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ENZYME DYNAMICS: THE STATISTICAL PHYSICS APPROACH

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1 INTRODUCTION

In spite of the fact that for a long time enzymes have been thought of as “floppy bodies,” namely as systems capable of lowering the free energy of the transition state by a convenient set of conformational fluctuations, the experimental evidence for such fluctuations and the significance of their role in catalysis are comparatively recent acquisitions.

The first experimental evidence for the presence of rapid (in the nano-second range) structural fluctuations in a large number of globular proteins and enzymes comes from experiments on fluorescence quenching carried out in 1973 by Lakowicz & Weber (39). Almost contemporarily it was suggested (9) that the ability to correlate in time the fluctuations of some relevant conformational variables could be an essential kinetic property of the enzyme macromolecule. Since then, the notion of the enzyme as a fluctuating unit has gained credit and its experimental and theoretical basis has been progressively strengthened. We (10) have already offered a physical assessment of the observed statistical time events reported in the vast enzymologic literature, have discussed their identification at a molecular level, and have pointed out their possible significance for catalysis.

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The aim of this paper is to review the recent literature in the field and to explore further the scope and limitations of the notion of the catalytic event as a relatively rare spontaneous fluctuation, by which a sufficient amount of free energy is fed from the surroundings to the active site. For this reason particular consideration is devoted to phenomena occurring at the protein-solvent interface, where a critical hydration coverage is now known to be necessary for catalysis. After a brief updating of the experimental aspects, the role of fluctuations in catalysis is both discussed on general grounds and applied to specific cases of biochemical significance.

Before proceeding, let us illustrate the usefulness of a statistical approach to enzyme catalysis. Individual protein molecules are small systems that consist of relatively few discrete particles, in which the distribution functions of thermodynamic variables are not as sharp as those of macroscopic systems. Statistical physics can offer general expressions for the mean square fluctuations of a small system (the macromolecule) in thermal equilibrium with its bath (the solvent). A numerical computation (14) of the mean square fluctuation of the internal energy and of the total volume of a representative globular protein gives quite large values. Because of the small size of the single protein system, transient fluctuations are inevitable, even at thermodynamic equilibrium. This is not a unique property of globular proteins, but in globular proteins the occurrence of these large fluctuations in fact is relevant to their function as enzymes. This is because some conformational changes or strains are wanted in the enzyme-substrate complex, to allow the correct presentation of the appropriate functional groups at the active site to lower the activation energy. The problem is then the detailed description of how the macromolecular structure can use these random processes for a specific catalytic action. To this end one should consider the time scale of such processes and their possible coupling with the chemical events involved in catalysis. Note that by *coupling* we mean a statistical coupling, well defined in terms of time and space correlations of the relevant fluctuating variables rather than the mechanical coupling that always exists among directly bonded atoms. These notions of statistical physics complement the classical stereochemical and thermodynamic approach familiar to biochemists. The statistical viewpoint is indeed necessary to deal with the kinetics of a small system like an enzyme.

As is shown later in this review, the application of statistical physics to enzymology can proceed at two levels of approximation. The first one is the realization that the so-called relevant factors toward catalysis should be reconsidered in terms of statistical variables in a critical review of known facts. This can be accomplished for systems where the reaction pathway is well understood. Although it may seem to involve only a matter

of words, the proposed description of enzyme catalysis in statistical terms actually requires a qualitatively new evaluation of facts, by emphasizing their statistical meaning. The second level of approximation is the quantitative evaluation of some properties of statistical significance, such as the mean displacement from equilibrium of a random variable and its statistical correlation with other similar variables involved in the catalytic process. This second step is hard to accomplish because of the complexity of enzyme systems, as this review makes evident; one simple instance is the relation between the time constants of chemical events and the characteristic time of the random fluctuations. However, the present lack of quantitative detail must not be considered a weakness inherent in the statistical physics approach, but rather a consequence of the limitations of current experimental techniques.

2 SPONTANEOUS FLUCTUATIONS IN GLOBULAR PROTEINS: REVIEW OF EXPERIMENTAL DATA

Experimental evidence for the occurrence of spontaneous fluctuations in globular proteins has been reviewed already (10). Moreover, several results on the internal motions of proteins are reviewed in this volume (63a), with special consideration for experiments of hydrogen exchange kinetics, but also with sufficient coverage of the major techniques for the detection of fluctuations in the nanosecond region, such as NMR, fluorescence quenching, and fluorescence depolarization. For these reasons this discussion is limited to a summary of the observed statistical time events relevant for enzyme catalysis, and to review two novel approaches based on cryogenic techniques and computer simulation.

2.1 Statistical Time Events in Globular Proteins and Enzymes

Table 1 is reproduced from our previous summary of observed statistical time events (10). By *statistical time events* we mean a class of spontaneous fluctuations around equilibrium, the amplitude of which is distributed along a gaussian curve and decays with the same correlation time. A major step in the field has been made with the development of fluorescence quenching techniques (39). The use of the fluctuation-dissipation theorem makes it possible to include also results obtained by relaxation techniques.

The inspection of the data reported in Table 1 shows at least two points worth mentioning. First, a comparison of the events detected in natural

Table 1 Time events in globular proteins and enzymes^a

Determinants	Time (sec)
Protein surface	
Bound water relaxation	10 ⁻⁹
Side-chains rotational correlation	10 ⁻¹⁰
Proton transfer reaction of ionizable side chains	10 ⁻⁷ -10 ⁻⁹
Protein conformation	
Local motion	10 ⁻⁸ -10 ⁻⁹
Isomerization process	10 ⁻² -10 ⁻⁷
Folding-unfolding transition	10 ⁺² -1
Enzyme substrate complex in solution	
Encounter rate	diffusion controlled
Estimated lifetime of the transition state in covalent reactions	10 ⁻¹⁰
Change in metal ion coordination sphere in metalloenzymes	10 ⁻⁶ -10 ⁻⁹
Enzyme-substrate local conformational motion	10 ⁻⁹
Covalent enzyme-substrate intermediate lifetime	10 ⁻² -10 ⁻⁴
Enzyme-substrate complex conformational isomerization	10 ⁻² -10 ⁻⁴
Enzyme-substrate complex unfolding transition	10 ⁺² -1

^a Modified from reference (10).

systems with those occurring in models (10) (such as H-bonded polymers, etc) reveals that no fast ($\leq 10^{-7}$ sec) events have been found in proteins that cannot be found also in suitable model systems. Second, quite a number of different statistical events have a correlation time around 10^{-8} sec. These events are likely to be statistically coupled, although no direct experimental evidence exists for such statistical coupling. Most of these events occur at the surface of the protein molecule and particularly involve the relaxation of bound water, a fact relevant to subsequent discussion in this paper.

2.2 Computer Simulation

The dynamics of a folded globular protein (bovine pancreatic trypsin inhibitor) has been investigated by McCammon and co-workers (44) by Monte-Carlo techniques solving the equation of motion for the atoms in the neighborhood of its equilibrium conformation. This study involves the internal dynamics of the protein, and it is of even greater interest than the analysis of displacements along a suitably chosen isolated coordinate (for example, the opening and closing of the active site cleft in lysozyme reviewed below). Unfortunately, in this theoretical study an empirical energy function for interaction among atoms has been assumed without a discussion of the influence of the assumed potential on the results, and

the solvent contributions have been neglected. However, some features of the molecular dynamics of the protein in the short time scale (the picosecond region) have been clarified by this work. For instance, the average root mean square fluctuation of all atoms is found to be 0.9 Å, which is quite a large value; moreover the largest root mean square fluctuations occur in the side chains at the protein surface and in the two ends of the backbone, as expected. Most interestingly, the study of the time dependence of motions reveals a time constant of ~ 1 psec for the decay of atom fluctuation, the existence of correlated fluctuations (e.g. positive and negative dihedral angle structure preserving correlations), and the occurrence of concerted motions (e.g. rippling motions in the far infrared region for a strand of β sheet). Even more important is the occurrence of concerted transitions in the backbone surface, involving low activation energy and probably extending in the low frequency region above a picosecond. Quite rightly, an idealized model for proteins as a dense, hard-sphere fluid composed of particles connected by flexible links is proposed.

