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Biophysical Chemistry 105 (2003) 559–572

Biophysical Chemistry

www.elsevier.com/locate/bpc

Thermodynamic and extrathermodynamic requirements of enzyme catalysis

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Received 24 October 2002; received in revised form 22 January 2003; accepted 22 January 2003

Abstract

An enzyme's affinity for the altered substrate in the transition state (symbolized here as $S^{\ddagger}$) matches the value of k_{cat}/K_m divided by the rate constant for the uncatalyzed reaction in water. The validity of this relationship is not affected by the detailed mechanism by which any particular enzyme may act, or on whether changes in enzyme conformation occur on the path to the transition state. It subsumes potential effects of substrate desolvation, H-bonding and other polar attractions, and the juxtaposition of several substrates in a configuration appropriate for reaction. The startling rate enhancements that some enzymes produce have only recently been recognized. Direct measurements of the binding affinities of stable transition-state analog inhibitors confirm the remarkable power of binding discrimination of enzymes. Several parts of the enzyme and substrate, that contribute to $S^{\ddagger}$ binding, exhibit extremely large connectivity effects, with effective relative concentrations in excess of 10^8 M. Exact structures of enzyme complexes with transition-state analogs also indicate a general tendency of enzyme active sites to close around $S^{\ddagger}$ in such a way as to maximize binding contacts. The role of solvent water in these binding equilibria, for which Walter Kauzmann provided a primer, is only beginning to be appreciated.

Keywords: Transition state; Binding; Enzyme catalysis; Inhibition; Thermodynamics

1. Introduction

As he laid the foundations for the present understanding of protein folding, Walter Kauzmann identified the pervasive influence of solvent water on cellular processes, and the importance of considering solution thermodynamics before seeking more exotic explanations of the rates and equilibria on which life depends. As an undergraduate at Princeton, I had the good fortune of listening to Professor Kauzmann's lectures on physical chemistry. Those lectures offered a bracing antidote to the encyclopedic monotony with which organic chemistry was taught in those days. Walter did not gloss over the difficulties. In one of his more delphic utterances, he once exclaimed ''Anyone who thinks that he understands entropy is crazy!''

The visionary biochemist Fritz Lipmann, with whom I carried out my doctoral work, had already begun to teach biochemists the importance of free

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doi:10.1016/S0301-4622(03)00066-8

energy changes during metabolism. During that period, I also learned from William Jencks and Frank Westheimer that the reactivities of organic compounds could be described in the language of physical chemistry, bringing the genuinely organic chemistry of living systems within the reach—if not necessarily the grasp—of people with a quantitative bent. When I returned as an assistant professor to Walter's department, I benefited from his hunch that the future vitality of academic chemistry lies in its application to biological questions. The impact of his teaching, on me as on so many others, has been remarkable.

2. Thermodynamic requirements of an efficient catalyst

Enzyme–substrate interactions have long been recognized as representing an extreme expression of structural complementarity in biological chemistry. One of the earliest observations to emerge from studies of catalysis by enzymes, and from heat inactivation of enzymes in the presence of small molecules, was that enzymes bind substrates reversibly, forming complexes that appear to dissociate at concentrations usually slightly higher than those that are present physiologically. Unreactive structural analogs of the substrate are usually found to be reversible inhibitors. This suggests that substrates and substrate analogs vie for a place on the enzyme, in accord with the possibility that ES complexes are also formed during the catalytic transformation of the substrate (for a review, see [1]). This view led to the well-known proposal by Emil Fischer that substrates fit enzymes as a key fits a lock. Captivated by that image, medicinal chemists occupied themselves for many decades in designing substrate-like inhibitors, hoping that they would be strong and enzyme-specific.

But was Fischer's view correct? In considering that question, it is helpful to focus attention on the various stages through which a substrate passes as it undergoes chemical activation. To enhance the rate of a reaction, a catalyst must enhance the substrate equilibrium constant for attaining the transition state. As early as 1921, Polanyi recognized that a catalyst must bind a reactant with increasing affinity as the reactant is distorted toward the structure that it adopts in the transition state [2]. In his remarkable textbook, written only a few years later [3], Schwab explains:

> The energy barrier to be overcome is lowered in the adsorption layer because the activated state is strongly adsorbed and, therefore, in the adsorption layer, is less endothermic and therefore more often reached. Hence, it is not that the adsorbate is activated but that the adsorbate is (more) easily activated and is therefore, at equilibrium, present in the activated state to a greater percentage extent than in the free gas.

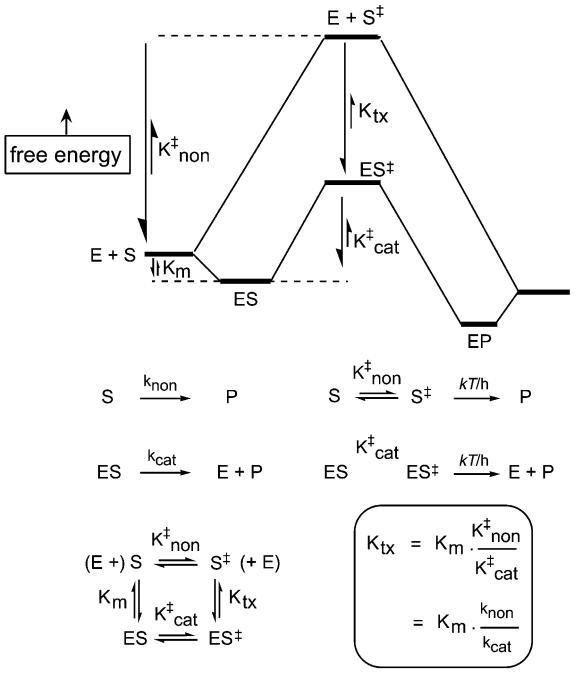
If 'active site' is substituted for 'adsorption layer', this statement contains the essence of our present view of how free energy changes accompany enzyme catalysis in aqueous solution.

