STATISTICAL TIME EVENTS IN ENZYMES: A PHYSICAL ASSESSMENT

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1. INTRODUCTION

1.1. Aim of the Paper

Enzyme action is the result of a large number of discrete steps involving a great variety of processes such as cooperative conformational changes, acid-base catalysis, nucleophylic and/or electrophylic attack from properly positioned groups, etc.; it is widely recognized that in order to be useful for catalysis, the various elementary processes must be space- and time-controlled during enzyme function. In the past decade great progresses have been made in understanding the chemistry and the stereochemistry of enzyme action, with particular emphasis on the role of the spatial effects. Obviously, an analysis of the temporal aspects of enzyme action is equally important. The ultimate goal is the description of the concomitance and/or sequence of individual elementary steps in the catalytic act. This ambitious but difficult goal can be approached by focusing the attention on the time constants of the various elementary processes and assessing their microscopic mechanism by comparative studies on representative model systems. This approach was introduced in enzymology with the development of fast relaxation methods and will be followed in this paper, with the understanding that it suffers from the same intrinsic limitations as an analysis of a musical piece restricted to a list of the sound frequencies occurring in it but devoid of any information about their temporal sequence and relative intensity.

Our aims are:

1. To review time events detected in enzymes using a proper physical framework, i.e., the theory of the random processes.
2. To identify these events at a molecular level by comparison with processes occurring in appropriate model systems.
3. To discuss the statistical significance of the detected events.

We shall start with the simpler model systems and shall then proceed to analyze situations of increasing complexity and eventually consider enzyme-substrate complexes. For each class of events some data will be critically reviewed and their relevance to enzyme catalysis stressed. All

data will then be comparatively discussed according to their time scale and some mechanistic conclusions will be derived.

The representative enzymes considered in this review were chosen among those which can work as separate entities in an aqueous medium because they are simpler and better known.

1.2. The Notion of Statistical Time Event

Individual rate constants in enzymes are generally measured by rapid mixing or by relaxation methods; quite recently an alternative, direct approach, i.e., concentration fluctuation spectroscopy, was successfully applied to the study of some simple cases[1-3] of chemical events.* Relaxation and concentration fluctuation spectroscopy are interconnected by the fluctuation-dissipation theorem,[4] which relates the average time course of the decay of spontaneously microscopic fluctuations to the time course observed after a small perturbation around equilibrium. Then, for a single process, the autocorrelation function of random fluctuations is an exponential which has the same time constant as the relaxation response of the system. Therefore each of the detected relaxation process observed in enzymes is representative of one class of spontaneous fluctuations[5] around equilibrium, their amplitude being distributed along a gaussian curve and decaying with the same correlation time. In order to identify one such class of fluctuations without necessarily specifying the molecular process involved, the term "statistical time event" has been used throughout this paper, since it requires only a measurement of time for its operational definition.

As it will become clear from the following discussion, it seems quite appropriate to assess the available data, which were mostly obtained by relaxation methods, in the frame of the random process theory. Here the correlation time, and for simplicity we shall limit ourselves to the autocorrelation time of a statisitically stationary variable, is the basic quantity of interest since it gives a direct measure of the time interval over which the variable involved is behaving more or less regularly and predictably. For longer time intervals the behavior becomes progressively more random. Therefore, in the operational definition of "statistical time event," the figure used to classify a given class of fluctuations has an inherent physical meaning, i.e., it expresses the time interval in which these fluctuations are persistent. This is most important when two or more classes of spontaneous fluctuations can be expected to interact among themselves, because, in order to do so (namely to become cross correlated), these different classes must have quite close autocorrelation times. It should be noted that this kind of analysis of random processes is based on the assumption that the variables involved are statistically stationary and linearly superimposed; this assumption may not be true for an enzyme where the different classes of fluctuations may interact in a nonlinear way, merging into new cooperative, nonstationary effects of great chemical interest! For this reason the expression "statistically stationary time event" would be a more accurate term to identify the kind of "time event" we are dealing with. Moreover, it is quite likely that during enzyme action even a single separate event can appreciably influence the following one because of the change induced in the macromolecule, namely the processes we are considering may not be markovian. The foregoing introductory discussion provides an indication of the difficulties to be faced before a correct time-

*The interrelationship between relaxation time τ, rate constant k, and frequency f are well covered in the literature.[1-3] Here we remind the reader that for a simple reacting system described by

$$N_A \underset{K_{21}}{\overset{K_{12}}{\rightleftharpoons}} N_B,$$

a perturbation imposed on the system decays exponentially as exp $(-t/\tau)$, and $1/\tau = k_{12} + k_{21}$. The frequency spectrum of the fluctuations per unit frequency interval (bandwith) is found to be[1]

$$[\delta N_A(f)] = \frac{4\tau \bar{N}_A[1 - (\bar{N}_A/N)]}{1 + (2\pi f\tau)^2},$$

where $N = N_A + N_B$ and the bar means the mean value. The autocorrelation function of the concentration fluctuations is defined as $\delta N_A(t)\delta N_A(t + t')$ and is a constant times exp $(-t'/\tau)$.

structure description of enzyme action can be achieved. Incidentally, these difficulties are quite similar to those met in information theory when dealing with human language.

2. MODEL SYSTEMS

2.1. Hydrogen Bonded Structures

The importance of hydrogen bonds in the stabilization of protein structure is a well-known fact. Each of these bonds is so weak that it can be readily broken and rebuilt at room temperature. This section discusses some hydrogen bound model systems where this statistical time event can be easily studied.

The ultrasonic relaxation frequency of several hydrogen bonded pure liquids (such as n-propyl alcohol, propionic acid, and acetic acid) is known[6] to be around 5MHz. This is because, although these quite different molecules are associated into hydrogen bonded chains of different degrees of polymerization, the relaxation process is due to the dissociation of a single hydrogen bond of the chain which is the kinetic process of interest for our purpose. In more complex hydrogen bonded structures, as in the case of glycerol where up to three hydrogen bonds per molecule can be formed, the dielectric and acoustical relaxation frequency increases to 50 MHz. This acoustical relaxation process is certainly due to the hydrogen bonding, since it is observed in N-methyl-acetamide but not in dimethyl-acetamide. Of course the rates are faster for hydrogen bonds weaker than those considered above.

The rate constants for the association and dissociation of some hydrogen bonded dimers in solution have also been measured from ultrasonic attenuation.[8,9] Since the association is essentially diffusion controlled, the dissociation rate constants were estimated to be in the $2 \times 10^6 \div 2 \times 10^7$ sec^{-1} range, depending upon the stability of the bond involved. Of particular interest is the case of 2-pyridone,[9] where the dimer is stabilized by two NH · · · O bonds, and the dissociation rate constant is 2.2×10^7 sec^{-1}.

The acoustical relaxation of aqueous solutions of nonelectrolytes provided with both an hydrophobic and an hydrophilic group, e.g., ethylamines and ethanol, is quite interesting. The process is quite complex because it involves the perturbation of equilibria with several water molecules. The experimental values of its relaxation frequency, are in the range 50 to 150 MHz.[10]

2.2. Proton Transfer Kinetics in Amino Acids, Peptides, and Other Model Systems

Intramolecular and solvent-protein proton transfer, as well as the conformational changes coupled with it, are of considerable interest in the context of the present paper.

In this section, some model systems suitable to the study of statistical single proton transfer will be discussed.

The proton transfer equilibrium between protein and the solvent is a familiar process, accurately described by the titration curve. Moreover, intramolecular proton transfer and the conformational changes which may be coupled to it are relevant to this equilibrium. There are some model systems where these single statistical processes can be studied as shown in the following.

The acoustical absorption relaxation of many single amino acids and oligo-peptides (such as di- and triglycine) in alcaline solution is characterized by a single relaxation process with a time constant ranging between 3×10^{-9} and 3×10^{-8} sec.[11] The pH dependence of this absorption suggested that the perturbation of the proton equilibrium between the α-amino groups and the solvent was involved. A similar process involving the α-carboxyl group has been detected in glycine acid solutions.[12]

Dissociable amino acids with side chains should be considered next. Ultrasonic measurements on arginine and lysine solutions at pH 9 show that some interaction occurs between the α-amino and at the side chain amino groups.[13] It is likely that this conclusion applies to polypeptides and proteins as well. In homologous acidic and basic polypeptides, the situation is complicated by the occurrence of a helix-coil transition, but it seems likely that proton transfer is the major (if not the only) source of absorption, as was clearly shown to be the case in poly-l-lysine solutions.[14]

Intramolecular proton transfer is a different kind of process. The case of o-aminobenzoic acid in methanol and acetone is a good model of cyclic hydrogen bonded system, the rate constant for the forward and reverse reactions of the classical zwitterion equilibrium were found[15] to be respectively, 1×10^7 and 1.4×10^8 sec^{-1}. Notice that the N-H-O distance in this system is 2.7 Å, i.e., very close to the one usually expected for intra-

molecular bridges of the proteins. Another interesting case where the solvent is not involved is the dimerization of ε-caprolactam in organic solvents, where the dissociation time constant is around 10^{-8} sec, and the association process is almost diffusion controlled.[16]

2.3. Polyelectrolytes in Solution

The existence of a counter ions atmosphere around the globular proteins is well known, and the relaxation of this atmosphere can interfere with some kinetic processes of catalytic interest. The dielectric properties of a solution of a macromolecule bearing a large number of charges were first investigated a long time ago, but only quite recently has a theory been developed[17] which satisfactorily accounts for the experimental data.

