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Enzyme Flexibility and Enzyme Action[1]

D. E. KOSHLAND, JR.
Biology Department, Brookhaven National Laboratory, Upton, New York

It has long been clear that enzyme action is intimately involved with the three-dimensional arrangement of amino acids. The specificity of the enzymes led Fischer (1894) to propose a "key-lock" model for the steric relations at the active site, and others showed that denaturation was correlated with changes in shape of the enzyme. The tools for measuring protein shapes are still primitive, and the physical description of that ill-defined area called "the active site" is even more complicated. Nevertheless, unsuspected tools are frequently uncovered under the impetus of pertinent questions and we should like, therefore, to attempt an answer to the question "Is the active site rigid or flexible during enzyme action?".

One source of information comes from the general studies on protein shape, which have followed almost from the discovery of proteins and protein denaturation. The first evidence that proteins were flexible as well as fragile probably comes from the studies of Anson and Mirsky ('34) on the reversible denaturation of trypsin. Since then the able and original work of a number of workers (e.g., Karush, '50; Kauzmann, '54; Lumry and Eyring, '54; Doty and Yang, '56; Linderstrøm-Lang and Schellman, '59) has led to a far greater understanding of this vital area. In addition to temperature, other reagents such as urea, *p*H, salt concentration, and organic solvents can be used to induce changes in the three-dimensional geometry of a protein. The changes in shape caused by these stresses can be measured by a variety of tools of which optical rotation, viscosity, sedimentation constant, deuterium exchange, and solubility are examples. A brief and over-simplified summary of all of this work is that certainly large portions of many proteins are flexible in the sense that they can be reversibly deformed. Urea, for example, will produce reversible changes in viscosity and enzyme activity in both trypsin and chymotrypsin (Harris, '56). With almost all these reagents, there appears to be a point of no return, after which irreversible changes are induced. If this limit is not exceeded, however, the evidence supports the conclusion that removal of the stress returns the flexible portions of the protein to their natural conformations.

The fact that large portions of the protein are flexible is by no means evidence that all portions of the protein are. Actually, fragmentary evidence exists that a change in certain portions of the protein always results in irreversible denaturation. We are left, therefore, with the conclusion that either a flexible or a rigid active site would be compatible with the general studies of protein properties.

Let us, therefore, examine the evidence for the template model of enzyme specificity that argues for a relatively hard and inflexible active site. To a chemist, one of the most astounding properties of enzymes is their specificity. The fact that a small group in the substrate, far from the bond to be cleaved, can decide whether an enzyme acts or does not act on a particular compound is almost incredible. Since studies of physical organic chemistry have clearly indicated the magnitude of inductive effects, we can conclude that such effects cannot explain the observed changes in velocity from substrate to non-substrate in most cases. Fischer therefore

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were too bulky to allow this association, no catalysis resulted. If the groups necessary for binding the substrate to the enzyme were absent, the substrate was not held to the enzyme and again no catalysis would result. Since the two postulated phenomena for the template hypothesis, i.e., steric hindrance and affinity by the formation of noncovalent complexes, were well substantiated in organic chemistry and since the development of enzyme kinetics supported the presence of an enzyme—substrate intermediate, this theory became widely accepted. In fact, it does suffice to explain the vast majority of the observed specificity patterns of enzymes.

Our own feelings that all was not quite so well with the template theory as might appear on the surface came when we were trying to explain the failure of muscle phosphorylase to catalyze an exchange between $P^{32}O_4$ and glucose 1-phosphate (Koshland, '54). In muscle phosphorylase, it had been shown that an acceptor was needed to observe exchange whereas with sucrose phosphorylase no acceptor was required (Doudoroff *et al.*, '47; Cohn and Cori, '48). Let us assume that the same mechanism is operating for muscle phosphorylase as was indicated for the sucrose synthesizing enzyme; i.e., that a group on the enzyme attacks from the back of the carbon atom to form a glucosyl—enzyme intermediate. The existence of this mechanism does not necessarily mean that exchange must occur. It could be said that the glucosyl—enzyme intermediate exists for so short a time that the inorganic phosphate is unable to leave and be replaced by a radioactive phosphate before the new covalent glucose—phosphate bond is formed. There is good analogy for this kind of kinetic variation in the neighboring group effect, in which the gamut from the formation of a completely stable bond, as in epoxides, to the transient interaction of a neighboring methoxyl group is observed. However, if such a process were going on and there were repeated formations of a glucosyl—enzyme intermediate, we might expect that periodically the water in the adjacent site would be able to react. Water should certainly be about as nucleophilic as the 4-hydroxyl group of the glycogen polymer, and it seemed unlikely that the glucosyl—enzyme intermediate being formed so rapidly and reversibly would not occasionally react with the adjacent water molecule. An alternative mechanism based on the S_{Ni} reaction (Koshland, '54) leads to almost precisely the same difficulty.