3. 'Adsorption of the activated state' by enzymes

Many years after Polanyi's paper, and with no apparent knowledge of its existence, Linus Pauling [4] and William Jencks [5] speculated that it might be possible to develop a powerful enzyme antagonist in the form of an unreactive compound, analogous in structure to $S^{\ddagger}$, the altered substrate in the transition state. Then, in 1969, the algebra in Scheme 1 was used to show that an ideal 'transition-state analog' should surpass a conventional substrate or product in its affinity for the enzyme, by a factor that matches or surpasses the very large (see below) rate enhancement that the enzyme produces [6].

Because of its startling implications for catalysis and inhibitor design, it seems worthwhile to consider some qualifications and questions raised by this scheme.

1. Must an enzyme bind $S^{\ddagger}$ more tightly than S? If enzymes did not stabilize transition states, no increase in rate would occur. The algebra of Scheme 1 tells us that to lower the free energy of activation, an enzyme must bind $S^{\ddagger}$ more tightly than S. Accordingly, the distinction that was once made between 'binding sites' and 'catalytic sites' appears meaningless, since catalysis depends on this transient increase in binding affinity. When two substrates are compared, specificity may appear in



Scheme 1. If equilibrium is maintained between the ground state and the transition state in dilute solution, then the formal dissociation constant of the altered substrate in the transition state (K_{tx}) is expected to be less than that of the substrate in the ground state (K_m), by a factor matching the factor by which the rate constant of the catalyzed reaction (k_{cat}) exceeds that of the uncatalyzed reaction (k_{non}). Effects of desolvation, charge separation or proximity in multisubstrate reactions can be considered to involve subpopulations of ES that depart from the mean in the usual statistical description of molecules in the ground state. At equilibrium, any of these subpopulations can be more reactive than ES, but can do so only to the extent that it is rare. Transition-state affinity may be underestimated if the mechanism of reaction in solution differs fundamentally from the mechanism of reaction at the enzyme's active site, or $k_{cat}K_m$ is limited by enzyme–substrate encounter. This relationship is not applicable to reactions involving quantum mechanical tunneling, and requires modification for reactions proceeding through covalent intermediates.

k_{cat} or in K_m, depending on the extent to which bonding differences in $S^{\ddagger}$ are already present in ES [6].

2. What happens on the path to the transition state? Transition state theory is silent about intermediates between ES and $ES^{\ddagger}$, leaving us free to speculate about points in between. Those intermediate species resemble a flock of sheep near a mountain pass. If we could illuminate the sheep with an instantaneous flash of light, we would observe that their population dwindled with increasing height. These sheep are not wandering with any purpose and have no inherent tendency to congregate near the most direct path to the transition state. The enzyme is designed in such a way that the rarer the species of the substrate (between S and $S^{\ddagger}$), the more tightly it is bound, near the path to the transition state [7].
3. Must the native enzyme be a pre-existing 'template' for the altered substrate in the transition state? No. Scheme 1 rests on no assumptions about the presence or absence of changes in the conformation of an enzyme during catalysis. Scheme 1 implies that an enzyme is a template that is in, or can easily adopt (i.e. without much distortion of the enzyme from its native structure, as expressed in free energy) a conformation complementary to that of the substrate in the transition state. The forces of attraction in the transition state are so strong that it would be surprising if some change in enzyme structure did not occur. Moreover, changes in enzyme conformation, as discussed below, are probably needed to reconcile maximal forces of attraction in the transition state (tending to involve a closed structure of the active site), with the conflicting need for rapid substrate access to, and product egress from, an open structure [8]. Structural evidence for such changes, obtained from the crystal structures of enzyme complexes with transition-state analogs, is now so abundant that it appears to be the rule rather than the exception.
4. What if the mechanisms of the spontaneous and enzyme-catalyzed reactions differ? Is tight binding of $S^{\ddagger}$ still required? Of the various pathways that may be available in water, the non-enzymatic reaction must follow that pathway that has the lowest free energy of activation. If that pathway differs radically in mechanism from the reaction at the active site, then it must have a lower free energy of activation than any reaction in solution that would more closely resemble that followed at the active site. The rate of the observed non-

enzymatic reaction would then be larger than the rate of any hypothetical non-enzymatic reaction that would be more appropriate for comparison. The rate ratio, and hence the binding affinity in the transition state, would have been underestimated accordingly. Thus, an unsuspected difference in mechanism does not weaken, but only tends to reinforce, the requirement that $S^{\ddagger}$ be very tightly bound [7].

