THE FLUCTUATING ENZYME

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ABSTRACT

The importance of random processes in generating conformational fluctuations in macromolecules which could be active towards catalysis is considered. The capability to correlate in time the fluctuations of some relevant conformational variables is proposed to be the characteristic kinetic property which can allow a macromolecule to work as an enzyme. Accordingly, it is suggested that the experimental study of delayed cross-correlations between variables specifying (sets of) conformations will prove particularly revealing. Reference is made to some open problems in biochemistry, and the case of lysozyme

is considered as a specific example. A relevant class of charge and conformational fluctuations in catalysis is indicated.

PRESENT VIEWS: THE OSCILLATING ENZYME

So far we do not have an understanding of the enormous specific rate accelerations which are brought about by enzymes. Some biochemists believe that "the reasonable extrapolations from known physical organic chemistry are insufficient to explain the catalytic power of the enzymes".[1] It has been often suggested that the induction of some sort of strain or distortion into the substrate by the macromolecule could be the main explanation for the action of the enzyme. Broadly speaking, after the fast process of formation of the initial enzyme-substrate complex, some conformational changes are supposed to occur to allow the right presentation of the appropriate functional groups at the active site (ES), and finally some bond-breaking and bond-making processes are supposed to yield the enzyme-product complex (EP). These last processes can be produced by conformational alteration of the substrate so as to increase the stability of the transition state, which may help to mobilize the transition state although such couplings have not been indicated by study of structural details. Then, it has been suggested that the oscillation of an enzyme between two conformations differing in their strain would provide an "ideal mechanism"[2] for catalysis if such oscillations could occur with some driving force. However, the nature of this driving force has not been identified, and, according to Jencks[3], "such oscillations would require a mechanism for the focusing or coordinating of thermal

energy in a cooperative manner which has not yet been clearly envisioned". Moreover one further difficulty often arises, and this is the need for several concurrent processes to occur at the same rate in order for the enzyme to perform a catalytic action. In the oscillating enzyme model this would mean the need of a strong and direct coupling between different parts of the macromolecule, a very unlikely fact considering known structural details.

The case of lysozyme is so simple and illuminating that it will be chosen as a standard throughout this paper. The mechanism of catalysis of this enzyme has been so well reviewed,[4] that we can limit ourselves here to a few remarks only. It seems clear that there are three different factors in the lysozyme catalysed hydrolysis of oligosaccharides, namely the substrate distortion induced by anchoring to six residues (including Asp 101) and then the participation of residues Glu 35 and Asp 52, the first one donating a proton and the second one bearing a negative charge that electrostatically favours the carbonium ion mechanism. It is not so simple to calculate quantitatively the rate enhancement anticipated for this mechanism as the product of the three above separate factors since the contribution of each one is quite difficult to assess, in a quantitative way. However, the conclusion has been reached that no single factor involved in lysozyme catalysis seems able to account for the observed rate enhancement, but it is evident that a concerted attack involving all three contributions might give rise to a rate enhancement of the order of magnitude which is observed".[4] As a matter of fact, a variable amount of substrate distortion is certainly not sufficient, alone, to produce the chemical change. And,

Fig. 1. Lysozyme and its substrate. A schematic representation, where only the residues mentioned in the text are shown. For more details see Reference 5.

inspection of the lysozyme structure[5] clearly shows that the two residues Glu 35 and Asp 52, and the substrate, are placed in well separated sections of the macromolecule, and that between these sections it is not easy to see any direct coupling at all. Then the oscillating enzyme model seems to fail in the case of one of the most simple and better known enzymes! The picture underlying this model is too mechanical to meet the real needs, even as an idealized case.

The aim of this paper is to reconsider the oscillating enzyme model on a statistical basis. We will suggest the possibility that a particular class of fluctuations in the macromolecule conformation can offer a mechanism for coordinating the thermal energy to produce a high free energy event. Then the capability to correlate in time the conformational fluctuations relevant for the catalytic act will be proposed as an essential kinetic property of an enzyme and will be discussed again for the case of lysozyme. Finally the design of appropriate experimental strategies facilitated by a statistical analysis following the Onsager theory of the thermodynamic fluctuations will be presented so as to render this important property detectable by suitable experiments.