The model consists of a sequence of charged rod-like subunits in an arbitrary but fixed configuration. It is assumed that a certain fraction of the counter ions is closely associated with the macromolecule. It is found that the high frequency dispersion and relaxation can be attributed to fluctuations in the distribution of bound counter ions along limited parts of the macromolecule, while the low frequency relaxation can be caused by the rotation of the entire molecule. The static electric permittivity can be explained in terms of fluctuations in the counter ion density over the whole macromolecule. This theory has been verified, in the range 5 MHz to 100 MHz, in solution of polymethacrylic acid and polyacrylic acid of a different degree of polymerization and in other polyelectrolytes, where the 2 relaxation times are respectively around 10^{-6} and 10^{-8} sec.[18] Of even greater interest is the case of aqueous solutions of poly-l-glutamic acid, where the 2 relaxation times are around 3×10^{-6} and 3×10^{-7} sec, respectively.[19] Also note that the mobility of the counter ions moving on an equipotential surface along the rigid subunits of the polyelectrolyte was not supposed (and experimentally confirmed) to differ significantly from the mobility of free ions. Ultrasonic attenuation measurements[20] in poly-l-glutamic acid solutions in the same frequency and pH range show a distribution of relaxation times which does not vary markedly with pH. These results were attributed[20] to several causes, such as the breakdown of the water structure around the polypeptide, some protolytic reactions, and possibly a contribution from the helix-coil transition.

An interesting case of chemical relaxation in zwitterions, which involves the exchange of protons, was recently shown[21] to occur in aqueous solutions of poly-l-proline after addition of formic acid at a pH less than 4. In this case, the pKs of the acid and of the base system were similar, and a further broadening of the dielectric dispersion with a relaxation time near 10^{-6} sec was detected with 10^{-3} *M* formic acid. This relaxation time is longer than the one observed in proton transfer reactions because of the contribution of the time of diffusion between the two partners in such a rather dilute system.

2.4. Segmental Motion of Macromolecules in Solution

Segmental motion means the local motion of a small group of adjacent atoms belonging to a much larger part of the macromolecule with an essentially rigid structure. This study started only recently thanks to the development of NMR.

Proton-decoupled, partially relaxed, Fourier transform NMR of carbon 13 in natural abundance has been used to measure the spin lattice relaxation time (T_1) of the individual carbon in solutions of complex molecules and polymers. From these experimental values for T_1 the correlation time for the various groups to which the atoms belong was calculated. The correlation time of methyl groups was thus estimated to be $\simeq 5 \times 10^{-12}$ sec,[22] that of the ring backbone of complex molecules (e.g., sucrose) to be 10^{-10} sec,[21] and the C_α nuclei of the central residues of glycine pentapeptides to be $\simeq 10^{-10}$ sec.[23] For polystyrene with a 10^4 molecular weight, the segmental motion correlation time is estimated to be around 6×10^{-10} sec,[24] while the correlation time for the internal rotation of the bulky phenyl side-chains is much longer because it is subjected to considerable steric hindrance.

Of even greater interest is the study of the rotational correlation times of the backbone and side chain carbons of poly (γ-benzyl-l-glutamate) in organic solvents.[25] The correlation time of the aliphatic side chains becomes progressively shorter as the distance from the backbone increases; for the internal rotation of the $C_\alpha - C_\beta$ bond it has a value of 2.5×10^{-10} sec. The correlation time is around 10^{-9} sec for the α-carbons of the backbone in the random-coil conformation, while it is essentially the same as the overall rotation time of the macromolecule in the helical conformation.

This means that no local segmental motion can be detected in helical polymers of molecular weight $\geqslant$ 7,000.[25]

2.5. Helix-Coil Transition Rates in Polypeptides

Although true α-helixes and true random coils seldom occur in enzymes, it is useful to consider these structures as limiting models particularly in the context of the helix-coil transition. In this cooperative process, the interaction of any helix unit (3.7 residues) following another helix unit is larger than that of any helix unit following a coil unit. Therefore a spectrum of relaxation times is to be expected because of the large number of possible states of the polymer chain involved in the transition, and what is actually measured is the mean of these transition times. The long lasting controversy over "fast" and "slow" helix-coil transition rates in polypeptides was finally settled on a theoretical[26] and experimental basis[27,28] in favor of the "fast" transition. Without entering into the details of this discussion, we merely quote as an example the relaxation time of 5×10^{-8} sec for poly-γ-benzyl-l-aspartate[27] and of 1×10^{-8} sec for poly-γ-benzyl-l-glutamate[29] in organic solvents which were measured by dielectric dispersion. From ultrasonic measurements on Poly-L-ornithine in water-methanol solutions, the relaxation time associated with the helix-coil transition was estimated to be 1.7×10^{-8} sec, while the rate constant for the formation of a helical segment at the end of a helical section was evaluated to be 7.4×10^{-10} sec.[30]

2.6. Hydrated Protein Powders

Some interesting events have been detected by NMR and dielectric measurements in protein powders during the hydration process. The NMR results have been recently and extensively reviewed,[31] therefore only a brief summary will be reported here. The dielectric data have not yet been critically analyzed and will therefore be considered in some detail.

The NMR technique is unable to distinguish between the different types of relaxations involving water molecules adsorbed on the protein surface. It can be shown[31] that even a small amount of nonmobile molecules can have a strong effect on the measured relaxation time. Unless an "a priori" distribution of correlation times for the adsorbed molecules is assumed, the observed relaxation time cannot offer any detailed information about the quantities of microscopic interest. Moreover, the theory usually applied in the treatment of data is valid only if an isotropic distribution of perturbations is assumed; this assumption may not be valid in an heterogenous system, such as the surface of a macromolecule.

Concerning dielectric data, a clear distinction must be made between the low ($\leqslant$ 10 MHz) and high ($\geqslant$ 10 MHz) frequency measurements. Starting with the low frequency measurements, a peculiar feature, displayed by all globular and fibrous proteins studied so far, is the sharp increase of the low frequency dielectric constant at a critical value of hydration h_c, where also a characteristic relaxation spectrum appears.[32-34] For instance, in hemoglobin[35] at constant temperature, this relaxation time decreases exponentially with increasing hydration below h_c, and retains an almost constant value of $\simeq 10^{-6}$ sec for $h > h_c$. Several explanations have been proposed for this strange and dramatic behavior, i.e., a Maxwell-Wagner type mechanism at $h < h_c$, the tumbling of the macromolecule,[35] or the orientation of the adsorbed water molecules[32] at $h > h_c$. Since the above explanations are not compatible with other processes which occur at the hydration layer (see Reference 30, p. 307), we analyzed these dielectric data in considerable detail and reached the conclusion that the sharp change observed at h_c must be due to an artifact. Full details are given below in Appendix 6.1 where the main phenomenon is attributed to the previously observed[36] exponential increase of the DC conductivity when h increases in a sample contained between two polarizable electrodes. By help of a simple model it can be shown that all the observations made at h_c can be reasonably accounted for.

The high frequency measurements are free from criticism and offer interesting results in several globular proteins.[37-39] In lysozyme,[37] two different dielectric relaxations with different time constants occur simultaneously in the whole range of hydration investigated up to 54% H_2O in weight. These two effects are attributed[37] to the relaxation of essentially two layers of adsorbed water molecules. The first layer is characterized by a process exhibiting one single relaxation time near 10^{-9} sec, with a negative activation enthalpy and entropy, and has been interpreted[37] in terms of one water molecule breaking its bonds with the protein, making new bonds with its neighbors, and thus giving off heat. This molecule, by reducing

the distortion in the bonds with the neighbors, fits better into the local hydrogen bonded network which becomes more ordered, so that the entropy of the group of molecules in the network is reduced. The second layer is characterized by a distribution of relaxation times centered around 2×10^{-11} sec; the activation parameters for this process have not been measured. Since the amount of the two types of water is difficult to assess, the correct interpretation of NMR is indeed difficult as previously mentioned.

Finally, we quote an interesting but isolated result[40] obtained by a nonconventional technique which suggests that the relaxation time on the surface of the hydrated protein is shorter than in the dry protein, i.e., that hydration increases surface motility. By the use of γ-ray directional correlation studies of ^{133}Ba ions attached to a carbonic anydrase powder, the perturbing effect of the motion of the water bound to the protein surface was detected as a lowering of the correlation time to about 10^{-7} sec at 20% hydration. This was interpreted as an interaction with the ordered bound water layer, since in ordinary water, which has a correlation time of about 10^{-11} sec, the time-dependent interaction should not be felt by the radioactive probe.

3. GLOBULAR PROTEINS IN SOLUTION

3.1. Intramolecular Modes

Raman spectroscopy is a familiar technique to detect high frequency modes in a medium, whenever modes can couple with and thus modulate an incident light beam. More exactly, it can be shown[40] that the light beam interacts with the oscillator fluctuations of the medium, and that the Raman intensities can be directly calculated by applying the fluctuation-dissipation theorem according to the response function theory. In other words, the Raman scattering spectrum can be considered as the spectrum of the spontaneous fluctuations of the medium at optical frequencies which must be determined by measurement performed on the associate radiation field.