5. What if the mechanisms of the spontaneous and enzyme-catalyzed reactions are similar, but the transition state for the enzyme reaction arises from a change in enzyme conformation, or the release of the product from the enzyme–product complex? If the rate of the enzyme reaction is limited by a physical event of that type, we are led to suppose that 'chemical' changes in the substrate occur more rapidly. Thus, the enzyme's ability to stabilize the altered substrate in the transition state for its chemical transformation, and therefore its binding affinity for that species, will once again have been underestimated.
6. Scheme 1 describes a reaction involving a single substrate. How large is the transition state affinity generated during one of the many enzyme reactions that involves a second substrate or a coenzyme? Scheme 1 is easily adapted to multisubstrate reactions, incorporating the likelihood that simple juxtaposition of two substrates—in a configuration appropriate for reaction—may go far to enhance the rate of such a reaction. Transition-state affinity for a multisubstrate reaction can be estimated by comparing the third-order rate constant $k_{cat}/K_{M(A)}K_{m(B)}$ with the second-order rate constant (k_{non}) for the uncatalyzed reaction between two substrates, A and B [7]. In such a case, it may be difficult in practice to determine whether chemical activation is actually occurring, because the upper limit of the advantage that could in principle be gained by restricting the rotational and translational motions of substrates in relation to each other is not yet well defined, but is almost certainly very large [9]. Preliminary experiments in the author's laboratory on hexokinase, peptide bond formation in the ribosome, alcohol dehydrogenase, and methyl transfer from SAM to amines, indicate that positive effects on the entropy of activation play a major role in enzyme catalysis of such reactions. In contrast, enzymes that catalyze single-substrate and hydrolytic reactions tend to do so by effects that are mostly enthalpic (see below). In these latter reactions, catalysis by approximation is not an option.
7. What is the meaning of transition-state affinity in an enzyme reaction during which covalently bound derivatives of the enzyme are formed, or in which there is quantum mechanical tunneling of protons? If an enzyme reaction involves the formation of a bond between the enzyme and part of the substrate (e.g. reaction of a serine residue at the active site of chymotrypsin with a peptide substrate, to form a serine ester that is hydrolyzed later), transition-state affinity cannot be estimated in the ordinary sense. Nevertheless, an equilibrium constant for 'transition-state interchange' can be estimated by comparing the enzyme reaction with the rate of reaction of the substrate with a model nucleophile [10], and numerous transition-state analog inhibitors have been prepared that resemble tetrahedral intermediates in the formation and breakdown of the acyl enzyme. In an enzyme reaction that, unlike its uncatalyzed counterpart, involves quantum mechanical tunneling of hydrogen atoms, the rate of reaction is substantially faster than it would be in the absence of tunneling [11]. Such cases are not amenable to the formalism in Scheme 1, unless the depth of the tunnel below the free energy barrier that would apply in the absence of tunneling can be estimated. If, in some particular case, the magnitude of the tunneling effect were relatively minor compared with other catalytic effects, Scheme 1 would tend to apply.
8. Is Scheme 1 adaptable to reactions that are susceptible to 'catalysis by desolvation'? All the species in Scheme 1, as it applies to enzymes that act in watery surroundings, are assumed to be present at equilibrium in dilute aqueous solution. It seems self-evident that water must be displaced from the substrate and from the enzyme's active site when they com-

bine to form an enzyme–substrate complex. Further changes in free energy of solvation occur during enzyme action, and during the course of the benchmark reaction in water. Thus, there can be little doubt that solvent water plays an important role in the catalytic effect that is observed [12]. In those cases in which transition states and their analogs are less polar than the starting materials, these transition states and their analogs are expected to be very tightly bound relative to the substrate in the ground state. Enzyme reactions involving thiamine pyrophosphate [13] and SAM [14] offer particularly clear examples of this kind of behavior. It should be remembered, however, that desolvation in the ground-state ES complex usually exacts a heavy penalty in free energy [15]. Thus, desolvation may provide a way of increasing k_{cat} and K_m, but tends to leave k_{cat}/K_m unaffected. The additional possibility exists, at least in principle, that solvent relaxation effects (as distinct from equilibria of solvation, which we have just considered) might limit the rate of a nonenzymatic reaction [16]. An enzyme might catalyze such a reaction by removing the substrate from water and reducing 'solvent friction' of this kind. In such a case, the enzyme's transition-state binding affinity might be overestimated, based on simple comparison of rate constants. However, solvent water is known to relax very rapidly, and solvent relaxation effects have been reported mainly for fast reactions such as photolysis [17]. In contrast, most biological reactions proceed very slowly indeed in the absence of enzymes (see below). Although the possibility cannot be ruled out, it would be surprising if solvent relaxation offered a major impediment to the progress of biological reactions in the absence of a catalyst.

9. Might distortion of the substrate, or its confinement to a high-energy configuration, enhance the reactivity of some fraction of substrate molecules in solution above the ordinary level? There are certainly reactions in which physical distortion of the substrate seems to play a role. Jencks has identified the strong inhibition of proline racemase by a planar proline analog as a possible example [5]. However, the rigidity of enzyme active sites is probably limited [18], so that major physical distortion—in which the substrate resembles a victim of Procrustes [19]—no longer seems likely to provide a very general mechanism for enzyme catalysis. In Schwab's words, quoted above, ''it is not that the adsorbate is activated, but that the adsorbate is easily activated and is therefore, at equilibrium, present in the activated state to a greater percentage extent than in the (unbound state)''.
10. How is transition-state affinity expressed thermodynamically? Structural evidence from crystal diffraction and NMR spectra of enzyme complexes with transition state analogs, and the kinetic effects of mutating enzymes and substrates, indicate that enzyme–substrate complexes usually form new H-bonds or electrostatic interactions in the transition state that were not present in the ground state. That tendency appears to be consistent with the observation that in reactions involving a single substrate, enthalpies of activation are consistently more favorable for the enzymatic than for the uncatalyzed reaction [20]. With one exception [21], entropy changes tend to be relatively small and unpredictable in sign. That does not mean that they can be neglected. The entropy loss associated with bringing two molecules together in water can be offset substantially or completely by the entropy gained when site-bound water molecules are released into solution [22–24]. Thus, entropy is gained with the formation of salt bridges between oppositely charged groups, or the mating of two hydrophobic surfaces [25].