A NEW PROPOSAL: THE TIME CORRELATED FLUCTUATIONS

It is commonplace to say that the enzyme reduces the activation free energy by subdividing the reaction path into discrete steps, each one requiring a small free energy barrier. It is widely believed[6] that specified changes of the macromolecular configuration occur via stepwise conformational transitions (not to be confused with the

conformational changes around the same equilibrium position. Here we consider only one of the steps after the binding of the substrate to the enzyme has occurred, and we are still faced with the problem of its enormous rate, which certainly requires the concurrence of different processes. To answer this question some biochemists "turn to the more speculative hypotheses: The precise orientation factor, the strain factor, the push-pull factor, the microscopic environment factor. It is also possible -- and perhaps more probable -- that each of these sources of catalysis can play a role."[1] If we make the reasonable assumption that each of the factors mentioned above is connected with the macromolecule conformation, and therefore must be subjected to small statistical changes in time, it is conceivable, and this picture we propose, that the "secret of an enzyme" lies in its ability to let the relevant conformational fluctuations occur with a well defined time correlation. By this we do not mean a rigid coupling between those relevant variables but instead a statistical correlation in their rate of change around the equilibrium fit to increase the yield of the rate process which occurs in the catalytic act.

In a broader sense, we believe that an enzyme must be characterized not only by its spatial but also by its temporal structure, and that the two structures must be interrelated to allow some particular configuration to be reached in the phase-space by virtue of a fluctuation. Then the probability W of this event is[7]

$$W \approx e^{-\Delta G/KT}$$

where ΔG is the change in the thermodynamic potential of

the enzyme, T the temperature of the medium and K the Boltzmann constant. Of course, if the temperature and pressure of the enzyme are different from the corresponding quantities of the medium, then G must be replaced by the function R min, which is the minimum work required to carry out reversibly this change in the thermodynamic quantities of the macromolecule.[7] If this is not the case and moreover if the enthalpy change is negligeable, then we recover the concept already introduced by the biochemists that enzymes work as "entropy traps", namely by reducing the entropy at the active site.[8,9] But in our opinion this is true only during the short time of the fluctuation, where indeed a kind of "freezing" or "togetherness" can well be reached, and is better described in terms of the time correlations between the variables which characterize the system. Then the notion of correlated fluctuations allows two entirely different proposed pictures, namely the "floppy" and the "rigid" enzyme[9], to merge together. In other words, we believe that the correlated fluctuations are the driving force which cause the enzyme -- substrate complex to evolve along the chemical pathway, the space structure of the macromolecule providing for the "right" time correlations in its conformational variables. In this sense we say that an enzyme displays both a space and a time structure, and this concept will be clarified in the course of this paper. Since this correlation in time is needed to achieve the high activity the catalyst, and since the characteristic times involved are large compared to the time scale of ordinary thermodynamic fluctuations, we will limit ourselves to the consideration of a particular class of fluctuations that display the longer time constant; moreover, this fact makes them convenient for

experimental study. However, it must be understood that other classes of fluctuations with shorter time constants (as the ones connected with temperature and pressure) can be relevant for catalysis, and here too their time correlation can play a possible role. As a matter of fact, according to the fluctuation-dissipation theorem[7], we anticipate the existence of other classes of large amplitude fluctuations in those frequency regions where the macromolecule displays a large dissipation. Unfortunately the high frequency relaxation spectrum of a globular protein in an aqueous medium, as it can be studied by Raman scattering, is not yet fully understood on the experimental stage because it is likely to be dominated by the modes of the tightly bound water[10]. In our opinion, a discussion of the high frequency fluctuations as conformons[11] should be reserved for a later time and we will proceed below in a more detailed analysis of the charge-conformation fluctuations in the micro-nano second region.

AN INTERESTING CLASS OF CONFORMATIONAL FLUCTUATIONS

According to the basic principle of statistical mechanics the conformation of a macromolecule fluctuates around its equilibrium position, and the frequency spectrum of its "noise" can encompass the frequency range from the far infrared to the acoustical region. We can assume that these fluctuations originate from a stochastic process which is stationary and Gaussian. Then the majority of these fluctuations consist of displacements involving small free-energy changes, a situation which can be very useful in that it allows the best fit of the partners needed for catalysis. However, in order to induce a chemical change some free energy must

be available at the active site, even if only for a short time; thus, for catalysis, we need some active fluctuations that occur rarely and well away from the mean value of the Gaussian distribution. In our opinion this active fluctuation is the bottleneck for catalysis. We believe that an increase in the free energy of one order of magnitude above the average thermal energy (say, up to 5 Kcal/mole) should be considered sufficient if the enzyme action is not grounded on the "brute force"; moreover, this increase must be lower than the net free energy of structure stabilization of a globular protein, which is of the order of 10 ÷ 20 Kcal/mole. Therefore, the active fluctuations towards catalysis must occur with a probability factor around 10^{-5}, and if we take 10^{-3} sec. as a representative value for the enzyme turnover, we anticipate that some active conformational fluctuations must originate from that part of the frequency spectrum which is centered around 10^{-8} sec.