The existence of high frequency (above 500 cm^{-1}) intramolecular modes in proteins, detectable by Raman spectroscopy, is well known and will not be reviewed. The relevant point to be emphasized here is that the study of these modes in proteins does not reveal any feature which cannot be attributed to molecular vibrations of the individual amino acid residues or to some backbone modes typical of the polypeptide chains. Notice, however, that some of these modes are so sensitive to changes of the protein structure that it should be possible to use them in the future as probes for structural changes occurring in a time range lower than but near to optical frequencies.

The Raman laser spectrum of several enzymes has been recently investigated in detail, however, for the sake of briefness, only the studies on lysozyme and bovine serum albumin will be reviewed here. Most of the observed bands have been assigned in terms of contributions from the individual amino acids residues and from the skeletal or backbone modes typical of the various polypeptide conformation (e.g., the amide modes).[42,43] Small differences in the band intensity or frequency have sometimes been observed between crystals and solutions,[44] but the interpretation of these fine spectral details have been criticized on experimental grounds.[45] Therefore it seems impossible at present to attribute any of the few still unassigned bands to a specific high frequency event in globular proteins. On the other side, clear changes have been detected in some bands,[46] i.e., the amide I and III bands or the disulfide stretching vibration of the cystine residues in lysozyme when the protein was thermally denatured; this confirms that these bands are affected by protein conformation. If it had been possible to analyze the shape of these bands by Fourier transform, as can be done in the study of the molecular dynamics of simple liquids,[47] the autocorrelation time of these processes could have been measured up to the optical region! Then, and only then the occurrence of high frequency events, if any, in proteins could have been detected. Needless to say, such a goal is far beyond the possibilities of present techniques.

Finally we note that some conformation-dependent motions in proteins have been observed in several proteins at about 30 cm^{-1} by laser Raman spectroscopy.[48] These motions are thought to involve large portions of the polymer chain (cf. the accordion-like motion of the α-helix) and are therefore rather difficult to assign in structures of low symmetry such as globular protein. Moreover, the absence of this band in carboxy-peptidase suggests that the presence of this band is not necessarily a representative enzyme property.

3.2. Local Conformational Motions

Evidence for the existence of local conformational motions in the frequency range around 10^{-9} sec has been provided by both types of measurements recently carried out in this frequency domain, namely fluorescence decay and ^{13}C NMR. While fluorescence studies have offered general evidence in a large number of enzymes,[13] ^{13}C NMR applications have been more limited but the information thus provided is more specific.

The quenching of tryptophanyl fluorescence by oxygen was studied in a large number of proteins,[49] and found to depend on conformational changes occurring in the nanosecond time scale. The general conclusion is that proteins undergo rapid structural fluctuations which allow the diffusion of oxygen to all the regions of the proteins, even those normally considered inaccessible to solvent as in the case of aldolase. The fluorescence lifetime observed in several enzymes ranges from 2 to 4 nsec when an exciting light of 305 nm is used so as to minimize energy transfer among tryptophanyl residues. In the case of lysozyme, binding of the substrate analog $(N\text{-Ac-GlcN})_2$ reduces the number of effective quenching collisions, but still allows oxygen quenching of the tryptophanyl fluorescence at 24% of the level expected for a diffusion controlled process; this indicates that the fast conformational motility of lysozyme is somewhat limited but not abolished by the binding of the substrate analog. Denaturation by *6M* guanidinium chloride increases the number of effective quenching collisions because it exposes the whole of the protein to oxygen. The above results are in qualitative agreement with the more limited results obtained previously[50] with a bovine serum-albumin-dye complex, where a temperature-dependent relaxation with a time constant in the nanosecond range was observed in the time-resolved fluorescence spectrum. Similar results have also been obtained with chicken pepsinogen[51] where the decay kinetics of the tryptophanyl fluorescence indicated the occurrence of a fast conformational relaxation.

Concerning the ^{13}C NMR, the results obtained[51] with ribonuclease A will be reported first because in this study, the spin-lattice relaxation times T_1 recorded under various conditions are carefully analyzed and the rotational correlation time is consistently discussed. Partially relaxed spectra were used to obtain T_1 values for various classes of carbon atoms present in the protein molecule, and these values were studied as a function of pH both in ribonuclease A and in its oxidized derivatives. Applying the Solomon treatment (which is valid in the case of isotropic motion only), pairs of values of the effective rotational correlation time τ_{eff} were derived from the T_1 values of certain aliphatic carbon resonances; in several cases, one of the τ_{eff} values of each pair could be excluded because they were inconsistent with fluorescence depolarization data. Considering that ^{13}C relaxation is dominated by the local ^{13}C-^{1}H dipole-dipole interaction, the above analysis, suggests the following important conclusions:

1. The α-carbon nuclei of the backbone in the denatured protein display τ_{eff} values between 0.5 and 1 nsec which decrease in a steady way as denaturation progresses. Somewhat larger values are found in the native protein, where, however, the above detailed analysis was not feasible.
2. The τ_{eff} values for the other carbon nuclei are in the range 0.1 to 0.3 nsec, and are hardly altered by denaturation. This provided direct evidence for the greater motility of the side chains.

From the above information taken in conjunction with what was outlined in Section 2.4 on the segmental motion of macromolecules, the following picture emerges. In the nanosecond time range, the protein backbone displays a local motility which, at least in studied cases, decreases upon substrate binding and increases upon breaking of the tertiary structure. Nonbound side chain groups move with a time constant which is about one order of magnitude lower than that of the backbone and is independent of the denatured or native state of the protein. This is in agreement with the qualitative conclusions of hydrogen exchange studies[53] which suggest the occurrence of appreciable segmental motions having a relatively low activation energy; such motions would expose all exchangeable hydrogen atoms to the solvent, and should not be confused with reversible thermal unfolding which proceeds at a much slower rate. The importance of this local fast motility will become apparent later on in this article.

3.3. Proton Transfer Reactions

Various chemical processes have been proposed

to explain the early ultrasonic absorption properties first observed in protein solutions. Subsequent work over a wide pH range and a correlation with the study of model systems reported in Section 2.2. lead to the conclusion that proton transfer reaction is most likely responsible for the observed absorptions at least in the pH ranges below 4.0 and above 10.0.

The case of bovine serum albumin, which is so far the protein best studied from this point of view, should be considered. The frequency absorption dispersion at pH 3.5 can be fitted by a single relaxation frequency f_0 = 2.2 MHz, while at pH 7 the absorption in the range 1 to 100 MHz presents a broad spectrum of relaxation processes.[54] The relaxation observed in the acidic pH region has been shown to be certainly due to proton transfer to and from carboxylate groups

$$-COOH \rightleftharpoons -COO^- + H^+$$

and to be insensitive to protein conformation.[55] The strong ultrasonic absorption displayed at alkaline pH values has been attributed to proton transfer from water to amino groups.[55]

$$-NH_2 + H_2O \rightleftharpoons -NH_3^+ + OH^-.$$

A theoretical analysis[56] in terms of the known pK values of the individual side chains accounts in a qualitative way for the experimental absorption data in the acidic pH range but fails in the alkaline range. Perhaps, some coupling among individual reaction sites occurs (as suggested in the case of hemoglobin[57]) or the environment affects the pK of the individual groups.

The ultrasonic absorption in the intermediate pH region is certainly more complex and may consist of several processes superimposed on the proton transfer to buried groups. As a matter of fact, in this pH region, ultrasound absorption is sensitive to changes in the protein conformation.[55] The occurrence of internal proton transfer with a relaxation frequency of 20 ± 2 MHz has been proposed[58] in bovine hemoglobin. The ionization of the buried groups in lysozyme in the microsecond region has been detected by the temperature jump method.[59]

The importance of ionization processes occurring mainly on the protein surface will be discussed in Section 5.

3.4. Rapid Conformational Isomerizations

Fast conformational isomerizations in globular proteins have been widely investigated since the introduction of suitable rapid reactions techniques. The results obtained with more than 10 enzymes and other proteins have been recently reviewed.[60] Therefore, it will be enough here to recall that discrete kinetic steps referable to conformational transitions are generally observed in globular proteins in solution[60] with time constants ranging between 10^{-2} to 10^{-4} sec. It must be remarked that most of these processes were detected by the T-jump method, and that the introduction of other techniques may show the occurrence of other conformational changes characterized by different time constants. Therefore, a complete evaluation of the time distribution of conformational isomerizations coupled or not to other processes such as ligand binding and/or protomer association is premature. The complexity of the problem is illustrated by the representative case of hemoglobin.