4. Transition-state analogs vs. substrate analogs

Soon after the derivation of the relationships in Scheme 1, this scheme was first tested deliberately in the design of the potential transition-state analog 2-phosphoglycolate, as an inhibitor of triosephosphate isomerase, and showed results that seemed promising [6]. Later ^{13}C-NMR showed that 2-phosphoglycolate is bound as a species that is very

rare in solution, and that its dissociation constant from this enzyme is approximately five orders of magnitude lower than the K_m value for glyceraldehyde 3-phosphate [26].

By 1976, more than 60 transition-state analog inhibitors had been identified, targeting enzymes of every mechanistic class that was then recognized [27]. These inhibitors furnished a test of the general mechanism on which their design had been predicated, and a new tool that could be used to uncover the structural details of enzyme–substrate interaction, using exact structural methods. Several transition-state analog inhibitors have K_i values of less than 10^{-12} M, and in the remarkable case of methionine sulfoximine phosphate (an inhibitor of glutamine synthetase), exchange experiments seem to place an upper limit of 10^{-18} M on the dissociation constant of the E–I complex [28]. Nature has also been found to imitate art, in that microorganisms produce transition-state analogs as antibiotics that include the leupeptins (peptide aldehydes that inhibit proteases by forming enzyme adducts resembling tetrahedral intermediates in peptide hydrolysis) and coformycin (an inhibitor of adenosine deaminase that mimics a tetrahedral intermediate in the hydrolytic deamination of adenosine). The diurnal rhythm of CO_2 fixation in plants is now known to be controlled by fluctuations in the concentration of a naturally occurring transition-state analog inhibitor of ribulose 2,6-bisphosphate carboxylase. The continuing usefulness of this method for generating powerful inhibitors [7,10,27,29–31] has been matched by practical applications that include the herbicide Roundup™ (i.e. glyphosate, an inhibitor of aromatic amino acid biosynthesis), inhibitors of the angiotensin-converting enzyme that are used to treat high blood pressure (Capoten™ and Vasotec™) [25] and a group of statine-containing inhibitors of the HIV protease that are used to control the spread of HIV infection [30]. A practical advantage of transition-state analog inhibitors, as drugs, is that they tend not only to be very potent, but also to be specific for the particular enzyme whose activated complexes they resemble, just as the transition state is unique to that reaction.

In contrast, substrate analogs tend to be relatively non-specific and may inhibit any of the several enzymes for which a particular substrate serves as a substrate or product, sometimes in more than one pathway. Moreover, the substrate (or product) in the ground state must be relatively weakly bound if catalysis is to ensue, because an enzyme can enhance the rate of reaction only to the extent that it binds $S^{\ddagger}$ more tightly than S. Substrate analogs are therefore expected to be relatively weak inhibitors, and it is not surprising that few substrate analogs have been reported that are significantly more tightly bound than the substrates themselves [32]. That does not mean, however, that the enzyme–substrate complex is uninteresting. An important question raised by Scheme 1, for which there does not as yet appear to be a general answer, is how an enzyme manages to bind $S^{\ddagger}$ very tightly in the transition state but avoids binding S almost as tightly, given their many similarities in structure. In some cases, such as triosephosphate isomerase in the ground state, infrared measurements suggest that strain may be present in the enzyme–substrate complex [33]. In other cases, such as adenosine and cytidine deaminase, NMR experiments suggest that substrates are initially bound by enzymes in forms that are closely related in structure and energy to forms of the substrate that are most abundant in free solution [34].

5. Extrathermodynamic requirements of an efficient catalyst

5.1. Stereochemical inversion at the scissile bond

In the absence of enzymes, biological reactions take place very slowly (see below), indicating that their transition states differ markedly in energy and structure from substrates in the ground state. The stereochemistry of the transition state might be expected to be important in view of the need for exact structural complementarity to an enzyme's active site, which is itself asymmetric. In displacement reactions at sp^2-hybridized carbon, transition states with tetrahedral-like carbon atoms are generated, with an inherent chirality that is not present in reactants or products. This chirality can sometimes be detected by comparing the effectiveness of two diastereomeric transition-state analog