Following the Langevin approach, let us consider now one macromolecule in an ionic solution. Then the temperature and pressure fluctuations of the medium relax with a time constant much shorter than 10^{-8} sec., and cannot be relevant to our end. Instead, the relaxation time for the charge density fluctuations, under ordinary conditions of ionic concentrations, is expected to be 10^{-7} to 10^{-9} sec[12,13]. Therefore, this specific kind of fluctuation of the medium can induce a conformational change relevant to catalysis if the macromolecule can undergo a transition with a similar time constant and if this transition can be coupled to the electric field fluctuation originating in the bath. These two conditions can be fulfilled, as it is shown in the following. A number of studies of the excess acoustic absorption

in solutions of biologically important macromolecules at different pH have been carried out in recent years in the 10^{-7} - 10^{-8} sec. region, and these studies have attributed the origin of the observed sound absorption to the proton transfer reactions between the ionizable groups of the protein[14]. Moreover, quite recently, the possibility of internal proton transfers has been pointed out[15], and the likely occurrence of a coupling between the degrees of ionizations of the various residues near physiological pH has also been suggested[16]. These proton transfer processes are the ones which can provide the coupling between the charge density fluctuations of the bath and the conformational changes of the macromolecule. As a matter of fact, in virtue of the polyelectrolytic nature of the macromolecule the dielectric relaxation[21] of the ionizable groups must act in turn on the macromolecule conformation for quite a number of reasons (for instance by changing the electric field on some buried charge groups, by influencing the state of water bound to the backbone, and so on). Of course, the rate of these conformational changes must have a similar time constant, and this can well be the case, because the existence of an intramolecular source of relaxation in this frequency range is also shown in some studies carried out by fast kinetic techniques[17]. Actually, the two kinds of charge and conformation fluctuations cannot be separated, because one of them must induce the other one.

Kirkwood and his co-workers[18,19] have introduced the important concept of charge fluctuations on the surface of the macromolecule to derive some consequences about the dielectric properties of the proteins in solutions, and about the attractive forces, originated by

this fluctuating electric field, acting between the protein molecules and between the protein and the substrate. At that time most of these considerations remained speculative for lack of a detailed knowledge of the fine structure of the proteins, and, moreover, Kirkwood's analysis of the resulting chemical reaction mechanisms was perforce too abstract[20]. Here, instead, we propose that this fluctuating electric field can act on some charged residues to produce those conformational motions and those changes in the chemical affinities that, properly coordinated in space and time can let the macromolecule work as a catalyst. Notice that in this proposed mechanism the free energy rich fluctuation provides the driving force of the oscillating enzyme model, while the molecular structure of the enzyme is supposed to be such as to allow those factors relevant for catalysis to arise in a concurrent way under the action of the random field. A specific example, again the case of lysozyme, is considered below.

A glance to a map showing the lysozyme with its substrate[5] reveals that the acidic and basic side chains that ionize on contact with water are all on the surface of the molecule (and this is the case of Asp 101), with the only exception of the residues Glu 35 and Asp 52 which are very close to the active site. Each of these two residues is connected to a massive section of the macromolecule where the polypeptide chain is highly organized as a kind of helix and pleated sheet. Asp 52 bears a negative charge, is H-bonded to other residues and offers its charged oxygen to the substrate, while Glu 35 is not H-bonded or ionized and offers its H towards the substrate. According to the most recent views[22], Glu 35 acts to donate this proton to the departing alkoxy group but the timing of this proton

transfer is said to be "obscure", while the role of Asp 52 is said to remain an "enigma" in spite of its involvement in the mechanism of catalysis. Then one can venture to inquire if some kind of correlated fluctuations of these two residues can help to understand their role. As a matter of fact, the two catalytic carboxyls are strongly interacting as a dibasic acid, and they are subject only to a generalized electrostatic potential which depends on the ionic strength, from the rest of the enzyme[13]. Then, when this vectorial potential fluctuates, the pK of the two acids must fluctuate as well as in an opposite but correlated way. Moreover, the same fluctuating potential can displace the charged residue Asp 52. An alteration of the H-bonding length of Asp 52-Asp 59 causes some electronic charge to flow on the O^- of Asp 52, and by virtue of the properties of dibasic acids, this yields a change in the pK of Glu 35. This is a quite possible role for Asp 52. Then, the overall reaction appears to originate by a high free energy fluctuation in the charge density of the bath, which induces correlated fluctuations in the charge of both Asp 52 and Asp 101, both of which control the pK of Glu 35[23]. The charge density fluctuations of the bath may also induce conformational changes via the water bound to the enzyme.