This paper by McCammon and co-workers (44) will certainly have a decisive influence on the biochemists' community, to outmode the notions of proteins as very rigid molecules inspired by narrow-minded interpretations of crystallographic data. However, the possibility of reaching a complete understanding of enzymic catalysis by the computer simulation of atomic motions in proteins lies very far in the future. This is because enzymic catalysis certainly involves conformational motions in the longer time scale, solvent effects, and more than pairwise interactions at the active site, for which the descriptions of events are well beyond the possibilities now available.

2.3 *Cryogenic Experiments*

Evidence for the occurrence of a large number of conformational states has been found by Austin and co-workers (2) in the analysis of the dynamics of ligand binding to heme proteins at low temperatures. The nonexponential course of rebinding observed at these low temperatures and in solid samples after photodissociation has been explained in terms of a practically continuous spectrum of activation energy brought about by the existence of many conformational states in which the biomolecule remains frozen when the thermal energy becomes less than the kinetic energy barrier between states. The width of the distribution of the activation energy spectrum is solvent dependent and encompasses several kilocalories per mole. Further biochemically significant details on these distribution curves should be of great interest.

Mossbauer spectroscopy of iron proteins has been used by Dwivedi et al (18) to study the dynamic properties of the active center, and in particular

the mean square displacement of the iron in some respiratory proteins and metalloenzymes. Measurements on frozen solutions of the iron-sulfur protein rubredoxin and of myoglobin showed three common features: (a) The mean square displacement of the iron is practically constant for $T < 10 \text{ K}^\circ$ [$\langle x^2 \rangle = 44 \text{ (pm)}^2$ for MbCO] and increases linearly with T for $10 \text{ K}^\circ < T < 160 \text{ K}^\circ$; (b) for $T > 180 \text{ K}^\circ$, $\langle x^2 \rangle$ increases rapidly; (c) line broadening indicative of nonperiodic motion of the iron is observable at still higher temperatures. It is suggested that internal degrees of freedom are responsible for the broadening observed in frozen aqueous solutions.

3 SPONTANEOUS FLUCTUATIONS AND THEIR ROLE IN CATALYSIS

The notion of an enzyme as a free energy transducer is not new in enzymology. It is conceivable that within the time of the catalytic event the system must ultimately increase its free energy content (with respect to the reactant) at the expense of the surroundings, and that part of the enzyme structure must be involved in this rapid handling of ground-state energy. By considering the substrate and the solvent molecules both as ligands, one can offer a thermodynamic description of the catalytic event quite similar to the one used to deal with the multiple internal equilibria of proteins in the binding of several ligands (62). Since the multiple binding of one ligand in the presence of other ligands can give rise to cooperative effects, by interaction of protein-bound ligands, chemical-free energy can be added by proper transducing. To be able to work as a free energy adder, this property of a protein has been shown (63) to hold in multiple binding under equilibrium conditions and should hold as well in nonequilibrium transient displacements from equilibrium occurring during the catalytic events. The multiplicity of the effects involved, like the tautomerization of the peptide chain and the second- and higher-order interaction among the chains, suggests a truly kinetic picture where the flexibility of the protein structure is the peculiar property of the macromolecule to handle these multiple binding situations. In the case of an enzyme we may think that the macromolecule is constituted by a number of semi-independent domains (like stretches of α helix or β structures), that in each domain structural changes can take place with a small local activation energy that is solvent dependent, and that the position of the residues relevant for catalysis is controlled by the changing structure of the domains or in the positioning of the domains relative to each other. In this sense the very numerous small changes in the location of the solvent molecule at the protein surface in the nanosecond time region control the displace-

ments of the relevant residues at the active site; therefore the space-time fluctuating nature of the solvent-surface interactions affect the temporal course of the catalytic event. This picture of enzyme catalysis grounded on fluctuations of the macromolecule in its bath is consistent with the usual thermodynamic description, because the probability of a fluctuation in the canonical ensemble depends exponentially from the free energy change, which is precisely the quantity needed in the thermodynamic description, and the existence of a coupling free energy between ligands must require a nonvanishing average value of the cross-correlated fluctuations (9). Moreover, the kinetic picture can go one step further, because in the catalytic events also the time derivatives of the fluctuating variables can be time correlated, i.e. the velocities can cross-correlate as well as the space conformational variables.

The best way to treat the multibinding processes of a biomolecule in a bath would be provided by a stochastic approach. In particular, such an approach is necessary to obtain information about correlation functions and spectral functions. The system is described by a set of stochastic variables that denote the number of ligands in the several energy wells, and the number of ligands in each well is not fixed so that fluctuations can be handled. A probability is introduced for finding the system in a particular conformation, and the time dependence of this probability is determined by a master equation containing the transition rates of the ligands among the different wells. This kind of approach has been used recently (1) to treat the binding and migration of small molecules to proteins at high ligand concentration, such as, for example, the binding of carbon monoxide to myoglobin. Because of the analytical complexity of even this simple nonlinear system, a number of stringent assumptions must be made to obtain a closed solution. For example, one neglects the transitions in which two or more ligands within the same biomolecule jump simultaneously. Nevertheless, convenient numerical procedures exist for the solution of the full nonlinear stochastics in general situations (P. Hanggi, personal communication).

In our opinion the stochastic approach is at the present time too complex to treat the conformational fluctuations in transparent closed form, but for many situations a description within the methods of linear irreversible thermodynamics will be sufficient. Therefore a statistical approach, like the one offered by the Onsager phenomenological equations, is more useful. These equations express a linear coupling between the time derivatives of the statistical macrovariables (fluxes) and the entropy change connected with the variation of these macrovariables (generalized forces), thus offering the possibility to treat the kinetic coupling between the macrovariables themselves in the Fourier components of their time fluctuations. In the

case of conformational changes during the catalytic act, this means a coupling between the velocities as well as between the space conformational variables. Both the steady-state (11) and time-dependent aspect (9) of enzyme action can be expressed in the frame of this linear theory, provided the principle of detailed balance on a microscopic scale holds. This last assumption is certainly fulfilled for enzymes that operate reversibly around equilibrium. A more delicate (and less acceptable) assumption must be made when the small displacements of the conformational variables around equilibrium are used to describe the large fluctuations that occur during the catalytic events. This limitation makes this theory unable to handle the transition state, but if one is interested only in the time correlation of the fluctuations, it is enough to assume that the correlation must not depend on the amplitudes of the displacements themselves, a condition likely to be fulfilled. A major difficulty of the proposed approach, in contrast to the applications in linear irreversible thermodynamics, is the proper choice of forces and fluxes to make use of the Onsager relation. In the analysis of specific enzymes, because of the impossibility of keeping track of the huge number of microscopic variables in the system, the correct identification of the statistical macrovariables, namely of the variables that determine the free energy of the system and display statistical fluctuations, are our major concern.