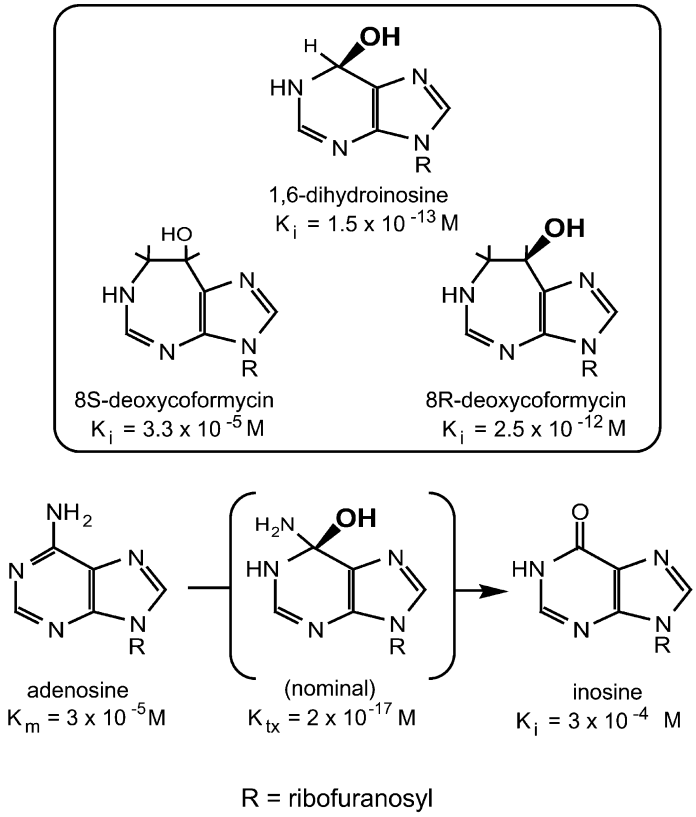


Fig. 1. Dissociation constants of ligands from calf intestinal adenosine deaminase.

inhibitors. A dramatic example is furnished by 2′-deoxycoformycin, whose 8*R*-form is bound by adenosine deaminase seven orders of magnitude more tightly than the substrate, whereas the 8*S*-isomer is less tightly bound than the substrate [35]. Fig. 1 shows these inhibitors, along with the 1,6-hydrate of inosine, which is even more tightly bound [36], presumably because it has the correct ring size. The chirality of the bound hydrate, like that of the 8*R*-form of deoxycoformycin, reflects the side of the purine ring from which zinc-bound hydroxide ion is believed to mount its attack [37].

At first glance, we might guess that the need for inversion of configuration in the transition state would vanish if, as in glycosidase reactions such as that catalyzed by hen egg white lysozyme [38], the product retains the same stereochemistry as the reactants. Such behavior usually implies, however, that a group at the active site forms a covalent intermediate by displacing a part of the substrate, which is then itself displaced by water. In such 'double displacement' [39] reactions, there are actually two transition states, formed on the pathway to and from a metastable covalent intermediate. Both transition states presumably require stabilization, and could be modeled in the design of an inhibitor.

5.2. Conflicting structural requirement of substrate access and product egress vs. the maximization of transition state affinity

The affinity of an enzyme for the altered substrate in the transition state, and its ability to distinguish between S and $S^{\ddagger}$, presumably depend on structural complementarity between the host

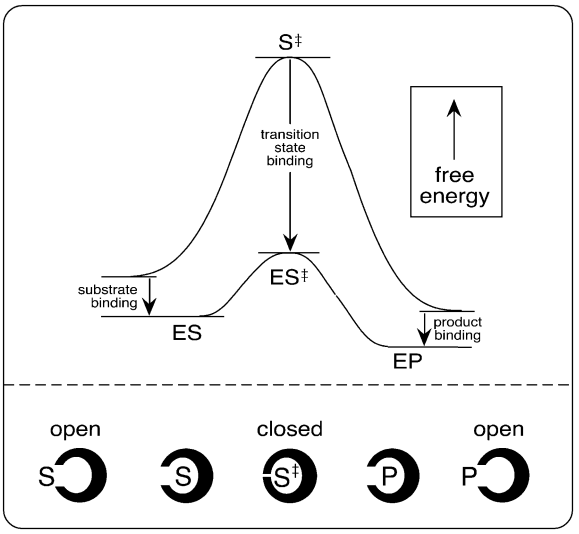


Fig. 2. A role for conformation changes in enzyme catalysis. An open configuration of the enzyme permits diffusion-controlled access of substrate and egress of product. A closed configuration of the enzyme allows maximal contact between the enzyme and substrate in the transition state.

and its guest. We might guess that optimal affinity would be observed if the enzyme's active site, in its native or most stable form, were rigidly designed to form a perfectly fitting template for $S^{\ddagger}$. It was, therefore, a surprise when, in 1970, the crystal structure of one of the first transition-state complexes revealed a tendency of the enzyme structure to change with inhibitor binding. Crystals of triosephosphate isomerase contracted by 7% along their major axis when 2-phosphoglycolate was bound, and expanded to their original size when the inhibitor was removed by dialysis [40]. The crystal structures of other enzyme complexes with transition-state analog inhibitors exhibit a similar tendency of the enzyme to surround the substrate in its activated forms [41–43].

Fig. 2 suggests a probable reason for that tendency [8]. Many enzymes are found to act with apparent second-order rate constants (k_{cat}/K_m) that approach the limits imposed by the diffusion of enzyme and substrate in solution. That implies that many enzymes tend to be open to substrate access most or all of the time, i.e. that an enzyme's active site tends to remain in an 'open' configuration before it binds the substrate. The movements mentioned in the previous paragraph would seem understandable if the substrate were first bound weakly by this open form of the enzyme, and the active site were then to change in such a way as to surround the altered substrate in the transition state (Fig. 2). That would allow maximization of the solid angle of contact, and of attractive forces of attraction, in the transition state. Thus, alternation of the enzyme between open and closed configurations might allow rapid substrate access to be reconciled with tight binding in the transition state. The only formal requirement would seem to be that this motion of the enzyme not be intrinsically costly from an energetic standpoint, i.e. that the enzyme be able to move easily between two structural extremes, as in the opening and closing of a first baseman's glove representing two domains of the protein. Examples of that kind of motion, first noted during substrate binding by hexokinase at low resolution [44], have now become commonplace. Structural studies, exemplified by Charles Carter's observations on cytidine deaminase [45], show how the potential energy of the active site's conformation changes as the reaction progresses.

