Analog Approaches to the Structure of the Transition State in Enzyme Reactions

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The effectiveness of enzymes as catalysts remains a vexing problem for biochemists 45 years after the crystallization of urease and the demonstration that this enzyme is a protein. Great advances have been made in the development of model reactions which suggest possible mechanisms of catalysis, the detection of fleeting events during enzyme catalysis, the characterization of relatively stable intermediates, and the determination of the exact structure of enzymes. It seems likely that a solution to the overall problem will eventually arise from a combination of these approaches, each of which has its own limitations.

X-Ray diffraction of single crystals provides an exact picture of the enzyme in its ground state, and there is evidence that the crystal structure for many enzymes is similar to the structure in solution. However crystallographic pictures are static, and convey no information concerning dynamic changes which occur during the catalytic event. Even if a true enzyme–substrate complex (with a typical lifetime in the neighborhood of 1 msec) could be subjected to this kind of examination, it would be likely to differ considerably in structure from the transition state for the overall reaction (with a typical half-life of 10^{-10} msec). Rapid reaction techniques, which do detect dynamic changes during catalysis, generally convey little detailed structural information about the protein.

There is need for a unifying approach, other than intuition or guesswork, which will permit these techniques to be combined. Rapid quenching techniques might be devised for trapping intermediates which could then be subjected to direct structure determination.[1] If the technical difficulties could be surmounted, this approach might provide useful information; however it appears that "the problems of achieving truly rapid quenching and of obtaining high enough concentrations of structurally identical reaction intermediates to produce detectable constructive interference of X-rays or thermal neutrons are, to employ a euphemism, very perplexing."[2]

This Account describes an indirect approach to unifying kinetic, chemical, and structural data in the study of enzyme mechanisms. This approach offers a rational basis for the design of potent and specific enzyme inhibitors, using mechanistic information obtained by other methods. It is similar to a method for transition-state characterization for chemical reactions proposed earlier by Kurz and applied by him to certain examples of acid–base catalysis.[3]

Theory

The function of an enzyme, like that of any other catalyst, is to make the transition state for the reaction easier to reach. It is difficult to imagine how this could be achieved if the enzyme did not possess an unusual affinity for the altered substrate in the transition state, exceeding that for the substrate itself. This view, originally advanced by Pauling,[4] has been discussed in terms of the possibility that some enzymes act by straining or distorting the substrate,[5,6] and it has been suggested that the unusual effectiveness of certain enzyme inhibitors might be explained on this basis.[4,6,7]

Potential applications of this concept, as a way of unifying the various approaches to determination of enzyme mechanisms, do not appear to have been recognized until recently. Let us consider the binding forces which are likely to exist during enzyme reactions. If the relative rates of a reaction in the presence and absence of enzyme are known, a conservative semiquantitative estimate of the tightness of binding of the altered substrate in the transition state may be made.[8] For simplicity, one-substrate reactions are considered first. These are common in biochemistry, and five examples can be found in the glycolytic pathway alone. Similar calculations for multisubstrate reactions (to be considered later) lead to the general conclusion that binding in the transition state is probably very tight indeed.

Glyceraldehyde 3-phosphate is converted to dihy-

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(1) M. Eigen in "Fast Reactions and Primary Processes in Chemical Kinetics: Proceedings of the Fifth Nobel Symposium," S. Claesson, Ed., Interscience, New York, N. Y., 1967, p 477.
(2) E. M. Eyring and B. C. Bennion, *Annu. Rev. Phys. Chem.*, **19**, 129 (1968).
(3) J. L. Kurz, *J. Amer. Chem. Soc.*, **85**, 987 (1963); J. L. Kurz, *Accounts Chem. Res.*, **5**, 1 (1972).
(4) L. Pauling, *Chem. Eng. News*, **24**, 1375 (1946).
(5) R. Lumry in "The Enzymes," Vol. 1, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, New York, N. Y., 1959, p 157.
(6) W. P. Jencks in "Current Aspects of Biochemical Energetics," N. O. Kaplan and E. P. Kennedy, Ed., Academic Press, New York, N. Y., 1966, p 273.
(7) G. J. Cardinale and R. H. Abeles, *Biochemistry*, **7**, 3970 (1968).
(8) R. Wolfenden, *Nature (London)*, **223**, 704 (1969).

Scheme I

Nonenzymatic

$$S \xrightarrow{k_N} P$$

Enzymatic

$$E + S \overset{K_S}{\rightleftharpoons} ES$$

$$ES \xrightarrow{k_E} E + P$$

$$E + S \overset{K_N^{\ddagger}}{\rightleftharpoons} E + TX$$

$$K_S \Big\downarrow\Big\uparrow K_{TX}$$

$$ES \overset{K_E^{\ddagger}}{\rightleftharpoons} ETX$$

$$K_S/K_{TX} = K_E^{\ddagger}/K_N^{\ddagger}$$

If transmission coefficients are unity

$$K_S/K_{TX} = k_E/k_N \quad (1)$$

In view of possible exceptions (see text)