From the foregoing example, the time correlation between the different concurrent factors towards catalysis appears as a kinetic property of the macromolecule, that can be reached by virtue of its own structure, and that expresses just the capability of a macromolecule to enhance a chemical reaction, namely to work as an enzyme. In principle, the knowledge of the macromolecular structure should allow one to calculate this property, translating into quantitative terms the qualitative argument we have shown above for lysozyme; but in practice the

complexity of the structure is such that the electrostatic field cannot be quantitatively assessed, even for such a simple enzyme as lysozyme[14]. Therefore, one must turn to an experimental approach to this problem, as shown in the next section.

THE EXPERIMENTAL SEARCH FOR THE TIME CORRELATIONS

Experimentally, one can often detect the position of a residue set at the active site by its interactions with convenient probes placed in its neighbourhood. For instance, in the case of lysozyme, it is known[15] that Glu 35, Asp 52 and Asp 101 interact with the tryptophanyl residues Trp 62, Trp 63, and Trp 108, and these interactions can be detected by U.V. absorption or fluorescence experiments. Now, in lysozyme these three tryptophanyl residues are firmly anchored to the substrate, so that if we choose as a reference system the substrate itself, then the relative displacements of the active residues versus the substrate may be inferred from the observable changes in these interactions. The experimental problem is far from simple, and a more detailed discussion is out of place here.

Actually, as we have seen above, the displacement (and the charge) of one residue is to be viewed as the result of several conformational and charge displacements on the other sites of the macromolecule, a very complex problem to be described at the molecular level and unnecessary, for our purposes, to be followed with so many details. Then to cope with this complex state of affairs, we introduce the concept of "relevant conformational variables" as those which describe the positions (and charge) of the active residues relative to the active

residues relative to the substrate. In the case of lysozyme, the three sets of relevant conformational variables are the ones describing the position (and charge) of Asp 101, Glu 35, and Asp 52. Then the important question concerning the correlation of the active groups in catalysis can be approached experimentally by searching for the time correlation between some observable quantities strictly related to the changes of the relevant conformational variables. However, we realize at once that in order to make this search in practice successful, we must not rely on those rare fluctuations which occur during the catalytic acts, but on all of the fluctuations that the system displays around equilibrium. In this last statement is implicit the rather reasonable assumption that the time correlation between the relevant conformational variables must not depend on the amplitudes of the displacements themselves.

As a matter of fact, to deal with the mean value of the Gaussian distribution instead of with the more energetic displacement, seems the best that one can do to handle our problem, still preserving the basic idea that correlated displacements must occur at the active site of the enzymes. Then, the chemical problem, to search for a few rare correlated events, may be reduced in a certain degree of approximation to the physical problem of finding the temporal structure of the correlations between the fluctuations of the relevant variables. This is a well defined problem, and provided the displacements from equilibrium of the conformational variables are small enough to let their rate of change to be expressed by the first order terms in the displacements, the statistical theory of fluctuations[7] can offer some important results. In the spirit of this theory,

the attention must be focused on the rate of change of the conformational and electrostatic free energy G around equilibrium (or of the function Rmin in the general case, see above). In the following we are going to consider this point in somewhat more detail.

Let x_i be a set of conformational variables, so defined that $x_i = \bar{x}_i = 0$ when the free energy G has a minimum, and let us assume the fluctuations x_i to be thermodynamic[7], and so small that we may restrict ourselves to the first order terms. Then, omitting the summation sign on i and K, we write as usual

$$\chi_i = \frac{1}{T} \frac{\partial G}{\partial x_i} = \beta_{ik} x_k \tag{1}$$

$$x_i^\circ = - \gamma_{ik} \chi_k \tag{2}$$

$$\chi_i^\circ = - L_{ik} x_k \tag{3}$$

where γ_{ik} are the symmetric Onsager coefficients. In these notations, the rate of the free energy change around its minimum is written as

