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The path to the transition state in enzyme reactions: a survey of catalytic efficiencies†

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ABSTRACT: A survey of k_{cat}/K_m values supports the view that most enzymes combine with substrates at rates that approach the limits imposed by diffusional encounter. The closeness of that approach precludes the obligate participation of any rare species of the substrate, or any rare species of the enzyme, in productive binding. Proceeding from the ground state through the transition state, the enzyme–substrate complex is assumed to remain in a state of quasi-equilibrium with the free enzyme and the unbound substrate in solution. Because the forces of attraction between an enzyme and substrate approach a maximum in the transition state for substrate transformation, the enzyme and substrate are also likely to approach a maximal state of distortion from their native ground-state structures at that stage in the reaction, furnishing a natural explanation of induced fit. There is now abundant evidence, from mutations of enzymes and substrates, that interactions between the enzyme and substrate, using ordinary chemical forces of attraction, are so strongly synergistic that they may suffice to explain the very high affinities that are achieved in the transition state. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: enzyme reactions; transition state; catalytic efficiency

WORKING WITH BILL JENCKS

Biological chemistry was originally the province of medicine, in which William P. Jencks earned his most advanced degree. The visionary biochemist Fritz Lipmann, with whom W. P. J. had done postdoctoral work, was also a physician who had begun during the 1930s to teach biochemists the importance of free energy changes during metabolism as a guide to understanding the orchestration of biosynthetic processes. Clear as his vision was, Professor Lipmann's powers of expression in everyday conversation tended to leave ordinary mortals in doubt as to what he had actually seen. In 1959, after spending some months as a graduate student trying to understand what Professor Lipmann meant, one of us (R.W.) expressed the hope that a year taking course work at Harvard might prepare him for work in the Lipmann laboratory. Lipmann answered, 'You don't need to take courses. You need to get into the lab. You learn something that way. There's a young man setting up his laboratory in Nate Kaplan's department at Brandeis . . . ,' and his voice faded as he walked away.

A year spent in the Jencks laboratory went far to instill confidence that the ideas and experimental methods of physical organic chemistry could be brought to bear on living systems, and that the action of enzymes would one day be understandable in the quantitative language of physical chemistry. What was really needed was to establish the experimental facts. Bill said, 'nothing is more important than a good number,' and, during that period, began to show his students how to obtain those numbers. His first students (Bruce Anderson, Giovanni di Sabato, Gene Cordes and R.W.) were cowed by his ability to scour the literature in the morning, retaining all that he had read (then and at earlier stages of his career) and then writing carefully all afternoon. His manuscripts did not make easy reading, but Bill himself remained accessible, and maintained a keen awareness and concern for the wellbeing of many of the students in the department. We were not surprised to learn that our young mentor has considered psychiatry as an alternate field of endeavor.

Not all of Bill's students grasped the full significance of what they were doing, including R.W., who, auditing a course on physical organic chemistry at Harvard, was fascinated to hear Frank Westheimer explain one morning how revealing it might be to study the effect of pH on acetyl transfer reactions of the 1-acetyl-3-methylimidazolium ion. After the lecture, R.W. was able to murmur the news that he had actually completed those experiments the week before, and saw a moment of incredulity flit across Professor Westheimer's face, followed by a delighted smile. It became clear that two of the great minds in the field really did think alike.

'Getting a good number' became a gold standard for W.P.J.'s students and many of his less direct scientific

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progeny. Here, we survey below some numbers that may have a bearing on one of one of the more tantalizing features of enzyme action, the formation of an enzyme–substrate complex.

