, Dr Hammes stated his agreement with the Animator's point that it is difficult, with enzyme reactions, to interpret correctly the temperature dependence of reaction rates; however, he did not see any difference in principle (from the standpoint of transition state theory) between enzymic processes and ordinary reactions in aqueous phase.

4. Role of enzyme flexibility in enzyme catalysis

The Animator, D.E.Koshland jr, USA, proposed the following topical questions for discussion:

What is the contribution of "strain" in enzyme reactions? Is the amount of conformational change significantly related to the catalytic action of an enzyme? Where is the dividing line between physical strain and electronic distortion?

He pointed out that no entirely satisfactory explanation has been found, as yet, for the enormous catalytic effectivity of enzymes and for their, often very narrow, specificities.

Taking into account the contribution of factors charateristic of ordinary coenzyme reactions in solution, such as the effect of propinquity of the reactants, general acid-base catalysis, solvent effects, there still remains a gap of many orders of magnitude (up to 10^9-10^{12}) between the velocities of enzymic and model reactions. This gap depends on the operation, in enzymes, of such specific factors as the correct orientation of reactants, conditions of strain which lower the energy of transition complexes, effects of the microscopic environment, push-pull mechanisms, etc. But even these factors fall short of entirely bridging the gap.

However, if we take into account that in the fixed structures of enzyme-substrate intermediates an optimal overlapping of the orbitals of interacting groups can be achieved, then a rational explanation of the efficiency of enzyme catalysis becomes possible. Dr. Koshland demonstrated this for a variety of model systems and also for an "artificial" enzyme – thiol-subtilisin. Replacement of the essential –OH group in subtilisin by an –SH group results, owing to the somewhat larger bulk of the latter, in a drastic reduction of the enzyme's activity. According to Koshland, it is the precise orientation of the electonic orbitals of interacting groups that makes the principal contribution to the catalytic activity of enzymes.

In the discussion, V.A.Yakovlev, USSR, presented evidence for the occurrence of conformational transitions, induced by substrates or inhibitors (including H^+ions), in the active sites of cholinesterase and chymotrypsin. B.L.Horecker, USA, reported data showing that the lower activity of rabbit muscle aldolase towards fructose-1-phosphate as compared to fructose-1, 6-diphosphate is due to a conformational change induced by the latter, apparently affecting orientation of the terminal –COOH group of the enzyme.

M.V.Volkenstein, USSR, D.S.Chernavsky and Yu. I.Khurgin, USSR, made conjectures on another possible aspect of enzyme "flexibility". The main idea of these speakers is that, in multi stage processes, energy released in preceding stages or cycles may be utilized to surmount the activation barrier in subsequent stages. Mechanisms were considered for the stockpiling of energy either in oscillations of the protein molecule as a whole, or in the form of elastic deformations of the protein globule. According to estimates by Chernavsky and Khurgin, the physical properties of the protein globule allow energy amounts of up to 10 kcal/mole to be accumulated in the form of energy of elastic deformation.

B.L.Vallee, USA, discussed the role of strain in enzyme catalysis. Investigation of the absorption and C.D. spectra of metallo-enzymes led him to conclude that the bonds formed between enzyme proteins and metals or metal chelates are strained, and this may be of advantage, thermodynamically, for the catalytic function. Such "entatic" states, in Vallee's opinion, are not confined to the metallo-enzymes, but rather

represent a characteristic feature of enzyme catalysis, which recurs in essential functional groups of the enzymes and in protein-bound coenzymes.

V.I.Ivanov, USSR, emphasized the importance of the notion of a "congruent model system", i.e., a model system wherein a reaction between low-molecular-weight components proceeds through the same covalent intermediates as in the relevant enzymic reaction.

A comparison between such model systems and the enzyme led the speaker and M.Ya.Karpeisky to assume that high efficacity of an enzyme reaction, as compared to its congruent model, is ensured by the following three factors: 1) stabilization on the enzyme of the ionic and/or conformational form (S) of reactants which have optimal reactivity for a given step of the reaction; 2) orientation of interacting groups along the reaction coordinate of the given step; 3) fulfillment of the first two requirements for all sequential steps of the enzymic reaction. The latter point implies that the enzyme is able to provide optimum conditions for each stage, as a result of structural alterations occurring in the course of the preceding stage. These assumptions were illustrated by results of work relating to the catalytic mechanism of aspartate aminotransferase. In multistage reactions this factor apparently plays the predominant role in determining the remarkable catalytic properties of enzymes. In the case of template-dependent polymerization processes catalysed by RNA and DNA polymerases, another mechanism may contribute to high efficacity, namely, specific coupling of exergonic and endergonic steps of two consecutive, interlocking cycles.