$$- \frac{1}{T} G^\circ = \gamma_{ik} \chi_i \chi_k = L_{ik} x_k x_i \tag{4}$$

In order to detect from suitable experiments both the static β_{ik} and the kinetic L_{ik} coupling matrices, we use the correlation function and its Fourier components

$$\Phi_{ik}(\tau) = \overline{x_i(t+\tau)\, x_k(t)} = \int_{-\infty}^{\infty} (x_i x_k)_\omega \, e^{-i\omega\tau} \, d\omega \tag{5}$$

$$(x_i x_k)_\omega = \frac{1}{2\pi}\int_{-\infty}^{\infty} \Phi_{ik}(\tau)\, e^{i\omega\tau}\, d\tau \qquad (6)$$

Then, according to the theory[7]

$$\Phi_o = \overline{x_i}\,\overline{x_k} = K\beta_{ik}^{-1} \qquad (7)$$

and

$$2\pi(x_i x_k)_\omega = K(L-i\omega\beta)^{-1}_{ki} + K(L+i\omega\beta)^{-1}_{ik} \qquad (8)$$

where K is the Boltzmann constant.

Provided the relevant conformational variables x_i are identified by experimentally detectable properties, then, the experimental study of their cross-correlations can give the desired information on the value of the static and dynamic coupling right in the free energy. Notice that in this way we can avoid the very difficult problem of evaluating the conformational free energy itself, because only the small changes around equilibrium of the relevant variables are needed, and this information can be inferred by the experimental study of the noise of some suitable physical properties. The non-zero values, if any, of the β_{ik} and L_{ik} coefficients express in a very elegant way the enzyme property to correlate the relevant time fluctuations.

Of great importance is an estimate of the cross-correlation time τ_o between the kinetic quantities, if we believe this to be the time in which the reaction occur. To see this, we write in the Langevin approach

$$\overset{\circ}{X}_i = L_{ik}\, x_k + Y_i \qquad (9)$$

where Y_i is a quantity fluctuating in time. Then theory[7] says that

$$\overline{Y(t)\; Y(t+\tau_o)} = K(L_{ik}+L_{ki})\; \delta(\tau_o) \qquad (10)$$

namely, that the cross-correlation between the fluctuating kinetic quantities do exist if $L_{ik} \neq 0$, but that this correlation is limited to an interval of time included in a δ-function, in practice over intervals of time τ_o of the order of that required to establish the incomplete equilibrium defined by x_i and x_k. It is important to realize that experiments feasible on a time scale τ, as those proposed above for the mean fluctuations of the relevant conformation variable, by detecting L_{ik}, can ultimately offer such an important information as the existence of a correlation of the kinetic quantities during the time scale $\tau_o \ll \tau$.

The above theoretical framework is quite in order,but now the major problem to solve is the correct identification of the quantities x_i on the experimental stage. The detailed analysis of some enzyme-substrate complexes shows that it is very difficult to find really good probes for Fourier analysis, namely probes that are both independent of one an other, and each one sensitive to a well defined relevant event for catalysis. This search is now in progress in our laboratory.

To end, let us notice that this physical approach is vastly different from the current experimental work of the biochemists, but it should not be too alien to their spirit. As a matter of fact, if the entalpy changes are

negligeable, the entropy changes become dominant, and then we recover the concept already expressed in biochemistry that "subtle conformational changes" as displayed by the entropy of folding are the most important time events for the catalytic processes[26].

SOME MORE SPECULATIONS

If the relevance of the correlated random processes for catalysis be proved, could we ever imagine a better microscopic structure than a globular protein to perform with a sufficient correlation all the specific atomic motions and charge interplays that are needed for the catalytic act, working at room temperature and in an aqueous medium? To our present knowledge, only such a macromolecule has a structure so designed as to make the best use of the random time event. On molecular terms, this means the increasing use by Nature of H-bonded structures in biopolymers, because these can act as ideal sources for mechanical and electrical fluctuations in the widest range of frequencies. This may be the physicists answer to the question so far left open by the biochemists: "Why are enzymes large molecules with a definite structure that must be maintained in tact in order that catalytic activity may occur"?[3] As a matter of fact, its large size reduces the trivial statistical fluctuations on the atomic scale, and allows its non-covalent bonded structure to perform reversible changes in a definite pattern. Moreover, if the capability to correlate relevant time events can be taken as a figure of merit for a catalyst, then one can eventually trace back the evolution of an enzyme as the progressive gain in this correlation from the poorly structured

primeval soup to the globular proteins we find today.

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