It would seem that either the displacement mechanism or the template theory was inadequate. This would hardly be sufficient basis for questioning the template hypothesis, but on reflection we thought the failure of water to react in a number of other instances (e.g., the hexokinase reaction) was equally puzzling. Moreover, evidence in support of the displacement mechanism increased, and an intensive search of the literature was therefore made for examples that could not be reconciled with the template hypothesis. An amazingly large number of instances were found (Koshland, '55, '58, '59), and since this material has already been published, only one example will be used to illustrate the type of reasoning involved.

Amylomaltase is a purified enzyme that catalyzes the hydrolysis of maltose but does not act on α-methylglucoside (Wiesmeyer and Cohn, '57). α-Methylglucoside has the same stereochemistry at the C-1 as maltose and the same type of bond to be broken; it differs only in that the methyl group has two hydrogen atoms where the remaining part of the second glucose ring would be placed. Since it could hardly be argued that these two hydrogen atoms would be unable to fit into the area on the template reserved for the full glucose ring, the failure of α-methylglucoside to react would, on the template hypothesis, have to be explained by a failure to be attracted to the enzyme surface. However, α-methylglucoside has been shown to be a competitive inhibitor. Hence it is known to be present at the enzyme surface and in the appropriate position, and yet no reaction occurs.

From examination of these and other examples, it was clear that the template theory would have to be modified. The reasoning that led Fischer to conclude that a steric interaction was required seemed unassailable. The theory was modified, therefore, to give the substrate a more-

positive role. It was assumed that the active site was not initially a negative of the substrate but became so only after interaction with substrate. This change in conformation of the protein occurred with the result that the final enzyme—substrate complex had the catalytic groups on the enzyme in the proper alignment with each other and with the bonds to be broken in the substrate molecules. This retained the idea of a steric fit proposed by Fischer but modified it in such a way that the failure of either too large or too small a compound to react could be readily explained. For example, the failure of water to react in the phosphorylase reaction would be explained by the fact that the small size of the water molecule did not provide sufficient buttressing action to lead to the proper alignment of catalytic groups. This, moreover, is in line with the observation of a minimum size for the primer in the phosphorylase reaction. This mechanism requires a flexible action at the active site, i.e., the protein changes shape under the influence of the substrate and returns to its original shape after the products have been released from the enzyme surface. This "induced fit" hypothesis also explained a number of other observations such as the synthetase-type enzymes that require the simultaneous presence of a number of substrates on the enzyme surface before any partial reaction occurs (Koshland, '55, '58, '59).

Some evidence of a different nature obtained by Dr. Harvey Levy and Dr. Nathan Sharon (Levy *et al.*, '59a) supports the postulated flexibility of the active site and the specific modification of protein conformation by the substrate itself. This evidence grew out of temperature studies on the enzyme myosin, the data for which are shown in figure 1. Recording the rate data on an Arrhenius plot gives a straight line if the activation energy and the PZ factor are constant. Such is observed to be the case for the myosin-catalyzed hydrolysis of ATP, which is linear over the experimental range of 30° to 0°C. It is to be noted, however, that both ATP in the presence of dinitrophenol (DNP) and ITP show a pronounced curvature. This is not a function of the experimental error or of the method of graphing. Careful repeti-

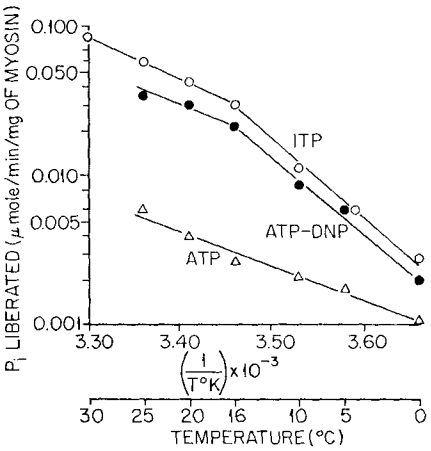

Fig. 1 Arrhenius plot for myosin with ATP, ITP, and ATP in the presence of DNP. Solution contained 0.01 *M* $MgCl_2$, 0.05 *M* Tris, 0.1 *M* KCl *p*H 7.3, and 0.005 *M* nucleotide triphosphate.

tion of the experiment and replotting of the data with various abscissa and ordinant ratios leads to the same conclusion. Actually, the two curves can be very well approximated by straight lines at each of the extremes of temperature, and these straight lines intersect in each case very near 16°C. This extrapolation should not be taken to imply that an abrupt discontinuity exists. However, the rather good agreement of the straight lines over a considerable range of temperature does tend to indicate that a shift occurs from a process at the higher temperature having an activation energy of ~12 kcal to a process at lower temperatures having an activation energy of ~25 kcal.