5. Spatial aspects of enzyme structures and function

A. *Physical approaches.*

Topical questions for the discussion of this theme were formulated by D.C.Phillips, UK, who was unable to attend the pre-symposium. The questions were: To what extent does the local amino acid sequence determine the conformation of the polypeptide chain in enzyme molecules? Are comparisons of enzymic activity involving (a) modified enzymes, or (b) modified substrates, useful without detailed knowledge of the structural consequences of the modifications?

These questions formed a basis for the talk by M.V.Volkenstein, USSR, who acted as Animator. He underscored that the viewpoint currently most favoured is the one considering three-dimensional structure of a protein as entirely determined (for a definite environment) by its amino acid sequence. This view is supported by the large amount of evidence concerning the consequences of single amino acid substitutions in the case of point mutations. In studies of the functional role of individual groups in enzyme catalysis, chemical approaches utilizing modification of groups either in the enzyme or in the substrate should be employed with caution, since chemical alterations at sites remote from the catalytic centre can markedly affect the structure and, hence, the activity of the latter. The mechanisms of such effects can, in principle, be elucidated by extensive use of a variety of physico-chemical methods, in the first place X-ray diffraction analysis, as well as ESR, NMR, spectrophotometric and spectropolarimetric techniques. Using several examples, Dr Volkenstein illustrated the effectiveness of the application of magnetic dispersion of optical rotation in the study of conformational changes in proteins. Development of new methods for the study of dynamic properties of proteins remains an important aim; neutronography of proteins may prove to be a promising approach.

O.B.Ptytsin, USSR, gave a communication concerned with the problem of the extent to which the local amino acid sequence determines the conformation of a protein molecule. Analysis of the distribution of amino acid residues in structurally ordered parts of the molecule reveals a definite relationship between the nature of amino acid side-chains and the type of structure. Assignment of a certain "helical potential" to each amino acid residue allows one to predict the type of secondary structure from the local amino acid sequence. Fair correlation between theory and experiment (80% coincidence of the predicted and real structures) confirms the predominant role of local interactions in determining the formation of the secondary structure. Since the secondary structure of proteins is also stabilized by interaction with distant parts in the tertiary structure, both types of interactions – local and non-local must be correlated so that they stabilize one and the same secondary structure.

A.R.Fersht, UK, described studies on the crystallization of enzyme-substrate complexes of chymotrypsin. Appropriate selection of substrates, of suit-

able concentrations of enzyme, substrate and reaction products allow one to provide conditions for high concentrations of definite enzyme-substrate intermediates in solution; such complexes can be crystallized. Preliminary X-ray crystallographic data indicate that the substrates are actually bound at the active sites of the enzyme molecules. B.K. Vainshtain, USSR, demonstrated an interesting possibility for investigation of the tertiary structure of protein macromolecules (catalase, phosphorylase, etc), at a resolution of about 20 Å, by electron microscopy followed by three dimensional synthesis based on diffraction patterns of monochromatic visible light from the electron micrographs of orderly enzyme monolayers.

Relationships between electronic and conformational properties of proteins were discussed in the paper by B.Atanasov, Bulgaria, in which he presented evidence demonstrating the existence of two conformers of myoglobin. The structural differences between these conformers are not large, but they extend over the whole surface of the molecule, and the two forms differ in oxygen affinity. The mechanism of the phenomenon is apparently connected with a small shift of histidine residue F8 relative to the heme iron atom. A shift of 0.2 Å can result in a large change of binding energy for the opposing sixth ligand, amounting to 5–8 kcal/mole.

Related aspects of enzyme catalysis were considered by O.L.Polyanovsky, USSR. He reported that the binding of substrate in the active site of aspartate aminotransferase results in alteration of the mobility of an iminoxyl-containing spin label attached to an –SH group located at some distance ($\geqslant 15$ Å) from the active site. From experiments with a series of inhibitors and substrate analogs, it was inferred that this effect is probably a result of neutralization of a cationic group of the protein by the ionized α-carboxyl of the substrate amino acid.

B. *Chemical tools for investigation of the active site*

Can the chemical topography of the active site at individual stages of an enzyme reaction be elucidated by chemical methods? The lecture of the Animator, A.Meister, answered this question positively. In a survey of his work on pure glutamine synthetase from mammalian brain, Dr Meister demonstrated how he was able to obtain, by means of inhibitor and substrate analysis, a detailed picture of the stereochemistry of the active site and an explanation for many peculiar features of this complicated enzyme. Glutamine synthetase, an enzyme with a M.W. of approx 500,000, consisting of eight subunits catalyses the ATP-energized reaction of amidation of the γ-carboxyl of glutamic acid by ammonia. By a comparative study of the substrate properties of the L and D isomers of glutamic acid, their α, β and γ *C*-methyl derivatives, and the geometric isomers of "cycloglutamic" (cyclopentane-1, 3-dicarboxylic) acid, it was made evident that the natural substrate is bound to the enzyme in the extended (*trans-trans*) conformation. Phosphorylated substrate is an important reaction intermediate. It was shown that the substrate anolog, methionine sulphoximine, irreversibly inhibits the enzyme and that it undergoes phosphorylation (at the imino N atom) in the active site; only one of the diastereoisomers of this compound, namely the L, (*S*)-form, displays inhibitory activity. Valuable information about the topography of catalytic and binding sites of glutamine synthetase was obtained from investigations of the stereochemistry of the substrates, their analogs and inhibitors by computer modelling.