As a first application of the Onsager formalism to the multiligand situation outlined early in this section, we express the kinetic coupling between the solvent-protein surface interactions and the chemical changes occurring at the active site of the enzyme in terms of two statistical macrovariables only, to be identified as the bound solvent concentration and the reaction coordinate (11). By this we mean that the two macrovariables are both independently fluctuating, but that time cross-correlated fluctuations may occur if the Onsager coefficient that expresses the cross-coupling between the macroscopic fluxes and forces is not vanishing. The analysis can be carried out further, following the model of the mechanochemical collagen engine of Katchalsky & Oplatka (34), to derive interesting relationships between the experimentally measured relaxation times of the processes occurring at the protein surface and at the active site. Of course this is a very crude model of the enzyme action, because of the minimum set of macrovariables used, but in our opinion it offers a sufficiently correct representation of the statistical process by which free energy available at the surface can be transformed into mechanical energy to displace the system along the reaction coordinate, thus letting the enzyme work as a reversible mechanochemical engine. Quite recently, a different model has been proposed (26) of the enzyme as an engine with a small number of elastic working elements, which makes use of structural fluctuations ener-

gized by random collisions with the solvent. One may wonder if one of the reasons enzymes are such large macromolecules is just that they thus have a better chance to undergo sufficiently large fluctuations at their wide solvent-protein surface.

Leaving for the next section the problem of identifying a set of macrovariables relevant for catalysis in some concrete cases, let us discuss here a general aspect of their possible kinetic coupling. The capacity to correlate in time the fluctuations of some relevant conformational variables has been proposed (9) to be the characteristic property that allows a macromolecule to work as an enzyme. By this is meant the time correlation among the different concurrent catalytic factors may be a "kinetic property" of the enzyme, which results from its peculiar macromolecular structure as selected by evolution, and which can be taken as a figure of merit of the catalyst. Here we may carry this speculative discussion one step further and consider that the time correlation between the fluctuating macrovariables can be induced at the transition state by the presence of the substrate itself. This consideration stems from the hypothesis that the catalytic activity is at a maximum when the structure of the enzyme is complementary to the structure of the substrate in the transition state, because in the transition state the free energy of binding of the substrate to the enzyme is largest (21). Then, it must follow that the probability that two macrovariables A and B reach by fluctuations configurations suitable for catalysis and requiring free energy changes, ΔF_A and ΔF_B , respectively, is increased by the presence of the negative free energy of binding, which is formally equivalent to a coupling term $-\Delta F_{AB}$, merely because of the exponential dependence of the probability of a fluctuation from its free energy change. This substrate-induced coupling between the different structural domains of the macromolecule should last only during the crossing time of the transition state. Since this coupling must require the cross-correlation among the relevant conformational variables during the life-time of the activated state, this fact can be rephrased as a manifestation of an extended space order in the region of the active site of the enzyme. Thus we recover the notion of the high entropic contribution to the rate constants of enzymatic reactions (47), a notion that stems from thermodynamic argument only, and that can be better expressed in the language of statistical physics as occurrence of space and time cross-correlations. Specific possible examples of these cross-correlations at the transition state are shown in the next section.

Another point of general interest is the identification, at the molecular scale, of the fluctuations by which the macromolecule can exchange free energy with its surrounding medium. It seems likely that the water weakly bound on the macromolecule surface can accomplish this task. Let us

mention here that an increasing evidence (5, 12) has shown that water binds on two different kinds of sites, the strong binding sites (the ionizable groups of the side chains) and the weak binding groups (such as the amide groups of the backbone exposed to the solvent). Since the free energy of binding on these weak binding sites is comparable to kT , the fluctuations in the occupancy of these sites must be large indeed. In molecular terms, this implies a continuous random perturbation of the backbone chain sections exposed to the solvent. This perturbation arises from the electron redistribution of the amide group upon hydrogen bonding on its carbonyl site, which in turn produces a variation on the amide plane geometry, namely a local conformational change at the protein surface. Since the amide water interaction is comparable to the amide-amide interaction (53), and because of the cooperativity of the amide hydrogen-bonding (53), the random conformational changes at the protein surface must propagate throughout the protein, affecting its secondary and tertiary structure and inducing at the active site those subtle changes in the positions of the active groups that are believed to be relevant towards catalysis. Thus the internal array of the hydrogen bonds is the transducing network that propagates the conformational fluctuations originated on the surface by the low free-energy interaction with the solvent. It is worth mentioning that this presents some analogy with the situation existing in the cellulose-water relationship, where the water weakly bound on the cellulose surface displays an intrinsic mobility and induces a mobility in the cellulose chains as well, an effect known as "plasticization" (24).

4 ANALYSIS OF SOME SPECIFIC CASES

4.1 *Lysozyme*

Lysozyme catalysis was reviewed in 1972 by Henderson & Wang (29) in the first volume of this series. A very detailed account of all its properties has been written by Imoto et al (31), and in 1974 in a book edited by Osserman et al (46). In the following presentation we assume the reader to be familiar with the preexisting background, most of which can be found also in current biochemistry textbooks.

Essentially three concurring factors are thought to be relevant in the overall catalytic effect: (a) acid catalysis in the protonation of the substrate glycosyl oxygen by Glu 35; (b) stabilization of the carbonium ion by interaction between the developing positive charge and the negative charge on Asp 52; and (c) ring strain of substrate residue D induced by substrate-enzyme binding. We believe that these three factors retain their validity, and we consider them on a statistical basis because they are controlled

in part by the fluctuating water molecules adsorbed on the protein surface. Moreover, to use the Onsager formalism, we must express them by means of three statistical macrovariables that determine the free-energy profile of the reaction.

To identify the first statistical macrovariable, let us look at the hydrogen-bonded network involving Asp 52 (Figure 1). Notice that all of the four residues constituting the network remain identical in the sequences of chicken and human lysozyme. It is evident that the negative charge must be placed on the oxygen atom O* of the Asp 52-carboxylate facing the substrate to stabilize the carbonium ion, whereas the charge must move away from it to help the destabilization process. And it is clear that the position of the negative charge is controlled by the two H-bond distances from Asn 59 and Asn 46, the shorter the distance the higher the formal negative charge on each oxygen atom of Asp 52. These two distances are certainly influenced also by the statistical binding of several water molecules to the Asn 59 side chain and to the amide backbone. The charge localized on the oxygen atom of Asp 52 facing the substrate thus can be identified as the first statistical macrovariable, and the electric potential acting on it can be identified as the generalized force.

To identify the second statistical macrovariable, we must consider the conformational changes that control the proton transfer from Glu 35 to

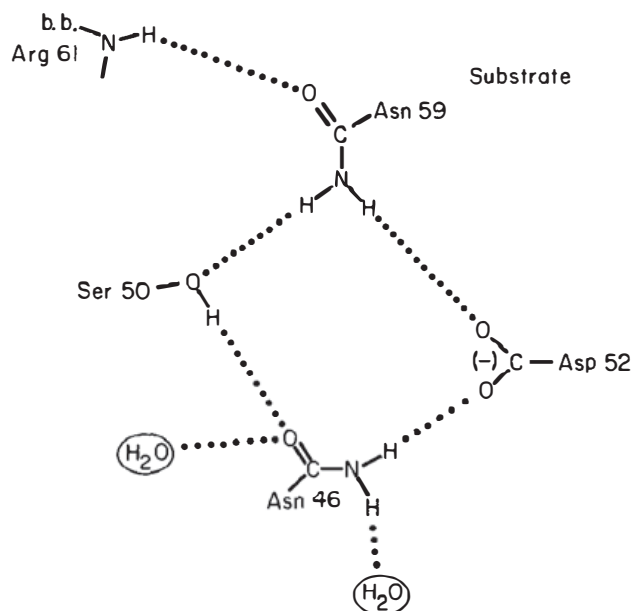


Figure 1 Schematic representation of the hydrogen bonding network that controls the charge distribution on Asp 52 in lysozyme. Encircled water molecules represent solvent molecules hydrogen bonded to Asn 46 side chain. [From (31).]