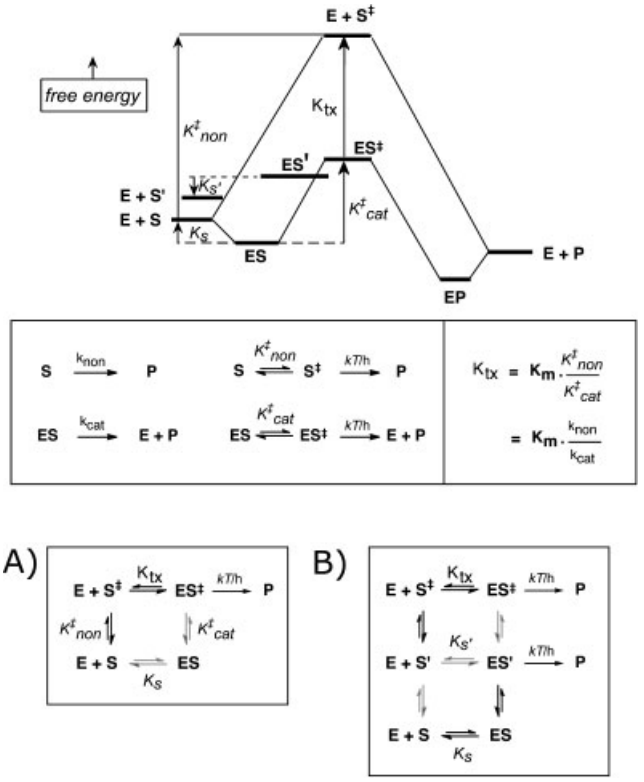
IS THE FORMATION OF AN ENZYME--SUBSTRATE COMPLEX NECESSARY?

Almost from the beginning, it has been clear that enzymes form non-covalent enzyme–substrate (ES) complexes, as indicated by the hyperbolic dependence of reaction rates on substrate concentration, and that view was confirmed in several cases by the ability of substrates or products to protect enzymes against heat denaturation. Henri identified two possible explanations of these findings, in which the ES complex might be either (1) productive, going on to form products, or (2) non-productive, interfering reversibly with productive bimolecular collisions between the enzyme and substrate.[1a] Many decades later, the development of transient-state kinetic methods has made it possible to determine whether ES complexes are productive or not. The literature discloses numerous examples of non-productive binding, particularly by proteases and glycosidases acting on artificial substrates. In the well-known case of lysozyme, a sugar trimer or tetramer may be bound 'out of register,' so that no part of these potential substrates occupies the active site in such a way that its glycosidic linkage is subject to cleavage.

In principle, it would seem possible for an enzyme, or any other catalyst, to enhance the rate of transformation of a substrate through a bimolecular collision process. It would be necessary for that process to have a very large second-order rate constant for catalysis to be effective, but most enzyme-substrate reactions do in fact proceed with very large second-order rate constants, approaching the limits imposed by diffusional encounter in solution (Table 1). What, then, is the purpose of forming an ES complex before the chemical transformation of the substrate begins, and is it necessary? An affirmative answer to the last question is suggested by a principle discovered in 1921 by Michael Polanyi,[2] the father of transition-state theory. According to Polanyi's formalism, shown in Scheme 1(A), a catalyst can enhance the rate of a reaction

Table 1. Values of k_{cat}/K_m at 25 °C compiled from the literature and the evidence (if any) that k_{cat}/K_m is near the diffusion limit