$$K_S/K_{TX} \geq k_E/k_N \quad (2)$$

binding ratio — rate ratio

droxyacetone phosphate in the presence of rabbit muscle triosephosphate isomerase, with a turnover number of 4.3×10^3 sec^{-1} in pH 7.9 buffer at 26°.[9] The system is represented in Scheme I, in which E represents the enzyme, S the substrate, ES the enzyme–substrate complex, TX the transition state for the nonenzymic reaction, and ETX the transition state for the enzyme-catalyzed reaction. The rate constant for isomerization at pH 7.9 without enzyme is approximately 1.4×10^{-5} sec^{-1}.[10] The rate constant for product formation from the enzyme–substrate complex thus exceeds the rate constant for product formation from the substrate alone by a factor of 3×10^8. If transmission coefficients are assumed to be unity for both reactions, it follows that the equilibrium constant $K^{\ddagger}$ for achieving the transition state in the enzyme reaction is larger than in the enzymeless reaction by a factor of 3×10^8. The virtual dissociation constant of the altered substrate in the transition state, K_{TX}, should therefore be lower than the dissociation constant of the substrate in the Michaelis complex, K_S, by the same factor (Scheme I, eq 1).

The apparent dissociation constant, K_m, for the substrate in the Michaelis complex is approximately 1.4×10^{-4} M;[9] thus the altered substrate in the transition state must be very tightly bound. For reasons discussed below, rate comparisons of this sort are likely to provide a maximum estimate of the dissociation constant of the altered substrate in the transition state. The dissociation constant of the altered substrate in the transition state is therefore expected to be lower than the dissociation constant of the substrate in the Michaelis complex by a factor—the "binding ratio"—at least as large as the ratio of the limiting rates in the presence and absence of the enzyme—the "rate ratio" (eq 2). If the transition state were not more tightly bound, *catalysis would not occur.*

This quasi-thermodynamic treatment suggests that during enzyme reactions the substrate is bound to the enzyme very much more tightly than in the Michaelis complex. This conclusion follows directly from the theory of absolute reaction rates. It rests on no assumptions regarding the specific nature of the attractive forces involved, nor does it require that the enzyme be rigid or flexible. Conversely, evidence which may be obtained in favor of tight binding of the altered substrate in the transition state cannot by itself be regarded as evidence for or against particular forces of attraction or changes in conformation which may occur in a specific case.

If correct, this theory provides a general explanation of substrate specificity which embraces more classical views. When two substrates of comparable chemical reactivity but differing enzymatic reactivity are compared, the more reactive substrate is simply the one for which the enzyme possesses stronger forces of attraction in the transition state. Specificity may in certain cases be manifested mainly in K_m (the classical case considered in Fischer's "lock and key hypothesis,"[11]) or in V_{max}, depending on the extent to which bonding differences between the two substrates in the transition state for their enzymatic reactions are *already present* in their respective Michaelis complexes. It appears meaningless to distinguish between "binding sites" and "catalytic sites" since catalysis actually requires an enhanced degree of binding. In addition, the theory suggests that it may be possible to design stable analogs approaching the structure of the altered substrate in the transition state and that such compounds should be extraordinarily potent enzyme inhibitors.

Possible Exceptions

Is the nonenzymatic reaction really suitable for comparison with the enzymatic reaction? It has been assumed that the substrate has a comparable structure in the transition state for both the enzymatic and nonenzymatic reactions. Of the pathways available to it, the nonenzymatic reaction must follow the pathway with the lowest free energy of activation. If this *differs* radically in mechanism from the pathway on the enzyme, it must have a lower free energy of activation than any pathway resembling that followed in the enzymatic reaction. The rate of the observed nonenzymatic reaction will therefore be larger than the rate of any hypothetical nonenzymatic reaction which would be more appropriate for comparison. For present purposes, the observed rate ratio therefore provides only a minimum estimate of the rate enhancement that the enzyme might have produced if both enzymatic and nonenzymatic pathways involved essentially similar structures in the transition state.

Many nonenzymatic reactions in aqueous solution are subject to acid and base catalysis. If this is not

(9) Calculated from the data of P. M. Burton and S. G. Waley, *Biochim. Biophys. Acta*, **151**, 714 (1968), using a molecular weight of 26,000 per active site.

(10) C. A. Lewis and R. Wolfenden, unpublished.

(11) E. Fischer, *Chem. Ber.*, **27**, 2985 (1894).

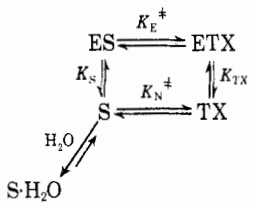
recognized in a particular case, the rate ratio will be underestimated for the reasons just considered. Reactions subject to specific oxonium and hydroxide ion catalysis can often be recognized from the dependence of rate on pH; the results may sometimes be useful in testing potential transition-state analogs, and will be considered later. Nonenzymatic reactions which appear to be pH independent may actually proceed through simultaneous but separate reactions catalyzed by protons and hydroxide ions; unless these are recognized, they will constitute exceptions of the kind just considered. Contributions to nonenzymatic reactions from buffer catalysis should of course be eliminated by the usual extrapolations to zero buffer concentration at constant pH.