O₅ at site D of the substrate. This proton transfer is critically dependent from the distance between the Glu 35 and O₅, which in turn is controlled mainly by the angle between the two lobes that enclose the cleft. The fluctuations of this angle are controlled in part by the statistical distribution of the water molecules bound to the backbone surface of lobe 1 and, most importantly, by those sitting near the hydrogen bonds between the substrate and the two lobes. Therefore we can identify the second statistical macrovariable as the proton concentration (produced by Glu 35) and O₅ at site D, and its conjugated force as the decrease of affinity of Glu 35 for its proton.

The third macrovariable can be identified by considering the statistical events that control the substrate strain through contacts between lysozyme and substrate. These contacts are certainly relevant for catalysis, since it has been shown recently (56) that the interaction of the enzyme with various parts of the substrate molecule change as the enzyme-substrate complex goes from the ground state to the transition state. The substrate is bound at the active site by about 10 hydrogen bonds exposed to the solvent, and involving some charged residues (Asp 101 and Arg 114). Therefore we expect that the water bound in the proximity partly control the interaction of the substrate with the enzyme. The strain of the substrate, defined by a suitable steric angle, thus can be identified as the third statistical macrovariable, and the derivative of the entropy with respect to this angle as the generalized force conjugated with it.

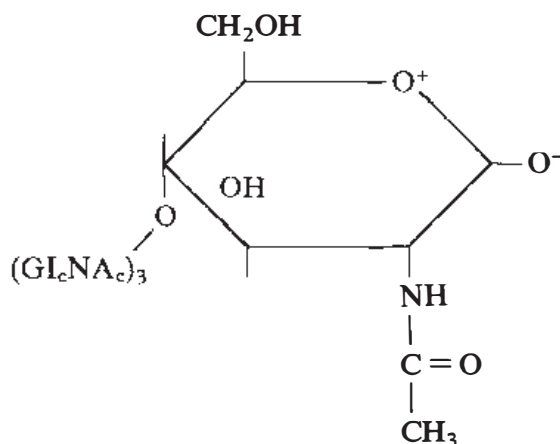
Having identified the three macrovariables, let us understand why their statistical fluctuations can cross-correlate at the transition state. If we consider the charge distribution in the substrate, we realize that the transition state is stabilized both by the negative charge on the Asp 52 O* atom and by the proton concentration near O₅, providing a better binding of the substrate to the active site of the enzyme. Therefore the angular velocity that describes the strain rate of the substrate becomes cross-correlated with the electric current flow from O* of Asp 52 and with the rate of increase of proton concentration generated by Glu 35 around atom O₅.

The concurrence of three factors in lysozyme catalysis thus can be described by the Onsager formalism in terms of three statistical macrovariables, which undergo hydration controlled fluctuations, and where the statistical cross-correlation is induced by the very nature of the transition state. However, the intrinsic limitation of this approach is also evident; we are describing the tight interdependence of three variables far from equilibrium as a mere statistical cross-correlation of their independent fluctuations around the equilibrium.

It has not been possible yet to work out direct experiments or theoretical

calculations to further develop a statistical formulation of lysozyme catalysis. Some recent papers are reviewed below to clarify present achievements and limitations of theoretical and experimental work towards this end.

THE NATURE OF THE TRANSITION STATE On structural grounds, inhibitors that may be expected to resemble the transition-state intermediates are not easily designed, because of the relative complexity of the oligosaccharide molecule and the instability of most of these complexes. In particular, the crystallization of suitable lysozyme-inhibitor complexes still presents problems, whereas attempts to bind tri-N-acetylglucosamine have been unsuccessful. Ford et al (22) have studied, at a resolution of 2 Å, the structure of a lysozyme-tetrasaccharide lactone complex, which is bound in sites A, B, C, and D. Analysis of the electron-density map for site D indicates that the lactone is planar in a conformation close to a sofa or a boot. The planarity of the lactone group is associated with the valence bond contribution of the resonance form that strongly resembles the structure of the proposed (31) transition state.



The polar nature of the bond between the substrate ring oxygen and the ω -carboxylic group of Asp 52 would lead to a distribution of the positive charge towards the O_5 . Thus the above X-ray diffraction studies provide support both for the role of strain in the proposed mechanism of lysozyme catalysis and for the peculiar charge distribution in the transition state.

Temperature-jump and stopped-flow methods have been used by Banerjee and co-workers (3) to study the temperature dependence of the reactions of lysozyme with N-acetylglucosamine oligosaccharides. Nonproductive and productive complexes were observed, with the productive complex being the product of a rapid bimolecular reaction and of two subsequent isomerization processes. For this mechanism, the complete free-energy

profile and an essentially complete enthalpy profile have been defined, with the standard free energy of the three transition states being about 15 kcal/mol within a range of 3 kcal/mol. The observed properties of the final complex suggest that during the rate-determining step a movement of part of the substrate or of the enzyme must occur to allow a more complete mutual interaction; this movement is additional to proton transfer at the active site. In other words, the final rearrangement of the complex, which results in full interaction of the substrate with the active site, apparently occurs in the rate-determining step and in concert with bond rearrangement.

HYDRATION EVENTS Quite recently a comprehensive correlation of infrared spectroscopic, heat capacity, diamagnetic susceptibility, and enzymatic measurements has been completed (G. Careri, E. Gratton, P. H. Yang, and J. A. Rupley, manuscript in preparation), and it suggests the following picture for the progressive hydration of the dry protein to the dilute solution state. At water coverage less than 0.25 g of water/g of protein, the interaction of water with the protein surface is best described as localized adsorption on hydrogen-bonding sites. The first water to interact with ionizable groups produces proton redistribution. There is a transition in the hydrogen-bonding interactions at 0.05–0.1 h (h = grams of water/gram of protein), which is associated with the change from water molecules dispersed about the protein surface to an arrangement of water molecules in two-dimensional clusters. These clusters must be seen as mobile arrangements that increase in size and number as more water is added, until at 0.2 to 0.25 h a major event in the hydration process occurs (see Figure 2), in coincidence with the onset of enzymatic activity and the attainment of the final value of the diamagnetic susceptibility, a property that monitors the electronic structure of the specimen. Monolayer coverage is complete at 0.38 h , a value substantially greater than that for first observation of enzymatic activity. The protein with a monolayer hydration shell must mesh simply with the bulk solvent, in view of the absence of further heat-capacity effects above 0.38 h .

A detailed gravimetric and infrared study of the hydration process of lysozyme films has been performed recently (G. Careri, A. Giansanti, and E. Gratton, manuscript in preparation). The aim of this work was to perform sorption experiments and contemporarily to follow by infrared spectroscopy the hydration status of some specific and representative sites of the protein, such as the acidic side chains and the carbonyl groups of the amide backbone. With a model that postulates the existence of two main classes of primary hydration sites with different binding free-energy and characteristic infrared spectral properties, the sorption isotherm of

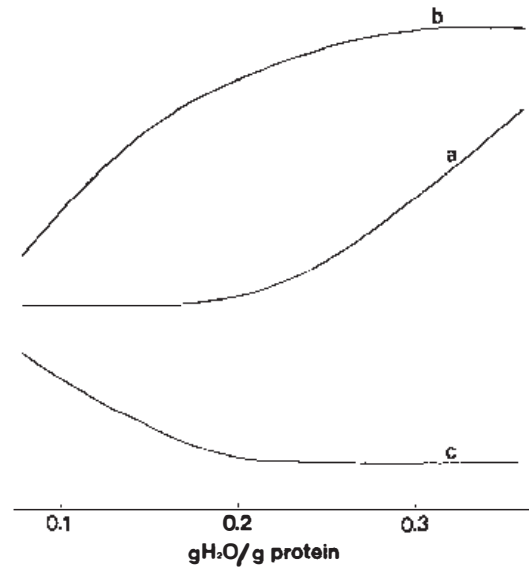


Figure 2 Lysozyme powder hydration events. (a) Enzymatic activity (arbitrary units); (b) amide backbone coverage (arbitrary units); and (c) diamagnetic susceptibility (arbitrary units) plotted versus protein hydration. Note the start of the enzymatic activity near the saturation of the backbone coverage and when the electronic ground state of the specimen has reached a final settlement. (From G. Careri, E. Gratton, P. H. Yang, and J. A. Rupley, manuscript in preparation.)

the sample has been decomposed to fit the two classes of infrared spectral changes undergone by the protein, as a function of hydration. With these data, and the reasonable hypothesis that the ionizable groups and the amide backbone correspond to the strong and weak binding sites, respectively, relevant thermodynamic information concerning the hydration sites can be derived from the sorption experiments. In this way the free energy of water binding to the strong and weak binding sites has been found to be 2.0 ± 0.2 and 0.4 ± 0.1 kcal/mol, respectively. The low value of these free-energy changes experimentally determined clearly shows that solvent-protein surface interactions can be an intense source of fluctuations.