To the extent that such behavior is general, it would probably be a mistake to attempt to design a drug to fit the native (or 'open') configuration of the enzyme, because much higher affinity is achieved in the transition-state complex, in its 'closed' configuration. The latter structure, rather than the open structure, would seem to offer a better template for improvements in drug design.

6. Does transition state affinity depend on a few or on many interactions?

Differences in structure between S and $S^{\ddagger}$ are apparently even more obvious to an enzyme's active site than they are to a chemist, viewing their structures on paper. The fact remains that many of the structural features of S are usually present in $S^{\ddagger}$. The differences between them are so few in number that an enzyme's ability to maintain such a sharp, quantitative distinction between these structures, in terms of binding affinity, still seems baffling. Confronted by the relative

binding affinities shown in Fig. 1, a reductionist might guess that any of those several structural features that distinguish the transition state from the ground state might, by itself, tend to confer very high binding affinity on a potential inhibitor. Does the ability of an enzyme's active site to distinguish between S and $S^{\ddagger}$ depend on a few local interactions at the sites of difference, or on an ensemble of interactions that involves every part of the substrate's structure?

The answer to that question, insofar as it has been learned from experiments with transition-state analog inhibitors, seems to be that enzymatic transition states exploit multiple interactions to the fullest extent possible. Experiments involving individual mutations of either the protein or the ligand show that elimination of any single binding interaction in one of these tight complexes can result in catastrophic losses of binding affinity, even if they are distant from the site of chemical transformation of the substrate. Conversely, the gain in binding strength that individual interactions derive from the fact that their binding determinants are properly connected in adenosine [46] and cytidine [47] deaminases approach the very large increments ($\sim 10^8$ M in effective concentration) that were estimated in theory by Page and Jencks [9]. The fact that catalysis is intensely dependent on the structural context of the substrate group that is being transformed reflects a long-recognized property of enzymes: their frequent failure to act on substrates smaller than their natural substrate, although these truncated versions of the substrates do not differ from it in inherent reactivity and are presumably capable of entering the active site. What is surprising is that seemingly irrelevant parts of the substrate (such as a ribose hydroxyl group in the case of cytidine deaminase [47] or the phosphoribosyl group in the case of OMP decarboxylase [48]) sometimes play an overwhelmingly important role in enhancing k_{cat} rather than K_m. The behavior of OMP decarboxylase is shown in Fig. 3.

Daniel Koshland, who was the first to recognize the oddity of this behavior, suggested that it might serve as a means of organizing the enzyme's binding site into a catalytically active configuration [39]. In relatively rigid substrates such as these, an unchanging scaffold of non-participating groups in the substrate appears to provide a setting that is essential for ideal expression of an enzyme's catalytic action [45]. That behavior seems understandable by analogy with binding phenomena such as the 'chelate effect', in which one binding interaction introduces structural constraints that greatly enhance the probability of a second binding interaction [9]. Individually, these interactions are so weak as to be unobservable in simple model systems in water. Together, they achieve great strength. In this way, extremely high affinities might in principle be generated from ordinary H-bonds, electrostatic attraction and non-polar interactions.

7. How large an affinity is expected of an ideal transition-state analog inhibitor?

Scheme 1 implies that the rate enhancement produced by an enzyme determines its susceptibility to inhibition by an ideal transition-state analog. Thus, the most reactive of several substrates should also yield the strongest inhibitor, a possibility that has been confirmed by comparison with the K_i values for several protease inhibitors [49–52]. Similarly, if the structure of the enzyme is altered by mutagenesis, the enzyme variant with the greatest catalytic power should be most sensitive to inhibition by an ideal transition-state analog inhibitor. This latter expectation offers a means of separating enzymes, using a transition-state analog inhibitor to elute enzymes in order of decreasing turnover number from a conventional substrate affinity column [53].

The slow progress of biological reactions in the absence of catalysts furnishes a standard by which to judge the catalytic power of existing enzymes, and their consequent susceptibility to inhibition by ideal transition-state analog inhibitors. By comparing different reactions with respect to the rate enhancements that enzymes produce, it should be possible to identify those enzymes that offer the most sensitive targets for inhibitor design. However, most biological reactions proceed so slowly in the absence of enzymes that their uncatalyzed rates in water have never been measured.