It has been assumed that the rate constant for the enzymatic reaction describes the making or breaking of bonds in the substrate, as in the nonenzymatic reaction. However, it is likely that even when the pathways of enzymatic and nonenzymatic reactions are mechanistically similar, the degree of bond making or breaking is different in the two transition states. It is of interest to consider extreme cases in which the rate of the enzymatic reaction is actually limited by some step or steps, such as protein isomerization or product release from an enzyme–product complex, which have no counterpart in the nonenzymatic reaction. In these cases, which can sometimes be identified by kinetic studies,[12–14] one is forced to the conclusion that the enzyme is subject to special restraints which set a limit on the turnover number. From the present point of view, the observed rate of the enzymatic reaction will lead to an underestimate of the ability of the enzyme to stabilize the transition state for bond making and breaking in the substrate. Therefore the observed rate ratio again provides only a minimum estimate of the binding ratio expected for an ideal transition-state analog.

Let us now inquire as to the possible effects of the solvent on the observed rate constants and binding equilibria. It has often been suggested that enzymes may accelerate reaction by desolvating substrates which are unreactive in the hydrated form. Consider, for example, the case diagrammed in Scheme II, in which the observed nonenzymatic reaction is due to a small population of unhydrated substrate molecules which react with a much higher rate constant than that observed for net conversion of substrate to product. The rate constant k_N, and therefore $K_N^{\ddagger}$, will clearly have been underestimated. However, the dissociation constant K_S will have been overestimated by the same factor, since both the binding reaction and the nonenzymatic reaction can be considered to draw upon the same small pool of unhydrated substrate. Equation 2 will tend to remain valid, although true values of $K_N^{\ddagger}$ and K_S are higher and lower, respectively, than the overall values calculated from experimental observations.

(12) A. Peterkofsky, *J. Biol. Chem.*, **237**, 787 (1962).
(13) K. Dalziel and F. M. Dickinson, *Biochem. J.*, **100**, 34 (1966).
(14) R. Wolfenden, *Biochemistry*, **8**, 2409 (1969).

Scheme II

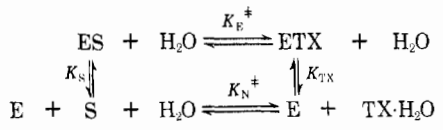


In another conceivable case, the transition state is more effectively solvated than the substrate, and therefore the nonenzymatic reaction is water catalyzed; this is likely to be true for reactions which proceed by ionic mechanisms in aqueous solution. Scheme III pertains to a nonenzymatic reaction in which the transition state is hydrated but the substrate is not. Water enhances the nonenzymatic reaction rate constant, k_N, above any value which might be observed if hydration did not occur in the transition state. By the reasoning used earlier, this is equivalent to the statement that water has a high affinity for the transition state. Water should therefore assist the release of TX from the enzyme, described by the virtual dissociation constant K_{TX}. Hydration of TX should thus produce a parallel enhancement of the values for $K_N^{\ddagger}$ and K_{TX} above those which would be observed if water were not present. These effects will compensate each other so that eq 2 will tend to remain valid.

Changes in the orientation of solvent without changes in the stoichiometry of solvation might in principle enhance the reactivity of some fraction of substrate molecules above the ordinary level. In the author's opinion, rates of reaction of these molecules should not be used for comparison with enzymatic rates, since they are only "snapshot" descriptions of points which depart from the mean in the usual statistical description of molecules in the ground state. To choose a special class of these for comparison with ES would be as arbitrary as to choose for comparison molecules at any other point on the rising limb of the free-energy diagram of the nonenzymatic reaction.

Many variations could be drawn on the above models for solvent participation, but the resulting factors usually tend to compensate each other so as to preserve the expected relationship between observable parameters, provided water is used as a common solvent for enzymatic and nonenzymatic reactions. In order to appreciate the true effectiveness of enzymes as catalysts, one should compare the rates of unimolecular reactions in the vapor phase with the turnover numbers of enzymes catalyzing similar reactions. For calculations of the present kind, however, any advantage would be lost because of the practical necessity of determining the free energy of solution of all species (includ-

Scheme III



ing the transition state!) in order to relate the calculated equilibria to observable enzyme-binding equilibria. Also, there would be serious technical difficulties in carrying out rate studies in the vapor phase for reactions of most biological molecules.

Forces of Attraction

Bonds responsible for tight binding of the altered substrate in the transition state, though collectively strong, must also be kinetically labile. It is therefore reasonable to suppose that they consist largely of noncovalent interactions, the enzyme serving as a superior chelating agent for the substrate in the transition state. Of the forces to be considered,[15] hydrogen bonds have received much study in model systems. In reactions which exhibit classical general acid and base catalysis, the properties of the reverse reaction (general acid catalyzed where the forward reaction is general base catalyzed, or *vice versa*) suggest that the proton is in the process of being transferred from the donor to the acceptor in the transition state, instead of returning to the donor when the transition state collapses to form products. In other cases, for example the intramolecular hydroxyl group assistance of the alkaline hydrolysis of esters, reversible formation of a hydrogen bond is believed to stabilize the transition state, the proton returning to the original donor as the transition state collapses to form products.[16]