THEORETICAL APPROACHES A theoretical study of the low-frequency hinge-bending mode in lysozyme, involving the two globular lobes (lobe 1, residues 5–36 and 98–129; lobe 2, residues 40–94) surrounding the active site cleft, has been done by McCammon and co-workers (45). Their calculations have been carried out for the free enzyme, the water molecules associated with the protein being ignored. By the use of empirical energy functions, the change in the conformational energy calculated for the enzyme alone is approximately 10 kcal/mol for an angular displacement of 10° . In the presence of solvent, the bending motion is determined by the Langevin equation for a damped harmonic oscillator and yields a

relaxation time near 2×10^{-11} sec. We anticipate this time constant to be considerably displaced toward the low frequencies when the substrate is in the cleft, because the substrate binds to both lobes (for instance, N-acetyl muramic acid in site F is hydrogen bonded to lobe 1 by residues 34 and 37, and to lobe 2 by residue 114 and the charged arginine 114).

Conformational energy calculations have been carried out by Pincus and co-workers (49) to predict the three-dimensional structures of enzyme-substrates and enzyme-inhibitor complexes of lysozyme. In this first paper, the lysozyme structure was held rigid and only the substrate was allowed to move in the region of the active site and to change conformation so as to minimize energy. It is unfortunate that the low energy minima for the oligosaccharides, which were taken as starting conformations for the substrate, were calculated (48) without taking the solvent into account.

Levitt (41) also studied the nature of the binding of substrate hexa-N-acetylglucosamine to lysozyme to minimization of an empirical energy function. These functions were obtained (40) by a method that minimizes the stereochemical potential energy, including nonbonding interactions. Actually, considerable uncertainty exists as to the best choice for these interactions, and the approximations listed by Levitt (41) seem rather crude. For instance, both charged and uncharged carbonyl oxygens, amide nitrogens of the charged residues, are considered equivalent, whereas it is known from the work of Janoschek et al (32) that a very large difference exists in the hydrogen bond energy between charged and neutral species.

In a subsequent paper, Warshel & Levitt (61) have devoted considerable effort to a theoretical study of the stabilization of the carbonium ion in the reaction of lysozyme. The aim was to consider the complete enzyme-substrate complex together with the surrounding solvent, and to evaluate the quantum mechanical energies associated with bond cleavage and charge redistribution of the substrate and the classical energies of steric and electronic interactions between the substrate and the enzyme. The electrostatic polarization of the enzyme and the orientation of the dipoles of the surrounding water molecules have been simulated by a microscopic dielectric model, and the solvation energy resulting from this polarization has been found to be a considerable factor in the stabilization of the carbonium ion intermediate. Apart from the uncertainty about the atomic coordinates at the active site and the energy functions, the evaluation of electrostatic polarization must be criticized for at least two reasons. First, the polarizability of the charged hydrogen-bonded Asp 52 should be evaluated better, since it is known from the work of Janoschek et al (32) that it can be as high as 100 times the atomic one. Second, the surrounding water molecules are treated as a uniform medium, bound to the protein by a kind of Lennard-Jones potential. On the light of the hydration experiments referred to above, this picture is too rough.

Computer simulations of the solvent structure around the lysozyme molecule in its triclinic crystal have been carried out. These studies use pair-wise empirical functions for the water-protein interatomic potential and can yield the equilibrium isoenergy contour maps for the water interacting with lysozyme (13) or the energy distribution of water in different environments (28). The occurrence of wide varieties of bound water is evident in both types of study, in line with the experimental finding for the absorption sites reported above. These studies suffer from the neglect of the dynamic properties of the solute molecule, which is considered to be rigid, as it appears from X-ray crystallography. Actually the charge redistribution that follows the peptide hydration (53) makes this neglect quite a crude assumption if one is interested in the details of the energy profile of the system.

4.2 *Serine Proteases*

Serine proteases contain a very reactive serine side chain that forms a transient covalent linkage with the carbonyl carbon atom in the susceptible ester or amide bond of substrates. These enzymes are all characterized by a precisely oriented catalytic triad, consisting of a carboxylate group of aspartic acid (Asp 102 in α -chymotrypsin), an imidazole ring of histidine (His 57 in α -chymotrypsin), and a hydroxyl group of serine (Ser 195 in α -chymotrypsin). Above pH 7, the hydrogen bond system between these groups is polarized by the buried negative charge of Asp 102, which makes the serine oxygen strongly nucleophilic. The reaction proceeds by a mechanism in which the trigonally coordinated carbon atom of the substrate is attacked by the approach of a fourth ligand to form a tetrahedrally coordinated species. In the transition state the negative charge of this form is stabilized in a hole by two hydrogen bonds [NH of Ser 195 and the NH of Gly (193)]. These three factors responsible for catalysis are called the charge relay system, the tetrahedral intermediate, and the oxyanion hole. This matter is discussed in standard textbooks and in recent reviews (4, 17, 38).

In the following we offer a description of serine protease catalysis that parallels the previous description of lysozyme, but which is more concise and less satisfactory because of some uncertainties (30, 42) in the mechanism itself and a less clear evidence for the role of hydration in catalysis (35). Our first task is again the identification of the statistical macrovariables and their coupling for catalysis. For the sake of simplicity, unless otherwise stated, in the following discussion reference is made to α -chymotrypsin.

The first macrovariable can be identified in the charge distribution on Asp 102, a fluctuating charge that controls the distance Z_2 between Asp 102 and His 57 (see Figure 3) and vice versa. This distance is critical

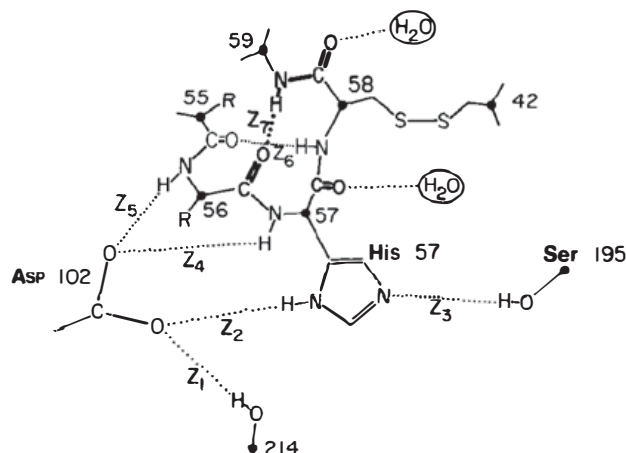


Figure 3 Schematic representation of the hydrogen bonding network, which controls the charge distribution on Asp 102 in serine proteases. Encircled H₂O represents solvent molecules hydrogen bonded to external backbone carbonyls. Critical hydrogen bond distances are indicated, as Z₁ to Z₇. [From (37).]

for the proton transfer. The hydrogen bonding network of the groups connected to Asp 102 is indicated in Figure 3, where the relevance of the amide backbone-bound water towards the charge distribution on Asp 102 is evident. At the transition state the Asp 102-His 57 distance can be reduced if the negative charge on the involved oxygen is increased, possibly by an hydration-induced conformational change of the backbone section near His 57, which in turn controls the charge distribution of Asp 102 via amide hydrogen binding. Note that the groups near His 57 are invariant in all serine proteases, as shown in Figure 4.