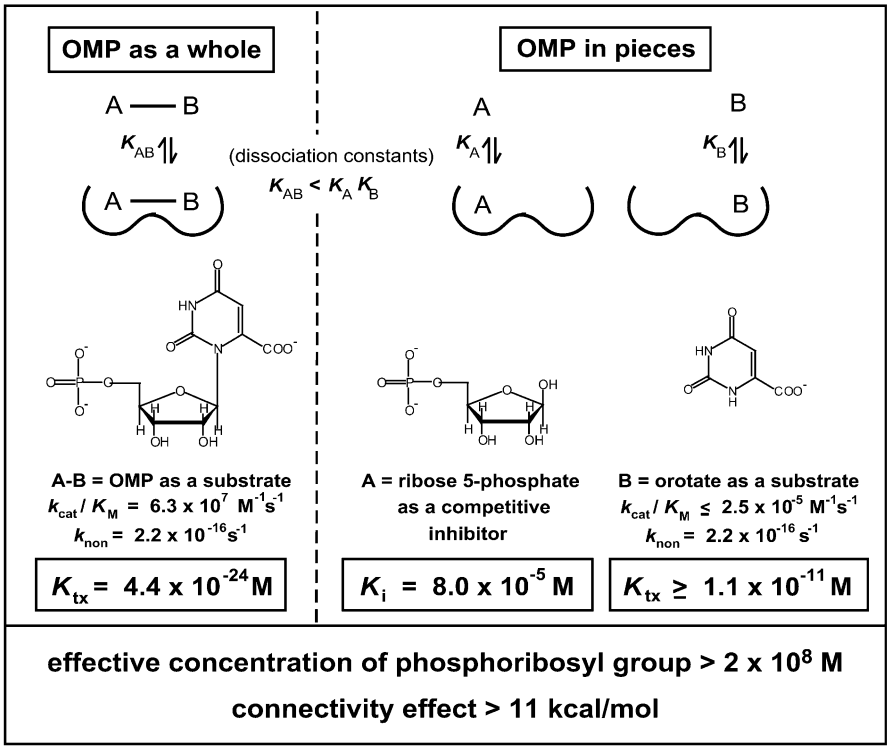


Fig. 3. Comparison of the binding affinity of yeast OMP decarboxylase for OMP, in the transition state for its decarboxylation, with orotic acid in the transition state for its decarboxylation, and for ribose 5-phosphate, the 'missing piece', as a competitive inhibitor. The effective concentration of the substrate in the transition state, $>10^8$ M, expresses the advantage the substrate gains by having its parts properly connected.

Many of these reactions would be impossible to observe, even at elevated temperatures, if they doubled in rate for each 10 °C temperature increases, as described by an ancient rule of thumb attributable to Harcourt [54]. However, recent experiments have shown that many biological reactions, proceeding spontaneously in solution in the absence of a catalyst, tend to become more temperature-dependent as their rates decrease. Instead of doubling in rate ($\Delta H^{\ddagger} = 12$ kcal/mol), the very slow decarboxylation of orotidine 5′-phosphate increases by a factor of 12.5 as the temperature increased from 20 to 30 °C ($\Delta H^{\ddagger} = 44$ kcal/mol) [55]. This tendency makes it possible to follow even very slow reactions in neutral solution in sealed tubes at high temperature, using Arrhenius plots to extrapolate the rate to room temperature. We have found that the progress of some uncatalyzed reactions is slow even on a geological time scale (Fig. 4). At pH 7 and 25 °C, typical half-times are 450 years for the hydrolysis of peptide bonds [56], 180 000 years for the hydrolysis of phosphodiesters [57] and 8 000 000 years for the hydrolysis of O-glycosides [58]. For the decarboxylation of orotidine 5′-phosphate, the last step in pyrimidine biosynthesis, the half-time is 78 000 000 years, implying an enzymatic rate enhancement of 10^{17}-fold and a dissociation constant of less than 10^{-23} M for the enzyme–substrate complex in the transition state [55].

8. Analyzing transition-state affinity: the confounding role of water

How is it possible for an active site to generate such extreme affinities in watery surroundings, and