V.K.Antonov, USSR, briefly surveyed the results of his investigation of chymotrypsin with the aid of quasi-substrates with restricted conformational mobility. The wotk allowed light to be shed on the stereochemistry of substrate bound in the enzyme's active site and the obtaining of convincing data in favour of the *cis* orientation of the amide side-group of the substrate. The suggestion was made that the substrate can undergo activation at the expenses of restriction of rotatory degrees of freedom upon binding of the molecule at the active site of chymotrypsin.

Studies of the physico-chemical and coenzyme properties of substituted analogs of pyridoxal (and pyridoxamine) phosphate, conducted by M.Ya. Karpeisky and associates, USSR, have provided valuable information concerning the nature of coenzyme-apoenzyme interactions, e.g. in aspartate aminotransferase, and allowed elucidation of both static and dynamic aspects of the topography of the active site. B.L.Vallee reported on investigations of the absorption and ORD spectra of enzymes selectively modified, under mild conditions, by treatment with various diazocompounds (diazoanthranilic acid, diazotetrazoles,

etc). The spectral characteristics of the modified enzymes provide insight into local conformational transitions in the protein and into the properties of micro-environment of the chromophoric groups.

J.Riordan, USA, illustrated a new chemical approach to the study of the dynamics of structural changes in aspartate transaminase associated with catalytic action of the enzyme. The speaker and Dr Christen have shown that nitration of the aminotransferase with tetranitromethane modifies one tyrosyl residue in the free holoenzyme and two residues in a transient enzyme-substrate intermediate, with concomittant inactivation of the enzyme. These results confirm earlier evidence (Turano; Ivanov and Karpeisky) on the presence of an essential tyrosyl residue in the active site of this enzyme; they allow one to identify, by using a variety of pseudosubstrates, the intermediate stage at which the essential tyrosine is most readily susceptible to modification.

6. Interrelations between the surface chemistry and function of the enzyme protein

The following questions were proposed for discussion by C.Tanford, USA: How precise are chemical methods or methods such as solvent perturbation of absorption spectra for determining which groups of the enzyme are at the surface, in contact with the environment? What are the effects of polas and hydrophobic areas upon the reactivity of specific functional groups? These questions were considered in the talk by M. Laskowski jr., USA, the Animator for this topic. He gave several examples showing that the method of differential spectroscopy allows the detection of chromophores with different micro-environments in a protein molecule or the characterization of the micro-environment of a chromophoric group attached to the protein. Recording of the spectral changes (shifts in position or broadening of a band) upon binding of ligands, alteration of the surrounding medium (the technique of solvent perturbation) or thermal perturbation helps one to ascertain which groups of the protein are in contact with the environment. However, interpretation of the data thus obtained is complicated by the lack of a quantitative numerical definition of "environment". Moreover, spectral methods give only an "averaged" picture of the distribution of identical chromophores in non-equivalent environments. That is why Dr Laskowski emphasized the importance of devising methods for the selective modification of proteins, i.e. methods which would allow one to substitute only one single group or to introduce one chromophore in a definite position. As an example of such a modification he quoted his work with trypsin inhibitors, where it was possible to study the effects of replacement of one single amino acid (e.g. lysine for arginine) in the active site. The speaker considers, however, that current techniques give a fairly correct picture of the distribution of chromophores in proteins, since the picture is often corroborated by the results of X-ray diffractometry.

One of the efficient methods for investigation of the state of the protein surface is the attachment of spin labels. G.I.Lichtenstein infers, from the results obtained in applying this method to various proteins (hemoglobin, lysozyme, myosin, etc.), that the protein macromolecules have a relatively rigid nucleus (or nuclei) and a conformationally more mobile cortical part.

The nature of hydrophobic interactions of chymotrypsin with substrates differring in polarity was investigated in detail by I.V.Berezin. Yu.I.Khurgin, USSR, studied the hydration behaviour of chymotrypsin. He took advantage of the capacity of this protein to dissolve in virtually anhydrous dimethyl sulphoxide with considerable retention of activity. On reduction of the residual water content of the enzyme, it was observed that its secondary structure became altered, and catalytic activity was abolished, only when removal of the tightly bound water was achieved.

The wealth of information presented at the Presymposium and its atmosphere of searching, critical analysis imparted to the participants a vivid impression of the vigorous current development and fascinating outlook of fundamental research in the field of Enzyme Catalysis.