SERINE PROTEASES

	53	54	55	56	57	58	59	60
CA Cow	V	T	A	A	H	C	G	V
CB Cow	V	T	A	A	H	C	G	V
E Pig	M	T	A	A	H	C	V	D
T Cow	V	S	A	A	H	C	Y	K
T Pig	V	S	A	A	H	C	Y	K
T Dog f.	V	S	A	A	H	C	Y	Q
ThBCow	L	T	A	A	H	C	L	L
TLE-SG	L	T	A	A	H	C	V	S

Figure 4 Amino acid sequences of serine proteases. Note the invariance of residues 55, 56, 57, and 58, which play a key role in Figure 3. H, His; C, Cys; L, Leu; M, Met; Q, Glu; D, Asp; V, Val; A, Ala; G, Gly; Y, Tyr; T, Thr; S, Ser. [Alignment and data from (16).]

The possibility that an undetected conformational change in the residues of the charge relay system could be responsible for the enzyme activity has been suggested by Freer et al (23), whereas Wright et al (64) proposed that the exposed imidazole of His 57 could ensure neutralization or delocalization of the negative charge of the carboxylate Asp 102 by solvent at the surface of the molecule. We believe our proposal to be simpler and more concrete, because the distance Z_2 , according to recent data by Kossiakof and co-workers (37) for trypsin, is sufficiently short to make this charged hydrogen bond sensitive to the water-controlled backbone conformations. The charge distribution on Asp 102 is the only quantity sensitive to hydration from one side and able to control the proton transfer from His 57 on the other; therefore it seems a reasonable choice for a statistical macrovariable relevant to catalysis.

A second macrovariable can be identified in a fluctuating geometric factor that controls the precise alignment of the polypeptide chain of the substrate in the binding site of the enzyme. Evidence for the importance of a very precise enzyme-substrate alignment is offered by the recent work of Hunkapiller et al (30), and Steitz et al (58) detected in the CO group of Ser 214 the conveniently placed hydrogen bond acceptor of the NH group of an amino acid of the substrate, if the carbonyl group is brought close to the side chain of Ser 195 of α -chymotrypsin. This suggestion was later confirmed by Segal (57) for other substrates. Robertus et al (50) noticed the extremely similar structural resemblance in the active side of subtilisin to the residues Ser 214, Trp 215, and Gly 216 of α -chymotrypsin, in spite of the different folding of the two macromolecules. Since these residues are exposed to solvent, it is reasonable to think that the hydration processes around this section of the backbone can control the position of the substrate in the active site, thus making geometric factors of a truly statistical nature. One can easily be convinced that this holds also for trypsin and elastase from inspection of their X-ray structures (52).

The third macrovariable can be identified in the fluctuating negative charge distributed in the oxyanion hole. As stated above, this is formed with the intervention of two backbone hydrogen bonds in all serine proteases except in subtilisin, where electron donation to the carbonyl oxygen atom occurs from the side-chain amino group of Asn 155 (43) via a strong hydrogen bond, which is allowed after substrate binding by a slight rotation of the Asn 155 side chain around the $C_\beta-C_\gamma$ bond. Asn 155 is located on the surface of the macromolecule (64). In α -chymotrypsin at the transition state it is reasonable to propose that a similar change occurs in the backbone section around Gly 123. This conformational change exposes to the solvent the backbone carbonyl of Met 192, which in the native

enzyme is located on the surface and is hydrogen bonded to the NH of Leu 143. Then the hydration of one critical amido group (Asn 155 in subtilisin and the backbone in all other proteases) can control the charge distribution in the oxyanion hole, thus making this quantity a statistical macrovariable. This hypothesis is attractive because it explains why the same function is preserved in otherwise different residues of the proteases.

Having identified a minimum set of macrovariables, their possible cross-correlation during catalysis should be investigated. Only a negligible strain is exerted on the substrate upon binding at the active site (51). According to Hunkapiller et al (30), the rate-limiting step for the hydrolytic reaction is the decomposition of the tetrahedral intermediate by the simultaneous transfer of two protons, presumably from Asp 102 to His 57 and from His 57 to the anilide NH of the substrate, with the His 57 remaining neutral but undergoing a tautomeric shift. In this way the unfavorable charge separation within the active site of the enzyme can be obviated, provided the substrate is properly positioned with respect to the catalytic residues. We may rephrase this statement in terms of the three macrovariables defined above by saying that at the transition state these macrovariables become cross-correlated, namely that the following statistical events occur simultaneously: (a) backbone desolvation near the oxyanion hole, which increases the length of two hydrogen bonds and makes this hole more negative; (b) water rearrangement on the backbone around the Ser 214, which contributes to the correct orientation of the substrate; and (c) water rearrangement on the backbone around His 57, which changes the length of the H bonds between Asp 102 and the backbone amide group, favoring the transfer of the proton from the neutral Asp 102 carboxylic group to His 57.

Notice that in the above scheme the charge relay process in the triad Asp-His-Ser is an important, but not unique, part of the catalytic mechanism.

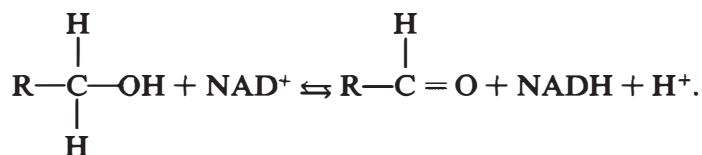
A theoretical computation of the above scheme is beyond present possibilities. The charge relay system in the triad Asp-His-Ser has been studied by molecular orbital calculations (25, 36, 60) to assess the conditions required for the concerted proton transfer. The contribution of the peptide backbone dipolar groups to the intraproteic electrostatic field, which had been neglected in the above studies, has later been found to be large enough in the case of α -chymotrypsin (33) to call for a proper consideration of the fluctuation related to hydration backbone even when studying the catalytic triad of proteases. More recently, Scheiner et al (54) have considered both the charge relay and the optimum model substrate orientation to derive the potential energy surface; the optimal reaction path suggested by these studies lead to the apparent conclusion that the above two factors

are nonconcerted. In a later work Scheiner & Lipscomb (55) have considered one water molecule bound the negatively charged oxygen atom of the tetrahedral adduct. This lowers some sections of the reaction profile by 25 kcal/mol. It is impossible at the present time to evaluate what would be the gain in the reaction rate when an amide group is hydrogen bonded to the charged oxygen atom and the true residues are used instead of the model so far employed.

4.3 The Case of Horse Liver Alcohol Dehydrogenase

To test the usefulness of the above approach we tried to apply it to a rather complex yet reasonably well-known system, horse liver alcohol dehydrogenase, a representative enzyme (8, 59) extensively studied from both the structural and functional view point (15, 20, 27).

The enzyme catalyzes the reversible transfer of hydrogen from an alcohol to the coenzyme NAD, according to the following scheme:



On the basis of available crystallographic, chemical, and kinetic evidence, the mechanism outlined in Figures 5 and 6A and B has been recently proposed (6, 19). This scheme refers to only one of the two subunits of the liver alcohol dehydrogenase molecule. The problem of cooperativity between the subunits is not discussed here.