The investigation of the hemoglobin-oxygen binding kinetics by the temperature-jump method[61] reveals the presence of three distinct relaxations with different time constants. The slowest effect ($\tau_1 \simeq 10^{-1}$ sec) is attributed to the dimer-tetramer equilibrium, while the other two ($\tau_1 \simeq 10^{-4}$ and $\tau_2 \simeq 10^{-3}$ sec) are related to oxygen binding. Investigations[61] by the electric field jump method revealed a faster event ($\tau_0 \simeq 10^{-7}$ sec), identified as an intramolecular process not related to oxygen binding; this event is thought to involve the cooperative function of hemoglobin because it is not observed with myoglobin. An effect with a time constant of the same order of magnitude has been recently detected by laser photolysis experiments,[62] the latter effect, however, was also observed with myoglobin, and has been attributed to structural changes occurring in the heme pocket upon ligand dissociation. On the other hand, the acoustical relaxation of oxygenated and deoxygenated hemoglobin, which was investigated[63] in the 0.5 to 1,000-MHz range at neutral pH, revealed a broad absorption with a clear cut-off at 10^{-9} sec, and consisted of at least 4 relaxation processes. Notice also that the effects detected by acoustical relaxation,[64] electric field jump,[61] and photolysis[62] show a similar pH dependence around neutral pH reminiscent of the Bohr effect. Finally, the dissociation rate of hemoglobin in subunits was recently measured[65]

by laser light intensity fluctuations, and was found to be around 10 sec^{-1}.

It is quite clear from the above that different external perturbations can affect different processes even in the same frequency range and that many complementary techniques are needed to yield an ultimate description of the complex series of conformational transitions which occur in a globular protein.

3.5. Reversible Unfolding

Folding–unfolding transitions in proteins are generally much slower than the enzyme turnover, and are therefore thought not to be relevant in enzymatic activity. Actually, unfolding in a highly cooperative system requires a nucleation process which can be very fast. Different experimental techniques can measure different time constants depending upon whether they detect the nucleation process and/or some intermediate steps, or the overall transition only.

For instance, the transition I of some pancreatic proteins has been studied by switching a thermostated microcell between two water baths within a few seconds.[66,67] The time-dependent change of the optical properties then yields a time constant which varies with the temperature in the range of 4 to 400 sec. Such a long time is in good agreement with the denaturation rate of native lysozyme inferred from the kinetics of the hydrogen-deuterium exchange, a process slow enough to be measured by the time-dependent absorption changes of the IR amide bands.[68] A study of the rate of tritium exchange in hemoglobin confirms the conclusion that reversible unfolding is a slow process, and the term "protein breathing" has been proposed to describe it.[69] The thermal denaturation of ribonuclease A, when described only in terms of "native" and "denatured" states, was also believed to be a slow process with a time constant of 2 to 200 sec; however, a more recent study with the temperature jump technique revealed the occurrence of a fast reaction of small amplitude in the millisecond range.[70] This behavior has been analyzed and fully accounted for by a sequential model involving a nucleation-dependent folding in a highly cooperative structure.[71] The occurrence of several steps with time constants in the millisecond range is confirmed by the temperature jump study of lysozyme unfolding monitored through the absorbance changes of a pH indicator.[59] One may wonder if a study carried out with a higher time resolution and by more sophisticated probes would not have revealed the occurrence of an initial phase of unfolding characterized by an even shorter time constant! For the time being we must confine ourselves with the statement that nucleation probably occurs in the time range of isomeric transitions (i.e., in the time range of the enzyme turnover), while the complete folding-unfolding process is much slower.

3.6. Dielectric Processes in the Hydration Layer

The complexity of the phenomena which occurs in the hydration layer and the lack of several adequate complementary techniques for the study of such phenomena prevent the identification (in protein powders) of events analogous to those which have been clearly detected by dielectric measurements on proteins in solution and which cannot be due to the tumbling of the macromolecule.

A relaxation effect at a frequency around 300 MHz has been noticed in egg albumin[72] and bovine serum albumin,[72,73] and an effect in the 500 to 1,000 MHz region has been detected in hemoglobin.[74] A possible explanation of these relaxations involves the relaxation of bound water in qualitative agreement with the results obtained in hydrated protein powders.[37-39] However, the possibility exists that these relaxations originate from the displacement of protons which are fluctuating among the different sites, because of the Kirkwood-Schumaker effect.[75] Detailed analysis[76] shows that this proton fluctuation can be detected by an independent dielectric relaxation time only if its time constant is higher than the rotational tumbling time of the protein. Moreover, the total number n of protons bound to the protein also fluctuates; it is important to realize that, because of the large number of ionizable side chains, the value of the mean fluctuation $\sqrt{\Delta\bar{n}^2}$ is appreciable. The mean fluctuation can be measured from the slope of the titration curve or from light scattering; in the case of bovine serum albumin at the isoionic point, its value is 3.6.[73] For a single ionizable group with a pK = 7, the autocorrelation time of the process $A^- + H^+ \rightarrow AH$ is about 10^{-3} sec when the inverse process $A^- + H^+ \leftarrow AH$ is diffusion controlled ($K_{ass} \simeq 10^{10}$ M^{-1} sec^{-1}). The autocorrelation time of the mean fluctuation $\sqrt{\Delta\bar{n}^2}$ has been estimated to be between 10^{-6} to 10^{-10} sec.[76]

It is most unfortunate that the above events cannot be described in detailed molecular terms at the present time; however, their occurrence in the hydration layer of globular protein is well established and their relevance will be discussed in Section 5.2.

4. ENZYME-SUBSTRATE COMPLEXES

4.1. Chemical Aspects

The events described in the previous section occur, with varying features, in all globular proteins, including enzymes in the resting state, i.e., in the absence of substrates. Events occurring in enzymes in the presence of their substrates should now be considered.

Detailed kinetic analysis and experiments carried out by rapid methods at high enzyme concentration in particular, have shown that enzymic reactions proceed through the sequential formation of several enzyme-substrate intermediates. Many of these intermediates have been identified as discrete chemical species which involve the formation of covalent links between the enzyme and the substrate. The lifetime of such intermediates generally ranges between 10^{-4} and 10^{-2} sec.[16,77,78]

The chemical nature of the enzyme-substrate intermediate complexes and the rate constants for their formation and transformation in different classes of enzymes has been the object of several excellent reviews.[16,77-81] As a representative detailed study we quote the beef heart lactate dehydrogenase system,[82] which includes a formal analysis of the Gibbs energy values.

The chemical aspects of the transformations occurring at the active site of enzymes will not be dealt with in this article. We shall instead focus our attention on the various types of statistical events reviewed in the previous chapters.

4.2. Covalent Transitions and the Lifetime of the Transition State

As stated above, specific covalent transitions occurring at the active site during the catalytic cycle will not be discussed here. In the context of the present review it is important to point out that although chemical events which modify the covalent structure of the enzyme-bound substrates occur with apparent first order constants of 10^2 - 10^4 sec^{-1}, it has been estimated that at least in the case of a key step in ribonuclease action, the formation and breakdown of the postulated pentacoordinate intermediate must have a rate constant greater than 10^{10} sec^{-1}.[16] Such a rare event obviously escapes all present detection techniques. In principle, however, it could eventually be studied by Raman spectroscopy, as shown in the discussion of intramolecular modes reported in Section 3.1.

4.3. The Binding Rate

The binding of a substrate to an enzyme can be considered a second order reaction, beginning with the diffusional encounter of the two partners.

Since at concentrations of $\simeq 10^{-4}M$ enzyme and 10^{-4} to 10^{-3} M substrate the reaction half-time was typically in the range 10 to 100 μsec,[77,83] the second order rates involved, approached the maximum possible value of $10^9 M^{-1}$ sec^{-1} expected for a diffusion-controlled reaction.[83] This last figure is evaluated by the well-known Smoluchowsky expression.

$$K = 4\pi N (D_A + D_B) R_{AB}, \quad (1)$$

where

D_A and D_B are the diffusion coefficients of the two species,

R_{AB} the distance between the active sites in the encounter complex and N is the number density.

The Smoluchowsky expression is derived assuming that all encounters produce a productive binding.

The validity of the Smoluchowsky expression for the encounter between a protein and a small molecule can be tested by measuring the protein fluorescence decay due to a quenching by oxygen, assuming the validity of the Stern-Volmer expression. As previously pointed out in Section 3.2, this expression certainly holds for the quenching of the tryptophanyl groups in some proteins by oxygen in the nanosecond time region.[49] It should be noticed, however, that oxygen is quite a small molecule and could easily reach the internal tryptophanyl groups during the fast conformational changes of the protein which govern the quenching process, and which just happen to occur in the same time range as that predicted by the Smoluchowsky expression for the oxygen concentrations used in the experiments (about 0.1 M). Had a larger molecule than oxygen been used, or had the tryptophanyl residues been more deeply

buried or had the protein molecule fluctuated less rapidly, then appreciable deviations from the Smoluchowsky limiting value would have been observed. As a matter of fact, the recent data on the oxygen quenching of the phosphorescence of liver alcohol dehydrogenase and of alkaline phosphatase[84] suggest that globular proteins differ markedly in the rate at which they undergo conformational fluctuations capable of exposing buried residues. These results will be discussed in more detail in Section 4.7.

The association rate constants for real substrates are reported to be in the range 10^7 to 10^8 M^{-1} sec^{-1}, which is somewhat less than the value expected for diffusion controlled processes.[60, 77,83] The molecular fit between the enzyme and substrate certainly affects the binding rate; a comparison of the apparent second order rate constants for the formation of the first detectable enzyme-substrate intermediate between aspartate-amino-transferase and a number of substrates and analogues shows that small differences in the geometry, size, and charge distribution of the substrate molecule greatly affect the rates of the interaction even when the equilibrium constant is not markedly different.