Curves that show such a change in activation energy have been observed before, and a number of different explanations have been proposed. Dixon and Webb ('58) have summarized these as follows.

(a) There is a phase change in the solvent. This idea is supported by the observation that the point of inflection apparently occurs in the same place for a number of different enzyme systems and by the existence of a transition point near 0°C. where a phase change in the solvent water is known to occur.

(b) There are two parallel reactions with different active centers. Dixon and Webb ('58) pointed out that such a mechanism could explain Arrhenius plots that

are concave upward but could not explain those that are concave downward.

(c) The enzymic process involves two successive reactions having different temperature coefficients. The idea that such a shift from one rate-mastering step to another was responsible for the transition in the Arrhenius plot of physiological processes was originally suggested by Crozier ('24) but was later seriously attacked by Burton ('39) and others. Burton showed that, in some systems having activation energies of the order of those found by Crozier, a consecutive-step mechanism would not lead to so abrupt a transition as was observed in the experimental cases. Burton was dealing with a system involving two different enzymes, however, and the mathematics accordingly is not precisely the same as that for two consecutive steps on a single enzyme surface. The sequence of events in the latter case are illustrated by equation (1) (fig. 2). Since the experiments reported here were done at enzyme saturation, we need only consider the steps in equation (1) having the constants k_1 and k_2. When the kinetics for this case are derived by using only the steady-state assumption and the condition that $(ES)_1 + (ES)_2 = E_T$, the relation shown in equation (2) is obtained. This turns out to be different from the kinetics treated by Burton; the observed values for an enzyme having activation energies similar to those of myosin with ITP are shown in figure 3. Since the observed rates for such a consecutive-step mechanism are seen to be a very good rough approximation for the ITP-myosin curve, it is clear that a consecutive-step mechanism cannot be excluded simply by the argument that the transition observed experimentally is too abrupt.

(d) The enzyme exists in two forms having differing activities. This suggestion was originally advanced by Sizer ('43) and can be formalized as shown in equations (3), (4), and (5). In this mechanism the protein changes from a high-temperature form, E_1, to a low-temperature form, E_2, in a reversible manner over a fairly narrow temperature range. The velocity of the over-all reaction is given by equation (6). This mechanism can give Arrhenius plots that are either concave

$$E + S \rightleftharpoons (ES)_1 \xrightarrow{k_1} (ES)_2 \xrightarrow{k_2} E + P \qquad (1)$$

$$\frac{d(P)}{dt} = \frac{k_1 k_2 E_T}{k_1 + k_2} \qquad (2)$$

$$(E_1S) \xrightarrow{k_1} E_1 + P \qquad (3)$$

$$E_1 \underset{}{\overset{K_1}{\rightleftharpoons}} E_2 \qquad (4)$$

$$(E_2S) \xrightarrow{k_2} E_2 + P \qquad (5)$$

$$\frac{d(P)}{dt} = \frac{k_1 K_1 + k_2}{1 + K_1} \qquad (6)$$

Figure 2

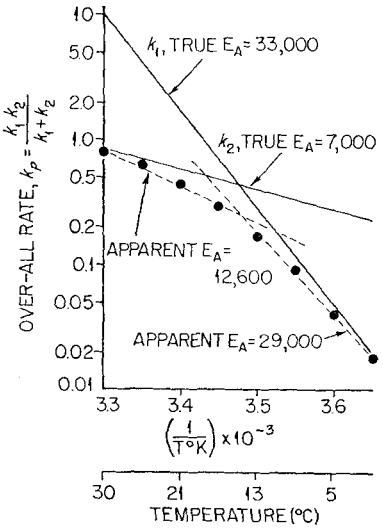

Fig. 3 Calculated velocities for a consecutive-step mechanism of the type shown in equation (1) (fig. 2). The true activation energies of k_1 and k_2 are assumed to be 33 and 7 kcal, respectively. The absolute magnitudes of the rate constants for these two steps are taken from the solid lines drawn with these activation energies. The rate of appearance of product at any temperature is obtained from these values by equation (2) and is shown by the points on the graph. These velocities, which would be the observed velocities in an experimental case, give apparent activation energies of 12.6 and 29 kcal in two rather linear portions of the curve.

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