According to the proposed mechanism, NAD^+ binds first, inducing a conformational transition that perturbs the pK of a Zn-bound water and

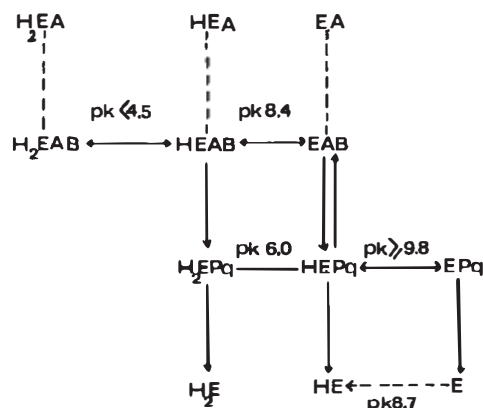


Figure 5 Proposed mechanism for horse liver alcohol dehydrogenase [compiled according to (7,19)]. E, Horse liver alcohol dehydrogenase; A, NAD^+ ; B, alcohol; P, NADH; q, aldehyde substrate.

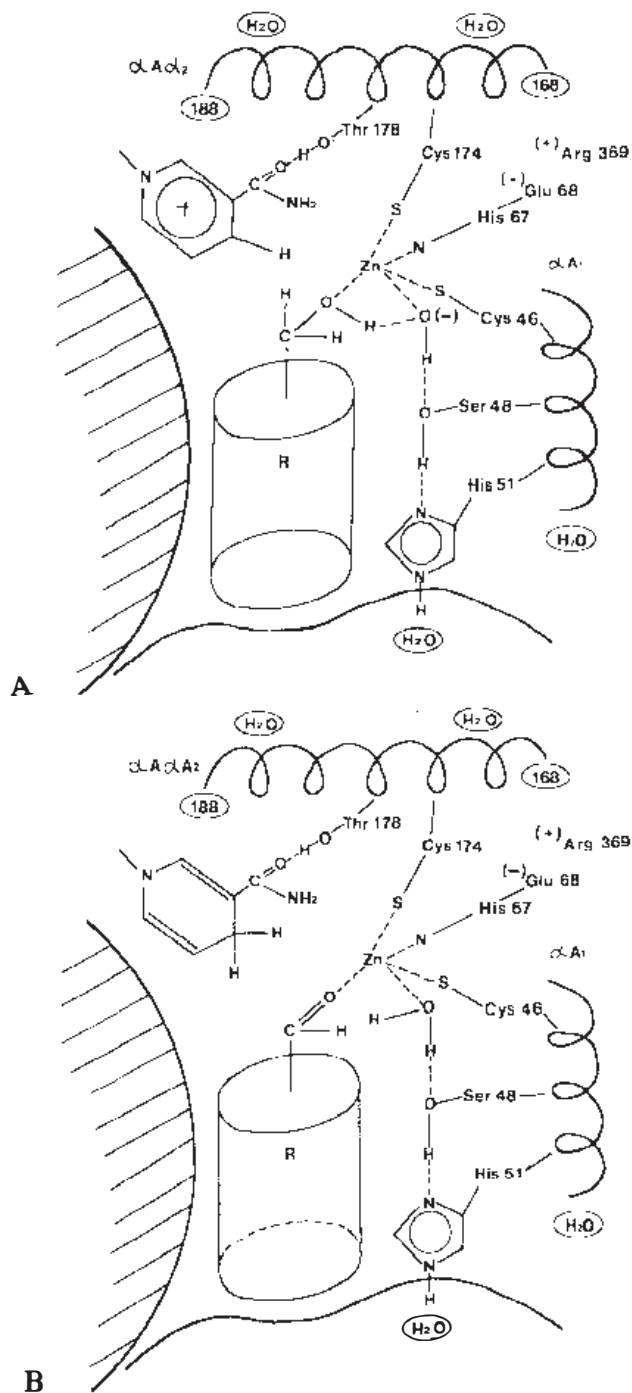


Figure 6 (A) Schematic representation of the active site in the alcohol dehydrogenase-NAD⁺-alcohol ternary complex. Encircled H₂O represents solvent water molecules hydrogen bonded to the exposed protein groups. The cylinder represents the substrate binding hydrophobic barrel (6,7). (B) Schematic representation of the active site in the alcohol dehydrogenase-NADH-aldehyde ternary complex.

hence causes the release of a proton presumably mediated by the hydrogen bond system of Ser 48 and His 51 (8, 27). In the next step, the alcohol substrate binds at the active site, the side chain of the substrate is positioned in a hydrophobic barrel, and the reactive hydroxyl group points towards the active site Zn (7, 20, 27).

According to the recent structure reactivity studies (19), and contrary to previous suggestions, no negative charge would develop on the substrate hydroxyl oxygen during catalysis, and hence the transfer of the proton from the alcohol to the enzyme active site would occur simultaneously with the hydride transfer from the substrate to the C-4 atom of the coenzyme nicotinamide ring. A general acid-base catalysis would be consistent with these views. In the enzyme-NAD-alcohol ternary complex the water hydroxyl ion is assumed to remain bound to Zn, whereas the neutral alcohol molecule adds as a fifth ligand to Zn (Figure 6A). The Zn-bound hydroxyl ion is postulated to be the general base catalyst for the oxidation of alcohols and the Zn-bound water molecule as the general acid catalyst for the reduction of aldehydes (Figure 6B).

It should be pointed out that this scheme has not been conclusively proved, and that a variation of the classical mechanism (8), which postulates the formation of a tetracoordinated alcoholate anion, cannot be excluded. Although the following discussion refers to the mechanism proposed by Dworschack & Plapp (19), which as mentioned above postulates a penta-coordinated enzyme-substrate-Zn complex, our considerations could be adapted easily to the classical mechanism as well.

We maintain that statistical fluctuations, related with solvent-water binding to critical parts of the enzyme surface, do occur and affect protein groups directly involved in the catalytic process. Moreover, we suggest that such fluctuations cross-correlate at the transition state. Let us examine the hydride transfer step. A schematic diagram of the situation at the active site is given in Figure 6A and B. At least three sets of enzyme groups are involved in the process: (a) the coenzyme nicotinamide ring and the protein side chains directly bound to it; (b) the active site Zn and its coordinated water; and (c) the hydrogen bond charge relay system extended from the active site water to the protein surface through the side chains of Ser 48 and His 51.

Available evidence shows that the coenzyme molecule binds in an extended conformation within a crevice of the enzyme. The adenosine phosphate moiety is firmly anchored to the enzyme. The nicotinamide moiety is positioned in the active site, with the A side of the ring facing the catalytic Zn atom and the B side facing the hydrophobic wall of the parallel pleated sheet of the coenzyme binding domain. The distance from the Zn atom to the center of the nicotinamide ring is 4.5 Å and somewhat

shorter to the C-4 atom of the ring. The only polar contact between the nicotinamide moiety and the protein is a hydrogen bond between the side chain of Thr 178 and the carbonyl oxygen of the coenzyme carboxamide group. Thr 178 is part of an α -helical stretch extended from residues 168 to 188 helices $\alpha 2$ and αA according to Branden's terminology (20). Two facts are noteworthy: (a) One side of the helix faces the solution whereas the other faces the active site; (b) Cys 174, one of the groups coordinated to the catalytic Zn atom, is on the same helix, just 1.2 turns away from Thr 178.