Enzyme	Substrate	k_{cat}/K_m ($M^{-1}s^{-1}$)	Rate-determining step	Ref.
Superoxide dismutase	Superoxide	7×10^9	Diffusion	13
Fumarase	Fumarate	1×10^9	Diffusion	14
Triosephosphate isomerase	Glyceraldehyde 3-phosphate	4×10^8	Diffusion	15
Cytochrome *c* peroxidase	Horse heart ferrocytochrome	4×10^8	Unknown	16
β-Lactamase	Penicillin	1×10^8	Partly diff.	17
Chymotrypsin	MocTrp *o*-nitrophenyl ester	9×10^7	Partly diff.	18
Quercetin-2,3-dioxygenase	Quercetin	7×10^7	Unknown	19
OMP decarboxylase	Orotidine 5′-phosphate	6×10^7	Not diff.	20
Cytochrome *c* peroxidase	Hydrogen peroxide	5×10^7	Not diff.	21
Phosphotriesterase	*p*-Nitrophenyl phosphate	5×10^7	Diffusion	22
Catalase	Hydrogen peroxide	4×10^7	Partly diff.	23
Alkaline phosphatase	4-nitrophenyl phosphate	3×10^7	Diffusion	24
HIV protease	Peptide	2×10^7	Not diff.	25
Fructose 1,6-bisphosphatase	Fructose 1,6-bisphosphate	1.5×10^7	Unknown	26
Adenosine deaminase	Adenosine	1×10^7	Partly diff.	27
Staphylococcal nuclease	DNA, pH 9.5	1×10^7	Diffusion	28
Acetylcholinesterase	Acetyl thiocholine	1×10^7	Diffusion	29
Chloride peroxidase	Chloride	7×10^6	Unknown	30
Carbonic anhydrase	Carbon dioxide	7×10^6	Partly diff.	31
Carboxypeptidase a	Furylacryloyl-Phe-Phe	7×10^6	Diffusion	32
Anthrinolyl-CoA oxygenase	2-Aminobenzoyl-CoA	4×10^6	Unknown	33
Chlorite O_2 lyase	Chlorite	4×10^6	Unknown	34
Linoleate 11-lipoxygenase	Linoleate	4×10^6	Unknown	35
Cytidine deaminase	Cytidine	3×10^6	Not diff.	36
Ribonuclease T2	GpC	2×10^6	Diffusion	37
Chorismate mutase	Chorismate	2×10^6	Diffusion	38
4-hydroxybenzoate 3-oxygenase	4-Hydroxybenzoate	2×10^6	Unknown	39
Mandelate racemase	Mandelate	1×10^6	Partly diff.	40
Aminocyclopropane synthase	*S*-Adenosylmethionine	1×10^6	Diffusion	41
4-hydroxybenzoate 1-hydroxylase	4-hydroxybenzoate	1×10^6	Unknown	42
Histamine *N*-methyltransferase	Histamine	7×10^5	Unknown	43
Catechol 1,2-dioxygenase	Catechol	6×10^5	Unknown	44
Aspartate aminotransferase	L-Aspartate	1×10^5	Partly diff.	45,46
Threonine dehydrogenase	L-Threonine	9×10^4	Unknown	47
Alcohol dehydrogenase	Ethanol	8×10^4	Unknown	48



Scheme 1. Equilibria that accompany catalysis (A) for combination of an enzyme with the usual form of the substrate in the ground state and (B) for combination of an enzyme with a rare or activated species of the substrate. In case (B), E must combine with S (not S′ or S‡) if k_{cat} approaches the limit imposed by diffusion of the enzyme and substrate in solution

only to the extent that it binds the altered substrate in the transition state more tightly than it binds the substrate in the ground state. It is that 'jump' in affinity on which catalysis depends.[3] This principle has become widely accepted by biological chemists as a result of the successful development of transition-state analogues, enzyme inhibitors whose properties were a subject of early conjecture by Linus Pauling,[4] A. G. Ogston[5] and W. P. Jencks.[6]

If tight binding is important in the transition state, then it becomes of interest to ask whether natural selection would be expected to favor an efficient catalyst that bound the substrate weakly or tightly in the ground-state ES complex. Does an enzyme, in its most abundant form in solution, combine with the substrate in its most abundant form in solution?

It is evident from Scheme 1 that very tight binding of the substrate would detract from the catalytic power of an enzyme, since the value of K_s (the dissociation constant of the ES complex in its ground state) needs to be large relative to K_{tx} (the dissociation constant of the enzyme complex with the altered substrate in the transition state) if the enzyme is to enhance the rate of reaction. In an extreme case, if an enzyme were to bind its substrate as tightly in the ES complex as in ES‡, there would be no catalysis at all. In a less extreme case, if ground-state binding were so tight that the dissociation constant of ES was exceeded by the concentration of the substrate *in vivo*, the rate of reaction would be unresponsive to changing substrate concentrations, because it would be operating at the kinetic bottleneck corresponding to V_{max}.[7]

If Scheme 1 indicates that binding should not be too tight in the ES complex, it is of interest consider the other extreme, in which an enzyme might bind its substrate as weakly as possible. A change in that direction would increase the value of k_{cat}, but would increase the value of K_m to the same extent, so that the enzyme would be fully active at higher concentrations of substrate than before. A powerful counterargument to increasing the kinetic parameters, without limit, is suggested by the fact that catalysis depends on an enzyme's ability to discriminate between two species (S and S‡) that resemble each other closely except with respect to a difference in one or two bond angles or distances. The magnitude of that power of binding discrimination is already so remarkable that there would seem to be no advantage in making the task even more difficult. That very difficulty probably explains why arrangements that approximate bimolecular collision appear to be so rare, and why K_m values have usually been found to exceed the concentration of the substrate *in vivo* by only a small factor.

AVID COMBINATION WITH AN ACTIVATED FORM OF THE SUBSTRATE?