Regardless of whether the proton undergoes net transfer during reaction, hydrogen bonding could contribute negative free energy to the binding of the transition state in enzyme reactions, and might thus be exploited to enhance the reaction rate. Coulombic forces are also likely to be involved, especially in reactions in which charge is generated in passing to the transition state; the specific effects of salts on certain nonenzymatic reactions have been explained in this way.[17] Although coulombic and hydrogen-bonding interactions are usually so weak as to be difficult to detect in an aqueous environment, they may collectively become quite strong, especially in the relatively "dry" cleft in which the substrate binding site of many enzymes is situated. Hydrophobic interactions, evident in certain model reactions,[18] are also likely to be important. It is of interest that molecular shape and hydrophobic interactions appear to be of overriding importance in other cases where small ligands are tightly bound in biological systems. These include the binding of haptens,[19] odoriferous substances,[20] insect attractants, steroid hormones, and alkaloids to biological receptors.

There is evidence that precise orientation of the substrate in relation to amino acid residues at the active site is a critical determinant of the catalytic efficiency of enzymes. In subtilisin, the rate of nucleophilic attack on bound substrate by serine at the active site and the rate of deacylation of acylated enzyme are drastically reduced if the serine oxygen is replaced by sulfur.[21,22] This effect contradicts what might be anticipated from the relative reactivity of thiols and alcohols toward carboxylic acid derivatives in simple bimolecular reactions. The loss of activity is most marked in the case of specific peptide and amide substrates, as contrasted with *p*-nitrophenyl acetate for which the native enzyme produces less impressive rate enhancements for hydrolysis.

In terms of the present approach, these results suggest that the powerful attractive forces between enzyme and substrate in the normal transition state fall off drastically if the substrate or binding residues shift slightly in position. This is understandable if binding in the transition state arises from multiple forces, the cooperative effects of which are disrupted by small modifications such as the replacement of oxygen by relatively bulky sulfur. These interactions are expected to be particularly strong and numerous in substrates for which the enzyme produces a large rate enhancement. The effects of a small change in structure therefore might be expected to be most pronounced in these cases. In terms of the number of forces involved in stabilizing the transition state, "the bigger they come, the harder they fall."

Can Suitable Analogs be Made?

It would be unrealistic to suppose that an ideal transition-state analog, perfectly resembling in binding properties the altered substrate in the transition state, can ever be synthesized. Stable analogs cannot reproduce the bond actually being formed or broken in the catalyzed reaction, and there are likely to be minor differences between the free energies of solvation of any inhibitor and of the transition state, which will disturb the binding equilibria. Nevertheless, such tight binding is predicted for transition-state analogs in comparison with substrates that even molecules possessing only a *part* of the total binding interactions which normally result in catalysis should be extraordinarily potent inhibitors, with affinities far exceeding that of the substrate.

If an enzyme catalyzes a reaction by stabilizing a carbonium ion, a carbanion, an enediol, or some other reactive intermediate, it is reasonable to suppose that a stable analog possessing similar steric or electrostatic properties could be devised. The approach seems less likely to be successful in cases where bonds of largely covalent character exist between the enzyme and the

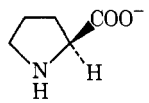
(15) For a recent review, see W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969.
(16) T. C. Bruice and T. H. Fife, *J. Amer. Chem. Soc.*, **84**, 1973 (1962).
(17) R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959, p 128.
(18) J. R. Knowles and C. A. Parsons, *Chem. Commun.*, 755 (1967).
(19) F. Karush, *Advan. Immunol.*, **2**, 1 (1962).
(20) J. E. Amoore, J. W. Johnston, Jr., and M. Rubin, *Sci. Amer.*, **210** (2), 42 (1964).
(21) K. E. Neet and D. E. Koshland, Jr., *Proc. Nat. Acad. Sci. U. S.*, **56**, 1606 (1966); K. E. Neet, A. Nanci, and D. E. Koshland, Jr., *J. Biol. Chem.*, **243**, 6392 (1968).
(22) L. Polgar and M. L. Bender, *J. Amer. Chem. Soc.*, **88**, 3153 (1966); *Biochemistry*, **6**, 610 (1967); *Advan. Enzymol.*, **33**, 381 (1970).

substrate in the transition state, as, for example, in formation or breakdown of an acyl-enzyme. To reproduce such bonds, an analog would also have to be bound covalently. In this case, which may be represented *reductio ad absurdum* by the products of reaction of an enzyme with an alkylating reagent or other irreversible inhibitors directed toward the active site, meaningful information regarding equilibria and mechanisms of binding is likely to be difficult to obtain. In cases where reversible covalent bonds (other than those formed or broken in the overall reaction) can be formed with analogs, useful information may, however, be obtainable.

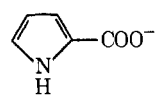
Suitable analogs should be powerful enzyme inhibitors and should reflect and confirm the structural characteristics of correctly postulated intermediates, thus providing evidence regarding transformations through which the substrate passes.