The second set of chemical groups at the active site comprises Zn and the ligands coordinated to it. In the proposed scheme the Zn is pentacoordinated to Cys 174, Cys 46, His 67, the oxygen of one water molecule (or of the corresponding hydroxyl ion), and the substrate oxygen. As mentioned above, Cys 174 is located on the $\alpha 2$ - αA helical stretch, relatively close to Thr 178, which is hydrogen bonded to the coenzyme carboxamide group. Cys 46 is located at one end of a helical stretch [helix 1 of reference (20)], which extends to residue 53 and comprises the residues involved in the charge relay system, namely Ser 48 and His 51. This stretch is exposed to the solvent only at its "C" end (Val 53 and, possibly, Val 52). The relative hydrophobicity of the side chains of the latter residues suggests that water may interact with the peptide backbone. Moreover, His 67, the third protein group coordinated to the active site Zn, is within interaction distance from the residue adjacent to Ser 48, namely Asp 49 (K. Branden, personal communication). This would provide another structural arrangement for coupling events that occur in different components of the active site. Finally, His 67 is adjacent to Glu 68, which forms a salt bridge with Arg 369, a polar group in a pleated sheet portion of the protein.

The third set of active site groups is the well-known proton relay system extending from the active site hydroxyl oxygen to the solvent through Ser 48 and His 51. As suggested by Branden (6), this system could exert a role in hydride transfer as well as on the proton release that occurs in the previous step, even though recent crystallographic data (K. Branden, personal communication) indicate that the orientation of the His 51 imidazole ring relative to Ser 48 may be altered in the holoenzyme complex.

Let us consider now the catalytic implications of the above situation at the active site and the events consequent to substrate binding. Structural and kinetic work (7) has shown that the R moiety of the substrate interacts with the hydrophobic barrel (cf Figure 6) and positions the alcoholic group of the substrate between the C-4 carbon of the coenzyme nicotinamide ring and the active site Zn. It is postulated that the latter atom coordinates the substrate oxygen. The hydride transfer from the primary alcohol sub-

strate to the coenzyme is stereospecific. Therefore, the orientation of the alcoholic group relative to the C-4 carbon atom of the coenzyme nicotinamide ring must be accurately determined in the enzyme substrate complex. According to Branden & Eklund (7), the C—H bond direction for the hydrogen atom to be transferred points to the C-4 atom of the nicotinamide ring forming an angle N-1—C-4 (coenzyme)—C-1 (substrate) of 100 degrees.

It is possible to tentatively identify several statistical macrovariables that determine the free energy profile of the reaction. One could be the charge distribution on the active site hydroxyl ion oxygen. It is evident from Figure 6A that the negative charge in question is controlled by the distances between all the atoms of the hydrogen bond relay system extending to Ser 48 and, possibly, to His 51. The latter interacts directly with the solvent water molecules fluctuating around the exposed part of the imidazole ring. Moreover, these distances may be affected by the fluctuations of water molecules binding at the end of the helical stretch $\alpha 1$.

A second macrovariable could be identified in the charge on the active site Zn. This charge and the electric field acting on it will be affected by the position of the ligands around the Zn, which in turn will be influenced by the statistical binding of water molecules to the helical stretch $\alpha 2$ and at the end of helix $\alpha 1$. Also, events affecting the distance between Glu 68 and Arg 369 could influence the charge on the active site Zn.

Other macrovariables could be identified in the distance and orientation of the C-4 atom of the coenzyme nicotinamide ring relative to the substrate H atom to be transferred. These geometric quantities are affected by the atomic positions and charge distribution in the H bond between the nicotinamide carboxamide group and Thr 178. The position of the latter will be influenced by the interaction between solvent water and the $\alpha 2$ - αA helical stretch. The C-4-substrate H distance and the N-1—C-4 (coenzyme)—C-1 (substrate) angle thus can be identified as the third and fourth statistical macrovariables.

A cross-correlation of the statistical fluctuations of these macrovariables at the transition state could be expected. As a matter of fact, the transition state is stabilized by a decrease of the distance between the coenzyme C-4 and the substrate H, by a transfer of the negative charge from the Zn-bound water hydroxyl ion towards the coenzyme nicotinamide ring, and by a redistribution of the Zn ligand field acting on the water hydroxyl oxygen and the substrate oxygen. Therefore, the velocity that describes the movement of the coenzyme towards the substrate should become cross-correlated with the electron flow out of the Zn-bound water hydroxyl and with the rate of redistribution of charges around the Zn atom.

Thus the concurrence of the catalytic factors could be tentatively de-

scribed by the Onsager formalism in terms of at least four statistical macrovariables, which undergo hydration-dependent fluctuations and may become cross-correlated at the transition state. The detailed examination of horse liver alcohol dehydrogenase suggests that in more complex systems, such as this dehydrogenase, the cross-correlation of the hydration-induced fluctuations could be facilitated by some peculiar features of the enzyme structure that are not present in the simpler hydrolytic enzymes previously considered. As pointed out earlier, in the case of horse liver alcohol dehydrogenase, some components of each of the sets of groups involved in each of the macrovariables are bound to one helical stretch that also contains components of a set of groups involved in another of the macrovariables, a kind of cross-connection that should help the establishment of space-time correlations between the statistical macrovariables.

Moreover, a conformational transition occurs upon formation of the enzyme-coenzyme substrate complex (7), which effectively seals off the active site from the environment, shielding it from random collisions with the solvent molecules. On the other hand, the components of the active site are attached to helical parts of the protein backbone partially exposed to the solvent, so that the random interaction with the latter can cause concerted fluctuations within the active site.

5 CONCLUSIONS

From the foregoing review a number of interesting features emerge, which are briefly summarized.

1. The notion of a fluctuating globular protein has been substantiated by theoretical and experimental studies. Since the energy of internal hydrogen bonds, which should preserve the secondary and tertiary structure, has been found to be nearly the same as the energy of the water-protein hydrogen bonds, one is justified to figure out the polypeptide chain moving in the solvent while preserving the overall shape of the globular protein, in a time scale as short as the nanosecond or less.

2. Since a critical role of the solvent in lysozyme catalysis is exerted upon completion of the layer of water weakly bound to the backbone, one can believe that the mobility of this weakly bound water plays an important function by inducing fluctuations on the backbone itself. These backbone fluctuations are likely to propagate to the enzyme active site, helping catalysis by an essentially entropic effect.

3. The identification of the statistical macrovariables in different enzyme families reveal great analogies at the molecular level. The hydration of an hydrogen bonded network controls the charge distribution both on

lysozyme Asp 52 (Figure 1) and on serine protease Asp 102 (Figure 2), and presumably of His 51 of liver alcohol dehydrogenase (Figure 6). Intramolecular hydrogen bonding controls the correct substrate positioning at the active site in all cases. Water-amide hydrogen bonding controls specific conformations of solvent-exposed backbone, in the proximity of His 57 and of the oxyanion hole in serine proteases and of Glu 35 in lysozyme. Since the hydrogen bonding free energy can be comparable to the thermal energy, it is quite reasonable that hydrogen bonding controls most of the statistical factors relevant for catalysis.

4. The qualitative application of the Onsager treatment to enzyme dynamics by selecting only a few statistical macrovariables fluctuating around equilibrium is a useful tool to offer a unified view of quite different molecular situations, and to identify the different statistical factors that emerge together at the transition state.

5. Cross-correlation among relevant fluctuations for catalysis has not been observed and is unlikely to be observed in the laboratory because of the short duration of the transition state where these correlations are more likely to occur. Besides the biochemical problem of placing intrinsic or extrinsic probes in meaningful sites of the enzyme, the spectral analysis of the cross-correlations in the nanosecond region is a major problem of experimental physics. Cross-correlations at the transition state also are unlikely to be theoretically proven because of the difficulties in solving the quantum mechanics of many bodies. The relevance of these cross-correlations for catalysis is still speculative, but, we believe, quite suggestive.

In our opinion the statistical physics approach to enzyme action provides a valid interpretation of known facts, based on better physical grounds. Moreover, although difficult at the present time, further developments to produce quantitative analysis, both at the experimental and theoretical level, will eventually take place.

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