The observed "less than diffusion controlled" rates for the binding of substrates to enzymes can reasonably be interpreted assuming that the measured values reflect at least two successive events: 1. bimolecular diffusion, and 2. one (or more) unimolecular conformational change of the protein and/or of the protein-bound substrate, a conformational change which is characterized by its own time constant.

Of course, events other than a conformational change can produce an apparent rate constant slower than diffusion-controlled for the binding of ligands or substrate to macromolecules. One possibility is that some collisions are unproductive because the appropriate binding groups are not correctly oriented relative to each other. Another possibility is that, under the conditions of the experiment, a fraction of the enzyme is in an ionic state unfavorable to binding, so that the effective concentration of reacting molecules is less than calculated. A study of the pH dependence of the observed rate can help in identifying such cases. Finally, the possibility of side reactions and nonproductive binding may also cloud the interpretation of the results. Moreover, it is generally difficult to demonstrate that a slower, first order isomerization is on the reaction path and not a side reaction.

The interpretation which postulates the occurrence of unimolecular conformational changes following substrate binding is supported by the well-known results obtained in ribonuclease.[85] A kinetic study of the interaction of this enzyme and cytidine 3′-phosphate as a function of pH by the temperature jump method showed the occurrence of three distinct relaxation processes related to the binding. One of the relaxation processes could be associated with the initial complex formation between cytidine 3′-phosphate and ribonuclease. The second order rate constant for this association reaction was found to be very large ($k_{on} \simeq 5 \times 10^8 \div 5 \times 10^9$ M^{-1} sec^{-1}). The other two relaxations (time constants ranging between 10^{-2} and 10^{-4} sec) were associated with isomerizations of the complex.

It is plausible that in many other experiments carried out until now, because of the experimental conditions and the instrumental time resolution, it was not possible to distinguish the two processes. Upper limits can be calculated, however, on the basis of the above assumption for the rate constants of the postulated unimolecular conformational changes related with the formation of the enzyme-substrate complex; such values generally range between 10^2 and 10^4 sec^{-1}. An interesting example of a clear-cut, substrate-induced conformational change has been reported[85] in the case of chymotrypsin. Here the occurrence of a conformational change affecting the functionally important formation of a salt bridge at residue ile-16 is supported by X-ray diffraction and chemical data. The process is cooperative, pH dependent, and somewhat slower than those described above, its time constant being about 1 sec.

We note in passing that the occurrence of an unimolecular process coupled with the binding process does not imply that the enzyme–substrate reaction is not "diffusion controlled". As a matter of fact, according to the original and correct definition, the overall reaction can be defined as "diffusion controlled" if the reactive association proceeds fast with respect to the diffusional separation of the product.[86] Moreover, a more general treatment for the kinetics of the diffusion controlled process in liquids under conditions where the Smoluchowsky conditions are not valid is available.[87]

4.4. Isomerization Transitions

These transitions are presumably analogous to the isomerization transitions considered in section 3.4. The rate of conformational changes in enzyme-substrate complexes has been measured in several systems by techniques for fast reactions and has been well reviewed.[83] The time constants range between 10^{-2} and 10^{-4} sec.

In the past years, with the improvement of techniques, faster isomerization has been detected in hemoglobin.[61,62]

In many cases, the occurrence of conformational isomerizations in enzyme–substrate complexes was postulated in order to explain relaxation effects detected in fast kinetic analysis; the exact nature of these conformational events has rarely been defined, although attempts in this direction have been made, making use of all available chemical and crystallographic evidence.

4.5. Proton Transfer Coupled with Conformational Transitions and Ionization Reactions

The study of the pH dependence of the relaxation times of conformational changes in enzymes and in enzyme–substrate complexes led to the identification of dissociable groups which control the conformational transitions.

A classical example is the previously quoted study of ribonuclease.[85] In the absence of substrates, ribonuclease isomerizes with a single relaxation time ($\tau \simeq 0.1$ msec) which depends on a single ionizable group of pK = 6.1 assigned to histidine-112, an important group at the active site of the enzyme. As previously mentioned, in the presence of the quasi-substrate cytidine-3′-phosphate, three distinct relaxation processes were observed. The characteristic relaxation times of these processes were studied as a function of pH, temperature, and concentrations. A quantitative correlation of all of the available data concerning this interaction can be made if the above mentioned pH-dependent isomerization of the enzyme is taken into account. It should be pointed out that the values for the standard activation enthalpy for the interchange of the two ribonuclease forms are surprisingly small (5 to 7 and 2.8 Kcal/mol). What is particularly relevant to this article is the finding that rapid isomerizations, coupled with the dissociation of groups at the active site, occur in this enzyme-substrate system.

The relaxation spectra of complexes of human carbonic anhydrase with aromatic sulfonamides was investigated by temperature jump and by laser-photolysis techniques.[88] A fast conformational effect occurs in the 200-μsec region. Two faster relaxation effects are seen in the 0.1- to 10-μsec range in photolysis experiments. The effect of substitution of the active site Zn^{2+} with various metal ions (Co^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}) on the slowest effect was systematically investigated. The time constant for the effect correlates with the catalytic activity of the variously substituted enzyme derivatives. This suggests that the greater catalytic activity of the Zn^{2+} and Co^{2+} enzymes is connected with their capacity to undergo fast changes in their coordination pattern determined by small conformational transitions of the protein. The spectral changes related with the slowest relaxation effect could be studied in some detail on the Co^{2+} enzymes. These changes suggest that the observed effect reflects a transition between distorted and undistorted tetrahedral structures. The faster relaxation effects are strongly influenced by the nature and concentration of the buffer in the medium and can be tentatively ascribed to the protonation of the essential group at the active site having a pK of ≃,7.

Another interesting case is presented by aspartate-aminotransferase,[89] at least two dissociable groups are known to be present at the active site of this enzyme, one contributed by the vitamin B_6 chromophore, the other by the protein.[90] Both groups, when bearing a positive charge, interact with anions present in the medium. The kinetics of the interaction between supernatant aspartate-aminotransferase (aldimine form) and Na or K-cacodylate, phosphate, and chloride were studied by the temperature jump technique, monitoring optical density changes at wavelengths corresponding to the B_6 chromophore absorption bands. Under all conditions, a single relaxation effect referable to a direct interaction between the anion and the enzyme was observed; relaxation times were less than 10 μsec with phosphate, 20 to 100 μsec with cacodylate, and 1 to 2 msec with chloride. The best fit of experimental data was obtained with models where the acidic component of the buffer directly donates a proton to the enzyme chromophore and the anionic component of the buffer binds to a positively charged group near to, but distinct from, the chromophore. The conformational transitions connected with the protonation of the protein dissociable group was studied under condi-

tions where the spectral changes of the B_6 chromophore is relatively slow (1 msec) so that it does not interfere with faster relaxations. The observed relaxation effects can be interpreted assuming a fast conformational transition coupled with a highly cooperative uptake of two protons by the protein. The pK of this protonation is 6.26 ± 0.05, which coincides with that observed in NMR experiments for the binding site of anions and is attributed to the essential histidyl residue. A conformational relaxation effect coupled with proton uptake is visible also in the presence of the substrate l-aspartate, but it disappears in the presence of an excess of the specific inhibitor succinate. It is worthwhile to note that in this system we also observe rapid conformational fluctuations coupled to the protonation of groups at the active site and, through them, to the interaction with ions from the medium.

The liberation of protons during turnover of liver alcohol dehydrogenase was recently studied using transient and inhibition kinetics.[91] Proton release occurred prior to, and uncoupled from, the catalytic hydrogen transfer step. The addition of saturating concentrations of NAD^+ to the enzyme resulted in a pH-dependent release of protons with 9.5 proton equivalents liberated at pH 7.6. Formation of a ternary complex of enzyme, NAD^+ and the substrate analog trifluoroethanol, resulted in liberation of 1 proton/equivalent of enzyme in the pH range 5.5 to 8.5. Trifluoroethanol binding to binary complex was dependent on an enzyme functional group with a pK of 7.6 with weak binding to the unprotonated form. The rate constants for proton release in burst reactions and from formation of ternary enzyme-NAD^+ trifluoroethanol complex (250 to 280 sec^{-1}) were compatible with a functional group with a pK of 7.6. The data have been interpreted assuming that protons are released from the enzyme during turnover as a result of the perturbation of the pK of a functional group on the enzyme from 9.6 to 7.6 and that the substrate alcoholic hydroxyl group binds directly to the basic form of the perturbed functional group. This scheme accounts for the required stoichiometry of proton liberation and is compatible with a concerted hydride transfer mechanism of catalysis and with the crystallographic evidence for a different conformation of the enzyme in the binary and ternary complexes.[92] It is important to note that also in this enzyme, rapid conformational changes coupled with the perturbation of the pK of a group at the active site occur during the turnover.