Analogs for One-Substrate Reactions

Proline racemase of *Clostridium sticklandii* is inhibited 50% by pyrrole-2-carboxylic acid (II) at a concentration 160-fold lower than that of the substrate L-proline (I).[7] Probably the α-carbon atom of the substrate assumes a near-planar configuration at some point in the reaction. Tight binding would therefore be expected for analogs, such as pyrrole-2-carboxylic acid, with sp^2 hybridization at this position.[6,7]

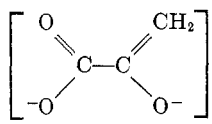


I

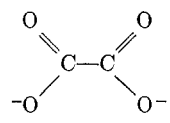


II

Oxaloacetate decarboxylase from codfish is potently inhibited by oxalate, with $K_i = 3.5 \times 10^{-6}$ M, as compared with the relatively high Michaelis constant of the substrate oxaloacetate, 1×10^{-3} M.[23] Other carboxylic and dicarboxylic acids are ineffective inhibitors. This enzyme has also been shown to catalyze stereoselective reduction of pyruvate by sodium borohydride (also inhibited by oxalate), and both this and the normal reaction are believed to proceed through generation of the enolate of pyruvate (III) by this enzyme.[24] The potency of oxalate (IV) as an inhibitor can reasonably be ascribed to its resemblance to III.

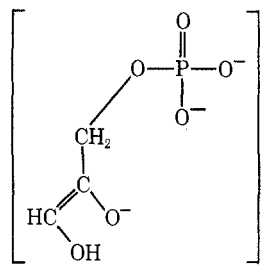


III

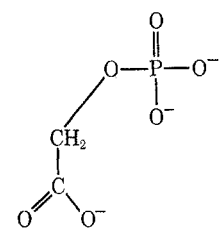


IV

The principles described above were recently used in a search for potential analogs of highly reactive intermediates in the action of triosephosphate isomerase, the structure of which is currently under study in several laboratories. It appeared particularly desirable to devise an analog of the cis enediolate intermediate (V) proposed earlier for this reaction by Rose[25] on the basis of isotope incorporation and stereochemical evidence. Of many phosphate esters examined, most were less tightly bound thatn α-glycerophosphate, a compound which resembles both substrates and is bound with a K_i value similar to the K_m of glyceraldehyde 3-phosphate. The single exception was 2-phosphoglycollate (VI).[8] The charge distribution and stereochemistry around both carbon atoms of this molecule are similar to part of the proposed intermediate, V, and the pK_a of its carboxylic acid group, approximately 3.3, is not far removed from the pK_a (4.2) of ascorbic acid, a stable enediol. The inhibition constant of 2-phosphoglycollate is 360-fold lower than the observed K_m value for *dl*-glyceraldehyde 3-phosphate and 330-fold lower than K_i for the substrate analog *dl*-α-glycerophosphate at pH 5.55. The carboxylic acid group of the inhibitor must be ionized for effective inhibition, and binding of the inhibitor is sensitive to the state of ionization of a "catalytic" residue or residues on the protein, whereas binding of substrates and substrate analogs is not.[26] These characteristics support the interpretation that phosphoglycollate resembles a reactive intermediate similar to V and provide evidence in favor of the mechanism proposed by Rose.



V



VI

In none of these cases has the actual binding constant of the substrate been determined; however, K_i values are much lower than observed K_m values for substrates (which may be higher or lower than the true substrate dissociation constants). In each case one inhibitor is bound very much more effectively than apparently closely related compounds which lack its resemblance to a plausible intermediate.

The theory advanced earlier suggests that any variable which alters the rate ratio should produce a parallel change in the binding ratio for an analog resembling the transition state in structure. For triosephosphate isomerase, effects of pH on the observed kinetic constants and on the binding of phosphoglycollate have been found to conform with this prediction if the nonenzymatic reaction, like other examples of the Lobry de Bruyn–van Ekenstein rearrangement, is base catalyzed. Ionization of a specific residue or residues on the protein affects V_{max} and inhibitor binding in the same sense, so that with changing pH the relative affinity of the enzyme for the inhibitor changes in parallel with

(23) A. Schmitt, I. Bottke, and G. Siebert, *Z. Physiol. Chem.*, **347**, 18 (1966).
(24) G. W. Kosicki and F. H. Westheimer, *Biochemistry*, **7**, 4303 (1968).
(25) I. A. Rose, *Brookhaven Symp. Biol.*, **15**, 293 (1963).
(26) R. Wolfenden, *Biochemistry*, **9**, 3404 (1970).

the apparent rate enhancement produced by the enzyme as catalyst.[26] Other tests with this inhibitor have shown that it protects the enzyme to the extent expected against inactivation by alkylating agents directed at the active site[27] and that it is an effective inhibitor of isomerases from all species tested, including chicken, rabbit, yeast, and *E. coli*.

Other possible examples, too complex for discussion here, include the inhibition of steroid Δ-isomerase by partially planar inhibitors[6,28] and the inhibition of acetoacetate decarboxylase by acetopyruvate[29,30] and by acetoacetone.[31]

Two-Substrate Reactions

The composition of the transition state in a two-substrate enzyme reaction depends on the detailed mechanism. In a reaction in which the enzyme reacts covalently, displacing a part of the first substrate and transferring it to the second, there appears to be no possibility of finding a truly comparable reaction in the absence of catalyst. It is not clear, at least to the author, how such a case can be approached theoretically. However, if the transition state were reached during the initial displacement reaction, for example in the formation of an acyl-enzyme, it is not difficult to imagine potential inhibitors which would be capable of forming enzyme adducts resembling tetrahedral intermediates in nucleophilic substitution.