There appears to be nothing in the formalism of Scheme 1 that would contradict the possibility that an enzyme might combine avidly with a rare, or activated, species of the substrate (S′), rather than with the form of the substrate that is most abundant in solution (S) [Scheme 1(B)]. It would only be necessary to postulate that an enzyme's *apparent* affinity for ordinary forms of the substrate actually represents the product of an unfavorable equilibrium constant for conversion of S to S′, multiplied by a favorable equilibrium constant for the binding of S′. Might S′ be the actual species with which an enzyme combines, most of the time, and might S′ actually approach S‡ in structure and energy in some cases?

Some substrates have been shown to undergo appreciable changes in carbonyl stretching frequency or ^{13}C chemical shift when they are bound by triosephosphate isomerase[8] or citrate synthase.[9] No such changes are observed when products are bound by cytidine deaminase or adenosine deaminase.[10] From these examples, one gains the impression that substrates may or may not be bound as rare species. However, these observations fail to indicate whether a rare species of the substrate might be required for productive encounter with the enzyme, because they refer to the situation after binding equilibrium has been established.

The very speed at which enzymes work may hold the key to an understanding of whether a rare species of the substrate (or the enzyme) is required for successful encounter. For most enzymes operating under physiological conditions, the concentration of the substrate is subsaturating, enabling the rate of substrate transformation to respond to changing substrate concentrations. The rate of product formation by an enzyme is then described by the second-order rate constant k_{cat}/K_m. An upper limit for k_{cat}/K_m is set by the second-order rate constant for encounter between an enzyme and a substrate in solution, which is probably in the neighborhood of $10^9\ s^{-1}\ M^{-1}$ in most cases.[11] Hence it is of interest to inquire whether enzymes appear to be efficient by that criterion. Such a property would be hard to understand if successful encounter required a form of the substrate or the enzyme that was not reasonably populous.

At the time when this argument was advanced,[12] only five values were available for k_{cat}/K_m. Not all of those were well established, and considerable progress has been made since 1974. Table 1 presents some values of k_{cat}/K_m that had been reported in the literature as of mid-2003. Also included is information about the physical significance of k_{cat}/K_m, as inferred from the observed effects of viscosogenic agents on k_{cat}/K_m. These catalytic efficiencies are seen to cluster in the range between 10^5 and $10^9\ M^{-1}\ s^{-1}$, with a mean value in the neighborhood of $10^7\ M^{-1}\ s^{-1}$ Many of the higher values apply to reactions for which k_{cat}/K_m has been shown to be diffusion-limited, whereas the lower values tend to refer to reactions in which k_{cat}/K_m is not diffusion-limited.

These large second-order rate constants, which allow reactions to proceed at useful rates at the limited concentrations ($<10^{-5}$ M) at which enzymes are present within the cell, suggest that enzymes tend to combine productively with forms of the substrate whose free energies approach the free energies of those forms that are most abundant in solution. The same argument applies to form of the enzyme that leads to reaction: thus, productive combination tends to involve forms of the enzyme that are both abundant in solution and accessible to the substrate.

These arguments against participation of rare species (of either the enzyme or the substrate) are, of course, only appropriate for reactions of the types shown in Fig. 1, whose k_{cat}/K_m values approach the diffusion limit. Nor do they imply that distorted forms of the substrate (such as S′) may not be present in the ES complex a moment after they combine. To choose any one of those distorted species as representing the 'typical' structure of ES