4.6. Local Conformational Motions

The fact that most of the inhibitors show a rotational correlation time, as measured by fluorescence polarization, which is close to the rotational tumbling time of the protein in solution has been said to be a good evidence that the binding at the active site is rigid. However, NMR studies, which are able to distinguish between the correlation times of the different groups of the inhibitor, can detect some groups which have a random motion faster than that of the protein. For instance, in the case of sulfonamide inhibitors bound to bovine carbonic anhydrase, the methyl groups protons were found to have a faster motion than the aromatic ring protons which display the same tumbling correlation time as the whole protein.[93] For this reason this technique seems particularly suited to the detection of the small but significant differences between the local motions in enzyme-bound inhibitors and active substrates; the clear cut example provided by pyruvate kinase[94] is discussed below.

A powerful method to study the substrate motion at the active site is to use NMR on enzymes having a transition metal ion present at the active site. This is the case of pyruvate kinase, where one Mn^{2+} ion is located at the active site and has a direct role in catalytic events. When Mn-^{31}P interactions were studied in some inactive ternary complexes containing analogs of phosphoenolpyruvate, the correlation time for phosphorus was indistinguishable from the rotation time calculated for the entire enzyme molecule (= 5×10^{-8} sec). This fact was believed to be a convincing example of immobilization or "freezing" at the reaction center which would permit subtle orientational effects to operate in enzyme catalysis. However, when the active quaternary pyruvate kinase -Mn^{2+}-*o*-phosphate-pyruvate complex was studied, a correlation time for phosphorous about one order of magnitude higher was found. This is because the geometry achieved at the active site, in the case of the active complex, allows more freedom to phosphorous. This example confirms that caution must be used before data obtained with inactive analogs can be extrapolated to the true substrate.

A recent comparative study of NMR and EPR data suggests that local conformational motions

might involve the coordination of Mn at the active site of pyruvate kinase.

^{31}P-NMR studies at one frequency of the ternary complex of muscle pyruvate kinase, Mn, and phosphoglycolate, a competitive analog of the substrate, give a Mn to P distance of 3.5 ± 0.3 Å, indicative of a distorted inner sphere complex. However, the Mn^{2+} EPR spectrum of the same system shows the presence of two separate species. The NMR and EPR data could be explained assuming that the NMR data represent the time average of two complexes (32% inner sphere (2.9 Å) and 68% second sphere (6.1 Å) and that the exchange between the two complexes occurs with a rate constant intermediate between the inherent frequency of NMR (5×10^6 sec^{-1}) and EPR (2×10^{10} sec^{-1}) measurements. The ternary complex with the real phosphoenol-pyruvate gives an EPR spectra very similar to those just discussed for the comples with the analog. The addition of the activator K^+ which seems to form a metal bridge between the enzyme and the carboxyl group of the substrate phosphoenol-pyruvate causes a re-orientation of the inner sphere complex. The formation of the E-Mn-phosphoryl bridge complexes could be catalytically relevant, being consistent with an associative or nucleophylic displacement on the phosphorous of phosphoenol-pyruvate by ADP.

4.7. Unfolding Transitions

The occurrence of conformational fluctuations has been detected in enzymes and enzyme-substrate complexes also by methods intrinsically slower than temperature jump or rapid mixing kinetics. Of considerable interest, because of its potential developments, is the study of phosphorescence. While the phosphorescence of aromatic chromophores in solution is normally quenched through diffusion of dissolved oxygen and other solvent-mediated processes, the phosphorescence of some proteins in solution is observed at room temperature. Tryptophan phosphorescence arises from residues which are hindered from interaction with oxygen by the folding of the polypeptide chain.

Measurements of the phosphorescence lifetime of horse liver alcohol dehydrogenase as a function of oxygen concentration indicate that internal tryptophan residues are periodically exposed to oxygen.[84] According to the assumed model, at high oxygen concentration the triplet lifetime of the internal residue tends to be a finite value; under these conditions quenching is rate-limited by the structural change which exposes the chromophore to oxygen with a rate near 10 sec^{-1}. The rate constant for the apparent conformational change which buries the same residue is found to be 7.8×10^6 sec^{-1}. This indicates that the equilibrium lies far in the direction of the closed conformation so that the protein exists only a small fraction of the time in the more open structure. It is of interest that isomerization of the enzyme-coenzyme complex has been proposed to occur[95] with a rate constant of 11 sec^{-1}, essentially equal to the rate constant for the "opening" conformational change.

It is worth noting that the previously quoted study of the influence of high concentration of dissolved oxygen on the fluorescence of aromatic residues in several proteins[49] had given values for the quenching constant close to the diffusion-controlled one; furthermore, the fluorescence lifetimes in these experiments tend towards zero. Both results indicate that in the globular proteins thus studied (albumin, lysozyme, ribonuclease) the chromophores were exposed to oxygen. A comparison of the latter series of data on fluorescence quenching[49] and those previously mentioned on the phosphorescence lifetime of alcohol dehydrogenase[84] shows that proteins differ markedly in the rates at which they undergo conformational fluctuations.

4.8. Overlapping Events

The picture emerging from the foregoing short review is the following: the substrate binds to the enzyme successively forming a series of intermediates, many of which differ in their covalent structure, with a lifetime of 10^{-2} to 10^{-4} sec. One intermediate is transformed into the next by going through a relatively rare (10^2 to 10^4 sec^{-1}) but rapid (lifetime 10^{-10} sec) formation and breakdown of a transition state.

It is important to realize that during the relatively long lifetime of each discrete intermediate (10^{-2} to 10^{-4} sec), several more rapid events (conformational oscillations, protonations and proton dissociations, local segmental motions, etc.) occur with time constants ranging from 10^{-5} to 10^{-9} sec^{-1}. The possible role of such rapid, statistical events in enzyme catalysis will be discussed in the next chapters.

5. DISCUSSION

5.1. The Time Scale of the Detected Events

A glance at Tables 1 and 2 shows how large the range of the detected events is, encompassing the region from 10^{-11} to 10^{2} sec. Intramolecular modes have not been included because these events are common to all chemical systems, as was discussed in Section 3.1. Longer times are associated with the cooperative motion of larger parts of the proteins, therefore one may get the impression that things move faster at the surface and move slowly at the core of the macromolecule.

5.2. Phenomenological Discussion

A phenomenological consideration of the data reported in Tables 1 and 2 lead to some rather general conclusions which are reported below.

1. The first point which arises from the comparison of statistical time events detected in proteins and in model systems is that no fast ($\leqslant 10^{-7}$ sec) events in proteins have been detected which cannot be found also in suitable model systems. So far, the time events which are characteristic of proteins are the slow conformational isomerizations (10^{-2} to 10^{-4} sec). Apparently, the complex entity which is the enzyme has been built by evolution so as to make use of different complex molecular structures, each of which retains its characteristic time constants. The inter-

TABLE 1

Time Events in Model Systems*

H-bonded molecules	
Breaking of one H-bond in associated liquids	$10^{-8}-10^{-9}$
Breaking of an H-bonded dimer	$10^{-7}-10^{-9}$
Proton transfer at terminal groups of amino acids	$10^{-8}-10^{-9}$
Proton transfer in zwitterions	$10^{-7}-10^{-8}$
Internal proton transfer in cyclic molecules	10^{-8}
Polymers	
Methyl group rotational correlation	10^{-12}
Backbone local motion	$10^{-9}-10^{-10}$
Tumbling of the entire macromolecule	$10^{-6}-10^{-8}$
Ionic atmosphere relaxation in polyelectrolytes	10^{-8}
Helix-coil transition in ordered polypeptides	$10^{-7}-10^{-8}$

*Representative values in seconds. For further details see text.

TABLE 2

Time Events in Globular Proteins and Enzymes*

Protein surface	
Tightly bound water relaxation	10^{-9}
Loosely bound water relaxation	10^{-11}
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	$10^{+2}-1$

*Representative values in seconds. For further details see text.

esting possibility exists that these events which have a recognized role in catalysis have a molecular origin in common with other events characterized by shorter time constants (e.g., local conformational motions). Since this possibility does not seem to have been reported in the literature, it will be considered in some detail in the following paragraphs.

In our terminology both the transitions between two states in equilibrium (e.g., an isomerization or an ionization reaction) and the small changes affecting only one, single equilibrium position (such as the segmental motion in a polymeric chain, or the vibrational mode of a bond length) are defined as "statistical time events" because they can be identified by a correlation time. The stochastic variables which describe the event by their time-dependent amplitude are in the first case the populations of the two states, while in the second case they are a convenient set of coordinates which identify the atomic positions.

Let us consider, the first case, i.e., a conformational isomerization with an equilibrium constant, K_e, close to unity,

$$A \underset{K_1}{\overset{K_1}{\rightleftharpoons}} B.$$

In this system the relaxation time constant for the concentration changes during the approach to equilibrium or the autocorrelation time of the spontaneous concentration fluctuations can be measured and according to the fluctuation-dissipation theorem these two times coincide (τ). It is well known that the reaction rate constants k_1 or k_{-1} can be derived from τ and K_e.

Let us now consider the second case. According to the transition state theory,

$$k_1 = A_1 \exp(-\Delta G^+/KT)$$

where

ΔG^+, is the standard free energy barrier
T is the absolute temperature,
K is the Boltzmann constant,
and A_1 is a quantity with the dimensions of reciprocal time which should be calculated from a microscopic analysis of the process responsible for the motion of the reaction coordinate.