There is less difficulty in situations of the simpler type in which the enzyme acts as a "marriage broker," providing an environment conducive to reaction between two substrates without itself becoming involved covalently in a displacement reaction. In such cases, it appears that binding of the transition state, which incorporates both substrate molecules, should exceed that which would result if their individual affinities for the enzyme were merely combined in a single molecule. This is apparent if one considers Scheme IV. Taking into account the same possible exceptions as in the one-substrate case, the dissociation constant for the transition state should be lower than the *product* of the dissociation constants for the two substrates by a factor at least as large as that by which the turnover number of the enzyme (the first-order rate constant for decomposition of EAB) exceeds the second-order rate constant for the nonenzymatic reaction under comparable conditions (eq 3).[31a]

Scheme IV

Nonenzymatic

$$A + B \xrightarrow{k_N} P$$

Enzymatic

$$E + A \overset{K_A}{\rightleftharpoons} EA$$

$$EA + B \overset{K_B}{\rightleftharpoons} EAB$$

$$EAB \xrightarrow{k_E} E + P$$

$$E + A + B \overset{K_N^{\ddagger}}{\rightleftharpoons} E + TX$$

$$K_A \uparrow\downarrow \qquad \uparrow\downarrow$$

$$EA + B$$

$$K_B \uparrow\downarrow$$

$$EAB \overset{K_E^{\ddagger}}{\rightleftharpoons} ETX$$

$$K_A K_B / K_{TX} \geq \frac{k_E}{k_N} \qquad (3)$$

binding ratio — rate ratio

This treatment could be expanded indefinitely for increasing numbers of substrates. It appears pointless to do so at present, since there are many ambiguities in relating chemical and kinetic mechanisms even for two-substrate reactions. Aside from theoretical considerations, the chances of achieving extraordinarily potent enzyme inhibitors tend to increase with the number of substrates participating in the overall reaction. Possible examples, in addition to those described in more detail below, include the potent inhibition of aminoacyl-tRNA synthetases by aminoalkyl adenylates,[32] of glutamine synthetase by a phosphorylated methionine sulfoximine,[33] and of lactic dehydrogenase[34] and pyruvate carboxylase[35] by oxalate. In each of these cases the inhibitor bears some resemblance to actual or conceivable reaction intermediates and possesses residues, not present in the substrates individually, which may serve as additional points of attachment.[35a]

(27) L. N. Johnson and R. Wolfenden, *J. Mol. Biol.*, **47**, 93 (1970).

(28) S. Wang, F. S. Kawahara, and D. Talalay, *J. Biol. Chem.*, **238**, 576 (1963).

(29) R. Davies, *Biochem. J.*, **37**, 230 (1943).

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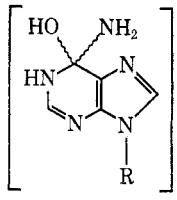
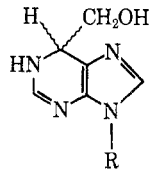
Inhibitors of Two-Substrate Reactions

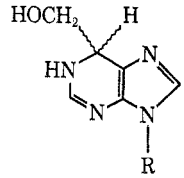
Adenosine deaminase catalyzes hydrolysis of amines, halides, alkoxides, oxygen, and sulfur leaving groups from the 6 position of purine ribonucleosides. Other mechanistic evidence suggests that these reactions occur by a mechanism analogous to that of nonenzymatic nucleophilic aromatic displacement reactions and that the transition state is reached during formation of a tetrahedral intermediate in displacement of the leaving group by enzyme or by enzyme-bound water.[36] It was hoped that compounds with a tetrahedral carbon atom at the 6 position might serve as suitable analogs for intermediates in direct water attack. Recently, diastereomeric 1,6-dihydro-6-hydroxymethyl adducts of nebularine (VIII and IX) became accessible through photochemical addition of methanol.[37] One of these compounds is an unusually potent inhibitor of mammalian and fungal adenosine deaminases, with a K_i



VII



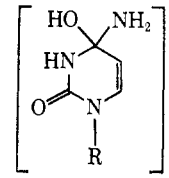
VIII



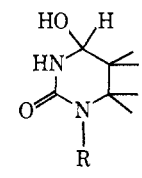
IX

value 200-fold lower than the K_i value for inosine (the substrate for the reverse reaction). Examination of space-filling models shows that the hydroxymethyl group of VIII can be rotated so that oxygen occupies the same position as it occupies in one diastereomer of the proposed intermediate VII for direct water attack. The exocyclic methylene group of VIII is in the position which would be occupied by the variable leaving group in VII. The other diastereomer, IX, is relatively noninhibitory, which is understandable if the enzymatic reaction involves stereospecific displacement from one side of the purine ring. The absolute stereochemistry remains to be determined; the same diastereomer, however, is observed to be most effective for both mammalian and fungal enzymes. This suggests that both enzymes, which differ greatly in physical properties and chemical composition, catalyze direct water attack from the same side of the ring. Substitution of one or two methyl groups for hydrogens on the exocyclic methylene group in VIII results in drastic losses in inhibitory power, indicating the close tolerance required for a good fit.