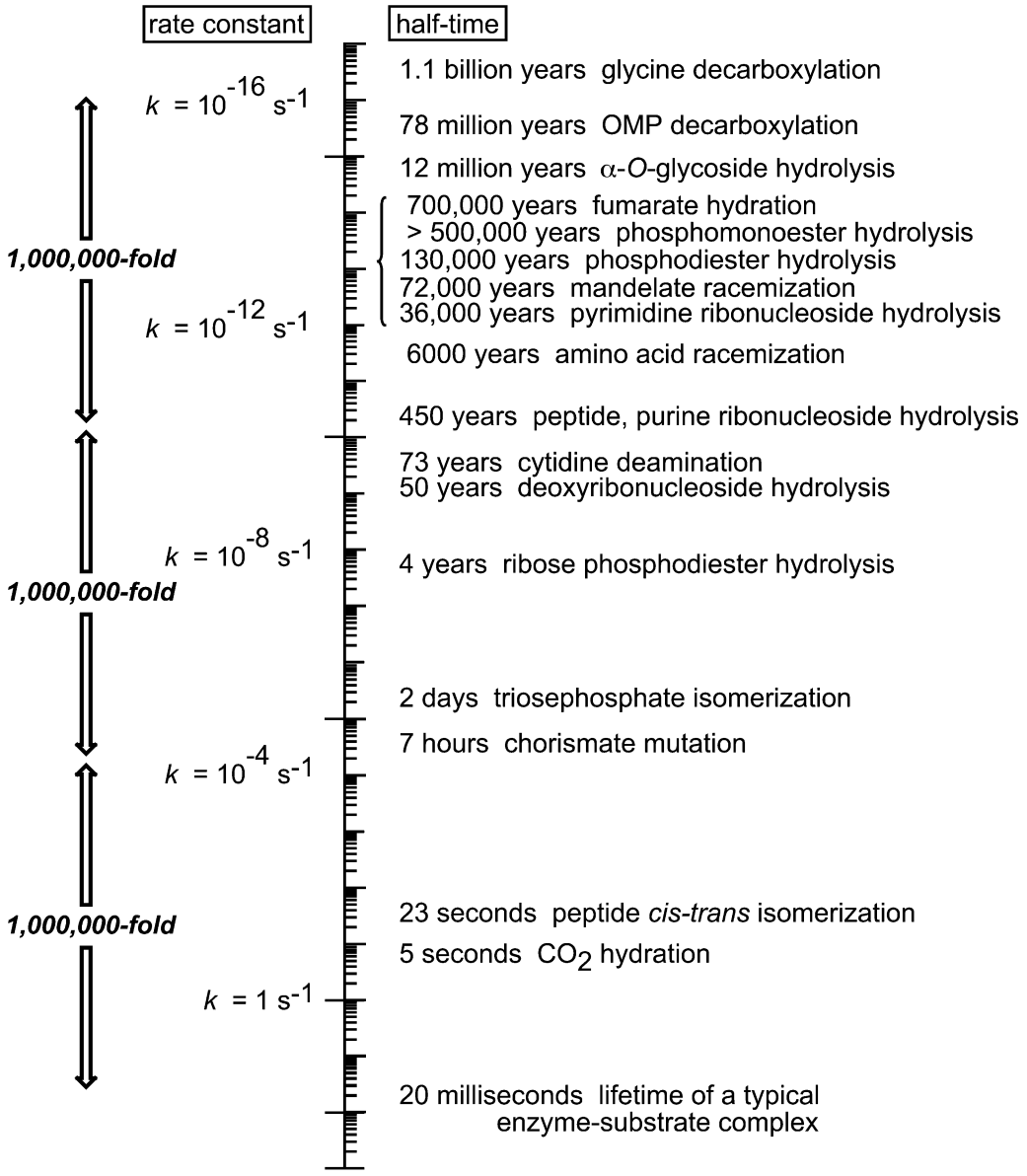


Fig. 4. Rate constants and half-times of biological reactions proceeding spontaneously in water in the absence of enzymes.

to exhibit such acute discrimination between similar molecules using conventional forces of attraction? That question seems baffling in view of the well-known weakness of non-covalent interactions in water, as indicated by the behavior of model systems. It can be addressed by measuring the binding affinities of ground-state and transition-state analogs and examining their enzyme complexes by exact structural methods. The chelate effect, mentioned in Section 6, appears to be capable of generating extremely high affinities from ordinary forces of attraction: H-bonds, electrostatic interactions and non-polar interactions. Observations on the transition-state binding affin-

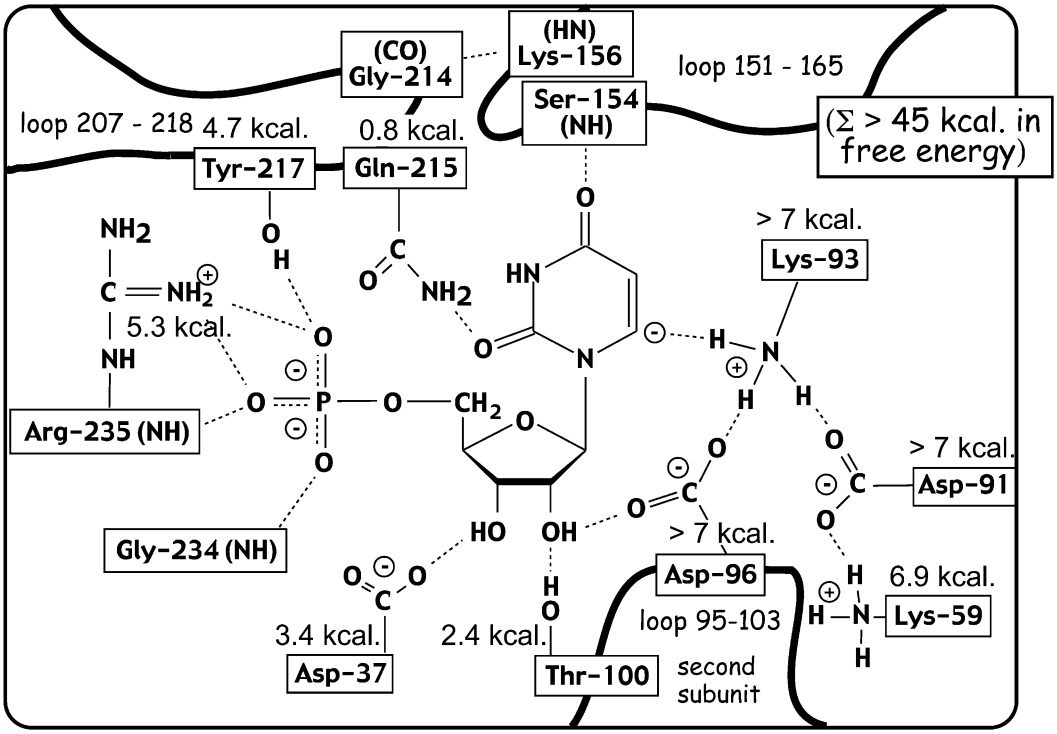


Fig. 5. Effects on the transition-state binding affinity of yeast OMP decarboxylase [59].

ity of OMP decarboxylase [59], shown in Fig. 5, indicate that any one of the forces involved in stabilizing the transition state, if it is eliminated by chemical alteration or mutagenesis, results in drastic losses in binding affinity. Conversely, the introduction of a new binding interaction in the transition state could in principle result in the very large increase in binding affinity that is needed to explain the rate enhancement that an enzyme produces.

To the extent that chemists can satisfy the delicate conformational requirements of these powerful interactions, major improvements in the design of stable inhibitors and man-made catalysts should be possible in future.

Acknowledgments

This work was supported by the National Institutes of Health research grant GM-18325.

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