Let us suppose that this process is so simple that the reaction coordinate can be monitored by a probe (e.g., a chromophoric group) suited to fluctuation spectroscopy. This probe will then reveal the fluctuations around the equilibrium position having a correlation time τ. Assuming that the isomerization reaction considered above results from a great amplitude fluctuation of the reaction coordinate around the initial free energy minimum which is a rare event belonging to the same gaussian distribution as the very frequent small-amplitude fluctuations around the equilibrium position, therefore the two types of measurements mentioned above (the former measures concentration the latter the reaction coordinate) concern two different statistical time events, which, however, have a common origin. To stress this particular point further, a simple model system is quantitatively treated in Appendix 6.2. Notice also that if the isomerization is a more complicated event that the one considered in the above model, i.e., it is the result of several concurrent processes, then the problem of time delayed cross correlations[5] among the fluctuations of individual process must be faced so as to select those correlations which lower the free energy barrier. The isomerization reaction can then be considered as a high amplitude fluctuation in this low free energy curve. Even in this more complicated case the possibility exists of connecting the rare event (the isomerization reaction) to the fluctuations of the reaction coordinates for the various processes involved in the reaction.

2. The second point that comes to our attention when looking at the tables of time events is the occurrence of quite a number of different statistical events having a similar correlation time in the 10^{-7} to 10^{-9} sec range. Such events mostly occur at the surface of the globular protein and are likely to be statistically coupled. By consideration of the pertinent model systems, these events can be assigned to kinetic processes involving counter ions from the medium and water bound to the macromolecule, to the ionization of side chains, to local motions of the protein backbone, and possibly to the breaking of internal hydrogen bonds. Since an important class of fluctuations (i.e., charge fluctuations of the ionic medium)[26] are known to occur with a correlation time of the same order of magnitude in the environment surrounding the protein, we wish to suggest[5] that the macromolecule and its thermal environment are likely to be in "speaking terms" on this

common frequency channel. This would mean that some free energy can be exchanged between the macromolecule and the environment through the coupling of events at the protein surface. A general discussion of this coupling between the macromolecule and the environment would also involve a consideration of the higher frequency channels through which temperature and pressure fluctuations become coupled by a mechanism which is largely unknown, and does not seem appropriate here. We shall, instead, further consider the statistical coupling in the frequency channel centered around 10^{-8} sec since such a discussion can be based on the experimental findings reviewed in the previous sections.

Let us consider the number density N of the ions in the aqueous medium containing the protein. Assuming an activity coefficient of 1, a simple application of statistical theory shows that

$$(\Delta N^2/N^2) = 1/N$$

which, for a dilute solution, becomes quite appreciable. The macromolecule is submitted to an intense bombardment of ions from the medium; ions hit the surface of the protein and diffuse back into the solvent. "Hits" have a Gaussian time distribution centered around the average time t required by an ion to travel over the mean distance between the ions in the medium and the macromolecule. This average time can be estimated by the Smoluchowsky expression to be around 10^{-8} sec for 10^{-1} *M* solutions. This figure provides a good starting point for our analysis. The modulation of these ions random movements by the changing electric field created by the fluctuations of the charges in the medium, should be considered next. It is known that the correlation time of the latter fluctuations is approximately 10^{-8} sec. Finally, it must be pointed out that processes occurring at the macromolecular surface can respond to these local external perturbations only if their dielectric response function has a characteristic time of the same order of magnitude. In the Section 3.6 we have shown that this is indeed the case. It seems, therefore, that good arguments exist suggesting the presence of an efficient frequency channel centered around 10^{-8} sec^{-1} through which electrostatic free energy can be exchanged between the environment and the macromolecule. The possible relevance of this type of free energy exchange for enzyme action can be easily appreciated considering that the active site of the enzyme often contains residues with a pK dependent on the net charge of the macromolecule. This is certainly true in the case of lysozyme, [27] where the ionization of the catalytic residue Asp 52 depends on ionic strength and on the total charge of the protein. It is therefore reasonable to expect that a large fluctuation in the ionic concentration of the medium will affect the active site.

Another efficient way by which free energy could be exchanged through the previously discussed frequency channel is provided by spontaneous fluctuations in proton transfer systems involving the macromolecular side chains described in Section 3.6. The relevance of these fluctuations in catalysis may derive not only from the change in the net charge of the macromolecule discussed above, but also from fluctuations in protein conformation resulting from a complex pattern of interactions among individual charged groups. Proton fluctuations are known[98] to be most ample at pH values coincident with the pK of the conjugate acids involved, however, electrostatic interactions tend to spread these maxima on both sides of the isoionic point, so that proton fluctuations should be relevant over a broad range of pH.

Finally, another source of fluctuations in the same frequency range is the reversible breaking of the hydrogen bonds involving the water molecules bound to the protein surface, and discussed in section 3.6. This process is important because it can induce conformational changes in the polypeptide chain where the water molecules are bound. It is immediately apparent that this process must also be coupled to the above mentioned charge fluctuations because of the large value of the water molecule dipole moment. As a matter of fact, the motion of the surface charges must involve bound water dipoles much in the same way as the mobility of ions in ice is controlled by the cloud of Bijerrum defects.[99] Reciprocally, the spontaneous fluctuations in the hydrogen bond network of bound water must influence the surface charge distribution, the correlation time of these processes being of the same order of magnitude. Actually, the local conformation of the macromolecular surface, the ionic charge distribution, and the bound water structure are in such a close interaction that they cannot be considered as separate processes. In order to carry this discussion further, we should now consider how local confor-

mational motions in the enzyme indicated above can be relevant for catalytic activity. Careri[5] recently proposed that the spontaneous occurrence of time delayed cross correlated local conformational fluctuations can exert a great catalytic effect. This property, resulting from a strategical distribution of various functional groups interlinked by a fluctuating array of hydrogen bonds, would be essential for activity. This hypothesis could be tested experimentally by recording the response (if any) of the groups at the active site to large charge fluctuations induced on the protein surface. Before such an ambitious experiment is performed, it should first be ascertained that a change of the surface charge occurring in 10^{-7} to 10^{-9} sec can affect the conformation of the enzyme active site.

The discussion in this section is somewhat speculative because sufficient experimental data are lacking; however, it was included in this review in order to suggest experiments which would contribute to a better understanding of the dynamic relations between the enzyme and its medium. If we assume that what has been discussed in 1. and 2. of the proceeding listing is real, the following picture of enzyme action emerges : fast (10^{-8} to 10^{-9}) ion and proton concentration fluctuations occurring in the medium are cross correlated with fluctuations at the active site, and when of a sufficiently high amplitude, they would constitute the slow (10^{-2} to 10^{-4}) free energy-rich event essential for catalysis. This dynamic description of enzyme action includes both the concept of the enzyme as an "entropy trap" (because cross correlations lower the entropy term of the free energy of activation) and that of the "floppy" adaptable enzyme.

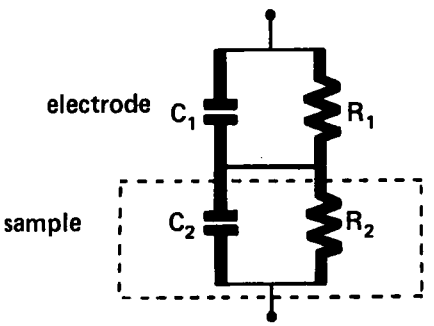
6. APPENDIXES

6.1. Some Remarks about the Dielectric Relaxation of Hydrated Protein Powders

The low frequency dielectric constant of the protein powders is reported in the literature to increase abruptly at a critical value of the hydration. Such a high value of the relaxation time of the bound water is in conflict with the data reported in Table 2. In the following paragraphs it will be shown that these dielectric data are probably affected by an experimental artifact.

Usually the dielectric constant of the protein powders is measured from the capacity of a plane condenser of known geometry filled with the powder. The dielectric constant is given by the familiar expression $\epsilon = Cd/S$, where d and S are the plates distance and surface, and C is the capacity. Two kinds of condensers have been used: (1) one employing conducting electrodes in intimate contact with the powder, and (2) one using electrodes insulated by a thin layer of teflon. Both arrangements display an intrinsic capacity due to the electrodes themselves. In system 1. the metal-protein contact resistence must be taken into account. The equivalent circuit is shown in Figure 1, and expressions for the total capacity as a function of frequency are easily obtained in terms of the sample and electrodes resistivity and capacity.[100]

It is also known[36] that the resistivity of the protein powder decreases exponentially with increasing hydration.[36] Using the above quoted expressions it can be shown that for this very reason the total capacity must increase abruptly at a critical value of the hydration. If the protein powder resistivity R_2 decreases with the hydration



$$C(\omega) = \frac{C_0 + C_\infty \tau_c^2 \omega^2}{1 + \tau_c^2 \omega^2}$$

$$C_0 = \frac{R_1^2 C_1 + R_2^2 C_2}{(R_1 + R_2)^2} \qquad C_\infty = \frac{C_1 C_2}{C_1 + C_2}$$

$$\tau_c = \frac{(C_1 + C_2) R_1 R_2}{R_1 + R_2}$$

FIGURE 1. Equivalent circuit for a condenser filled with a sample of protein powder.