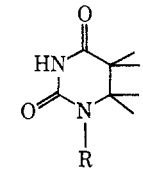
Similar studies with bacterial cytidine deaminase show that 3,4,5,6-tetrahydrouridine, kindly provided by Dr. A. R. Hanze,[38] is a competitive inhibitor (XI) bound with a K_i value approximately three orders of magnitude lower than the K_m value of the substrate cytidine.[39] In constrast, 5,6-dihydrouridine (XII) and the product uridine (XIII) are bound with K_i values higher than the K_m of cytidine. This enzyme is believed to act by a mechanism similar to that of adenosine deaminase, proceeding through a tetrahedral intermediate such as X. A tetrahedral carbon at the corresponding position distinguishes XI from XII and XIII. This presumably accounts in part for its unusual inhibitory properties and tends to support the proposed mechanism.[8] The inhibitor is also active against mammalian deaminases, but the pattern of inhibition appears to be more complex.[40]



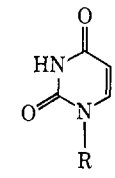
X



XI



XII



XIII

Several hydrolases for glycosides are powerfully inhibited by the corresponding lactones[41-43] and lactams.[44] Pig epididymal β-*N*-acetyl-D-glucosaminidase is inhibited by 2-acetamido-2-deoxyglucono-(1–5)-lactone with a K_i value of 5×10^{-7} *M*, whereas K_m values for pyranoside substrates fall in the range $1\text{–}3 \times 10^{-3}$ *M*.[43] This enzyme resembles egg-white lysozyme in many characteristics of the catalyzed reaction and may proceed by a similar mechanism. Model building shows that the lactone prefers a half-chair conformation (XV) like that adopted by the hypothetical carbonium ion generated in lysozyme action,[45] and the powerful inhibition by lactones may be due to steric and electrostatic resemblance to such in intermediate (XIV).[43]

In each of these reactions water is the second substrate, and its K_s value is unknown. If, as seems probable for the deaminases, the mechanism involves direct reaction between the substrates, and if K_s for water exceeds 1 *M*, this has the effect of raising the expected value of the dissociation constant of a transition-state analog (relative to K_s for the substrate hydrolyzed) *above* the value expected if water were more tightly bound (eq 3) and above the value which would be expected for a one-substrate reaction (eq 2). It is difficult to obtain information about water binding, due in part to the fact that it is not possible to change water activity as an isolated variable in water solutions.

synthesis with a twisted (and therefore strained) peptide bond [B. Lee, *J. Mol. Biol.*, **61**, 463 (1971)]. I am grateful to several of these authors for communicating their results in advance of publication.

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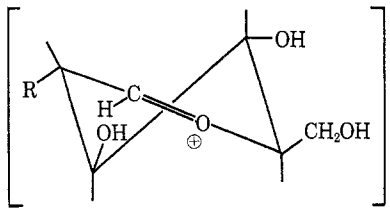
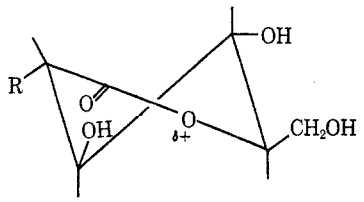


XIV



XV

Studies on these and other hydrolases in mixed solvents show that observed "V_{max}" values fall off with decreasing water concentration.[46] Interpretation of these effects is complicated by possible effects of changing solvents on several kinetic parameters;[47] however, they suggest that substrate water is not tightly bound.

Some hydrolases, such as myosin, show high specificity for water as opposed to alcohols in competition experiments;[48] however, it is not clear whether this is due to a selective binding site for substrate water or to details of the catalytic step which might serve to exclude alcohols. In the case of extracellular enzymes with a purely hydrolytic function, the pressure of natural selection might be expected to have resulted in the evolution of enzymes with high K_S values for water, permitting these enzymes to function efficiently (*i.e.*, without water saturation) in dilute aqueous solution. In cases where K_S for water exceeds 1 M and eq 3 applies, this will decrease the expected ratio of K_A to K_{TX}. However, the observed "V_{max}" may be much lower than that which would be observed if water concentration could be made infinite, so that the theoretical rate ratio may also be lower than that actually observed.

Do Enzymes Change Shape During Catalysis?

The transition state for enzyme-catalyzed reactions differs considerably in energy from the ground state and from the Michaelis complex. Crystallographic evidence suggests that structural changes occur when complexes with substrates or substrate analogs are formed by egg white lysozyme,[49] carboxypeptidase A,[50] and lactic dehydrogenase.[51] Changes in proton affinity and spectral characteristics, which may represent changes in protein structure, have been observed when ligands including substrates are bound by chymotrypsin[52] and by ribonuclease.[53] These changes may be related to changes which occur when enzyme–substrate complexes are formed.

In view of the likelihood that the enzyme constitutes a "super-chelating agent" for the altered substrate in the transition state, it is reasonable to suppose that additional changes in structure might occur during further reaction proceeding from the Michaelis complex. The powerful forces of attraction between substrate and enzyme presumably reach a maximum in the transition state. Unless the enzyme possesses remarkable rigidity, it seems improbable that these new forces could take effect without producing some change in the configuration of the enzyme.