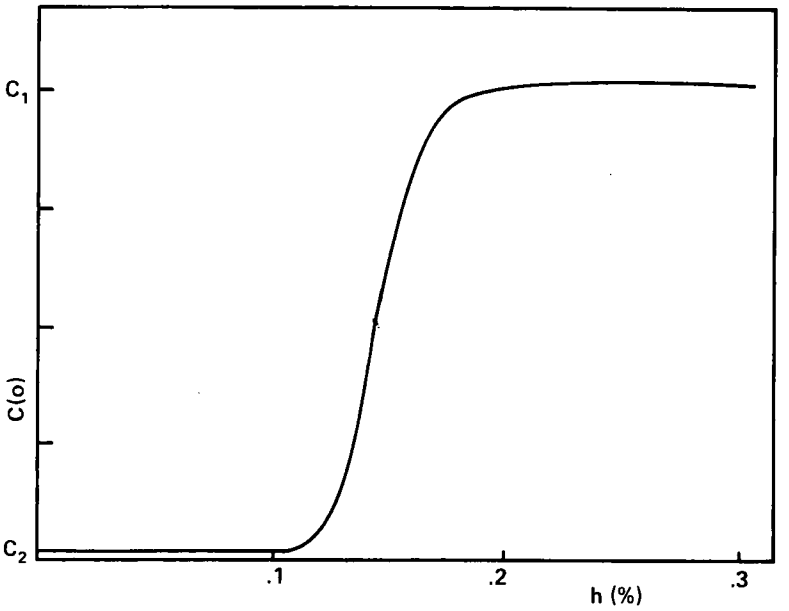


FIGURE 2. Computed DC capacity of the equivalent circuit shown in Figure 1 when the resistance R_2 of the protein powder decreases exponentially with increasing hydration according to Expression 1 (see text).

according to the empirical law for a condenser filled with a pellet a few millimeters high (as deduced from Reference 36), then:

$$R_2 = 10^{15} \times 10^{-40\,h}, \quad (1)$$

where R_2 is measured in ohms and h in (mg H_2O)/mg of protein). As shown in Figure 2, the capacity of the condenser as a function of hydration shows a rapid increase at $h_c \simeq 0.15$ (it was assumed for this computation that $R_1 = 10^9\,\Omega$, C_1 = 100pF, C_2 = 10 pF). In spite of some uncertainty in the empirical law, it is clear that such a break must occur when, because of hydration, sample resistivity reaches the same order of magnitude as electrode surface resistivity.

Alternatively, the relaxation time τ can be plotted against hydration h. Using the same empirical law with this plot, a break point is also obtained as shown in Figure 3. This behavior corresponds to that reported in the literature,[35] which had given the wrong impression of the existence of a critical hydration time, detectable at low frequencies, and tentatively explained as the slow relaxation of different water layers; such a fact, if true, would be of great interest in the context of this review. However, since the resistivity of the metal-protein contact could be responsible for the observed effects, we prefer not to rely upon the published data on the low frequency dielectric properties of hydrated proteins.

6.2. The Dissociation of a Diatomic Molecule in a Thermal Bath

In the following paragraphs it will be shown that two observable quantities belonging to the same model system can approach equilibrium with two different relaxation times as already indicated in Section 5.2.

Let us consider one of the simplest kinetic processes which require an activation energy, namely, the thermal dissociation of a diatomic molecule in an inert gas medium, e.g., bromine in argon. A complete treatment of this process is quite complex because both the rotational and vibrational degrees of freedom are involved,[101] but for the sake of simplicity let us assume that the dissociation takes place only because of high amplitude vibrations. This process can then be described by the truncated harmonic oscillator model[102] with a finite number of equally spaced energy levels such that level N is the last discrete level of the oscillator, the energy $E_{N+1} = h\nu(N + 1) = E_D$ is the dissociation energy and the activation energy for the unimolecular reaction, and ν is the natural frequency of the oscillator. The transition probabilities per collision $P_{n,n+k}$ =

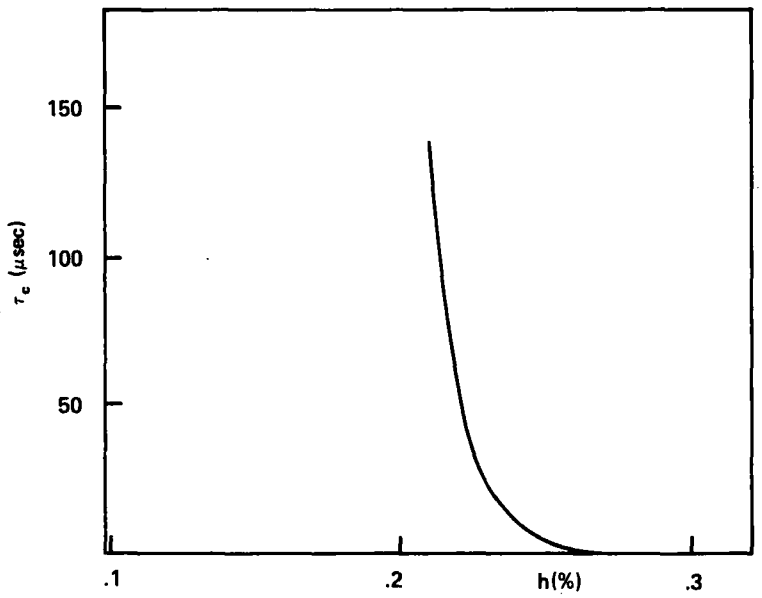


FIGURE 3. Computed relaxation time for the system described in Figure 2.

$P_{n+k,n}$ for the event which brings the oscillator from n to n+k level have been shown by Landau and Teller to be

$$P_{n+1,n} = P_{1,0}\,(n+1)$$

$$P_{n-1,n} = P_{1,0}\,n$$

where $P_{1,0}$ depends upon the interaction of the oscillator with the medium, and use has been made of the selection rule $P_{n\pm k,n} = 0$ for $k \neq 1$. The transition probabilities per collisions $P_{n+1,n}$ are related to the transition probabilities per unit time $W_{n+1,n}$ (which are the measured quantities) by

$$W_{n+1,n} = Z^* N^* e^{\theta} P_{n,n+1}$$

$$W_{n,n+1} = Z^* N^* P_{n+1,n}$$

so that $W_{n,n+1,n} = \exp\theta$, where $\theta = h\nu/kT$, as required by the principle of detailed balancing. Here Z^* is the number of collisions suffered by the oscillator per unit time when the gas density is one molecule per unit volume, and N^* is the total concentration of heat bath molecules.

With help of this model we can quantitatively evaluate two detectable "events" with two different time constants, one being the rate of creation of free atoms and the other the rate of change in the population of the lowest energy level, both events being due to the same physical process, namely, the transitions among the energy levels which are induced by collisions with the medium.

The rate of change in the populations at the lowest level (which can be measured for instance by the decay time of the vibrational spectrum) is easily seen to be $\tau_0^{-1} = Z^* N^* e^{-\theta} P_{1,0}$. The dissociation rate k_D can be calculated by the theory of stochastic processes such as the so-called "mean first passage time" of a random walker through an adsorbing barrier, and is found to correspond to the familiar Arrhenius expression,

$$k_D = A \exp(-E_D/kT)$$

where

$$A = Z^* P_{1,0}\,(N+1)\,(1-e^{-\theta})^2.$$

If, for the sake of simplicity it is assumed that the concentration of the free atoms is negligible so that the association reaction can be disregarded, then the time constant for the dissociation reaction (as measured by the increase in the concentration of the free atoms) is

$$\tau_D = 1/k_D,$$

and the ratio of the two relaxation times is

$$\frac{\tau_0}{\tau_D} = (N+1)\,(1-e^{-\theta})^2 \exp(-N\theta),$$

which is < 1 for N large and $N\theta \geqslant 10$, as required by the condition of statistical equilibrium.[102] Therefore, if the temperature of the medium is changed, the system will approach equilibrium with two different relaxation times according to the probe being measured. Similarly, if the autocorrelation function of the two probes is measured during the spontaneous dissociation process, two different autocorrelation times will be found. This is precisely the point we want to stress here.

The above model can be improved by allowing anharmonicity and the consequent possibility of transitions between non-nearest neighbor levels; however, when N is large, we shall still find that $\tau_D \gg \tau_O$. The use of quantization is not essential either because quite a similar conclusion could have been reached by considering the average time for a brownian harmonic oscillator to escape from its potential well in comparison with the autocorrelation time of the oscillator around the equilibrium position at the bottom of the energy through.

Finally we note that, in the real case, the contribution of the rotational degrees of freedom[101] pose the difficult problem of the cross correlation between the rotational and vibrational coordinates. The problem of taking proper account of the nonlinear superposition of these degrees of freedom for the largest displacements which produce dissociation is sometimes oversimplified by considering only one coordinate (the reaction coordinate) in an *"ad hoc"* potential profile, which reduces the free energy barrier to the appropriate experimental value. This same procedure is followed in enzymology, where, furthermore, the occurence of low activation energies may even invalidate the assumption of the equilibrium distribution which is needed to define a time-independent free energy profile.

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