There is little direct or unambiguous evidence to suggest whether structural changes do occur in enzymes during the events described by the turnover number. Many hydrolases exhibit zero or negative volumes and entropies of activation, and Laidler[54] has attributed the negative values to changes in enzyme structure or increased electrostriction of the solvent. For most hydrolases the additional possibility exists that V_{max} may actually describe the pseudo-first-order bimolecular solvolysis of an enzyme–substrate complex. Bimolecular solvolysis reactions are often accompanied by zero or negative entropies and volumes of activation;[55] it might therefore be possible to explain the observed results without reference to any special properties of the enzyme. More work of this type is needed, particularly for enzymes which catalyze reactions of other types, preferably reactions for which activation parameters in the absence of enzyme are known.

By trapping the enzyme in a mockery of the normal catalytic event, analogs of highly reactive intermediates might be expected to form stable enzyme complexes resembling to some degree the enzyme–substrate complex in the transition state. Since these complexes are stable, their structures can be determined by exact physical methods and might help to reveal the nature and extent of distortion which an enzyme may undergo during catalysis. Crystals of chicken muscle triosephosphate isomerase show negligible changes in X-ray diffraction pattern when soaked in saturating substrate

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analogs such as inorganic phosphate or the phosphomonoester of ethylene glycol. A small reversible contraction of the unit cell (1.7% by volume) occurs in the presence of saturating concentrations of α-glycerophosphate, an inhibitor which combines structural features of both triosephosphates and appears to be slightly more tightly bound than either. When crystals are soaked in saturating concentrations of 2-phosphoglycollate (the potent inhibitor VI which resembles the hypothetical enediolate intermediate), a major change occurs in the X-ray diffraction pattern, including a reduction of approximately 5% in the volume of the unit cell. Contraction occurs without major disordering of the crystal structure and is fully reversed when the inhibitor is removed.[27]

A change in unit cell dimensions may result from changes in tertiary structure or quaternary structure of the protein or merely from a rearrangement of molecules in the crystal lattice; further crystallographic studies will be required to decide between these alternatives. However, the magnitude of the change in crystal structure is positively correlated with the tightness of binding of inhibitors in solution and with the degree to which saturating concentrations of inhibitors protect the enzyme in solution against thermal inactivation.[14] It therefore seems likely that the contraction of the unit cell represents a change in protein conformation.

These findings suggest that this enzyme may undergo a small change in structure as it passes from the ground state to the Michaelis complex, followed by additional larger change in structure as it passes to the transition state. This is understandable if the altered substrate in the transition state (and its analogs) possesses additional points of interaction with the enzyme, not present in the substrate or its analogs.

Possible Developments

During evolution, enzymes have presumably been selected for structural complementarity to the altered substrate in the transition state of the reaction catalyzed. Exact structural techniques, in conjunction with appropriate analogs, may make it possible to define the approximate structure of this transition state. The strong forces of attraction should tend to stabilize analogs and protein binding residues in position, leading to sharp diffraction patterns. In conjunction with structural studies, equilibrium binding properties of analogs may also be helpful in elucidating the effects of pH, ionic strength, temperature, pressure, and allosteric effectors on catalytic activity.

Analogs of substrates which are intermediates in metabolic pathways would normally be expected to show affinity for two or more enzymes, including those responsible for substrate formation and breakdown. In contrast, analogs approaching the structure of the transition state for a particular reaction should often show a high and unique affinity for the enzyme or enzymes responsible for that reaction in a given organism. This may help to reduce undesirable side effects of antimetabolites, the design of which is based on these principles. The affinity and specificity of these analogs also recommend them for use as protective agents against heat inactivation and other forms of nonspecific destruction and as ligands for affinity chromatography. If analog affinity of this type can be combined with reactivity as a protein derivatizing agent, it may be possible to generate irreversible inhibitors with enhanced potency and selectivity.

It is a pleasure to acknowledge the work of Mr. R. M. Cohen, Dr. Ben Evans, and Mr. L. D. Byers, who have been responsible for the development of analogs in this laboratory. This work was supported by research grants (GM-12725 and GM-18325) and a career development award (AM-08560) from the National Institutes of Health.

Chemically Induced Dynamic Nuclear Polarization (CIDNP). I. The Phenomenon, Examples, and Applications

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Students of nmr spectroscopy are taught that, when presented with a spectrum taken under normal high-resolution conditions, they may expect to observe absorption signals with relative areas proportional to the relative concentrations of the nuclei under inspection. In the past few years, spectra taken of certain reacting systems have transgressed this rule with signal enhancements of several orders of magnitude over the expected intensities. Figure 1A illustrates such posi-

Harold Ward studied at Southern Illinois University and Massachusetts Institute of Technology and worked as a postdoctoral fellow with Andrew Streitwieser and Mark Whiting. He joined the staff at Brown University in 1963, where he is now Professor of Chemistry. His early research interests in hydrocarbon photochemistry have been ameliorated by combination with CIDNP applications.