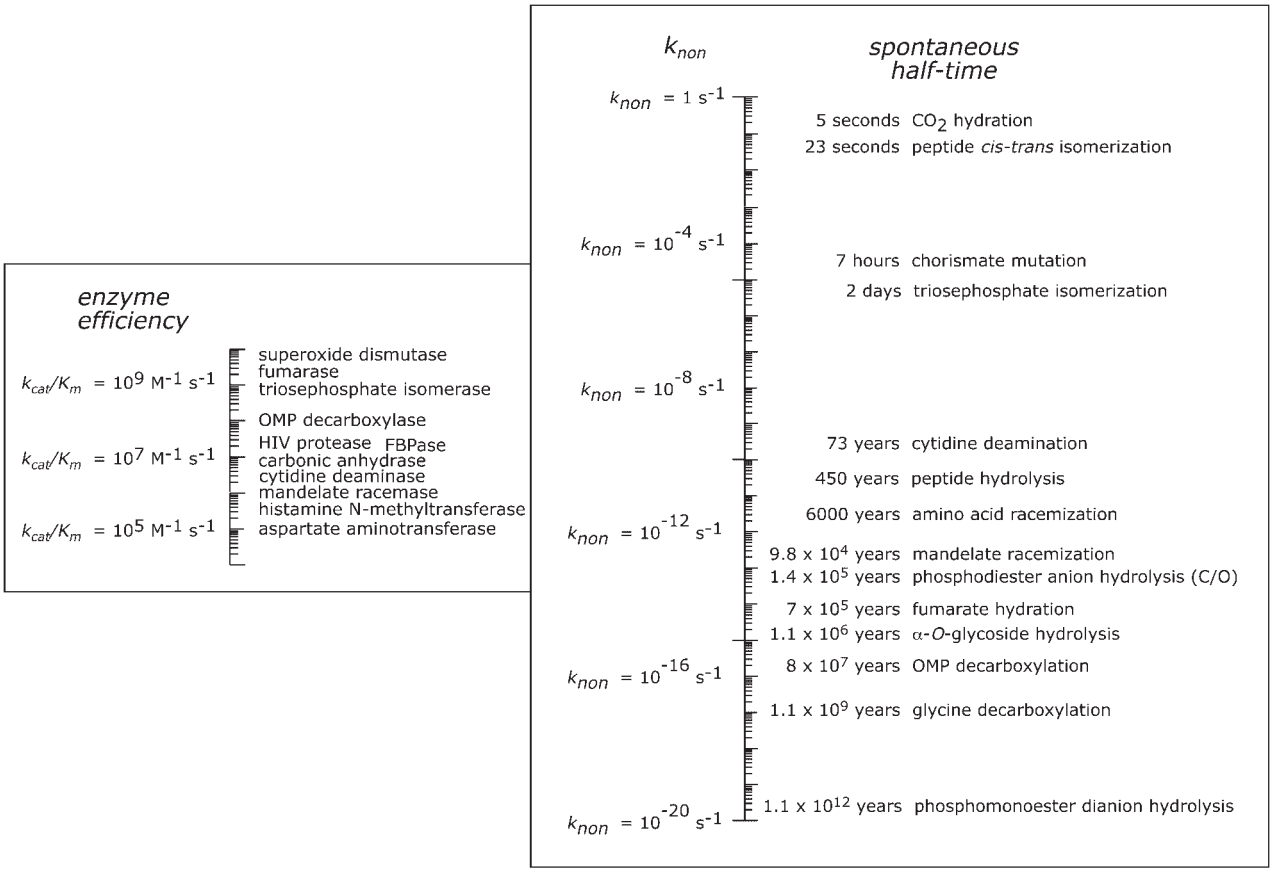


Figure 1. Logarithmic scales illustrating the range of rate constants observed for uncatalyzed reactions in water (k_{non}) and for some reactions taking place at the active sites of enzymes (k_{cat}/K_m) from Table 1

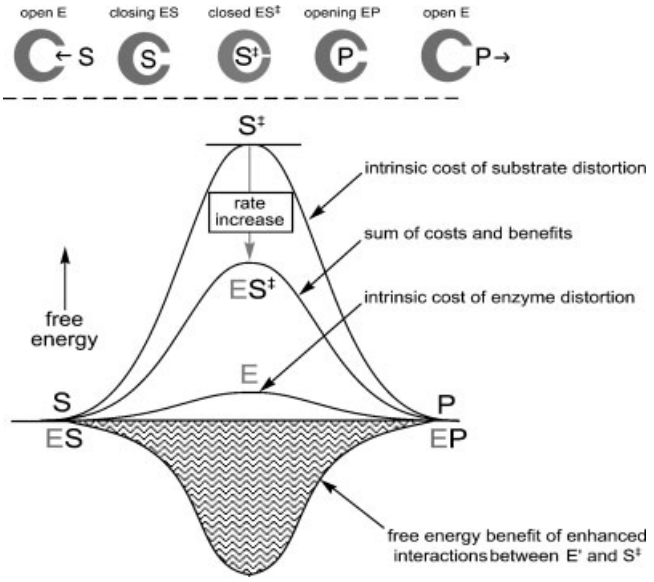


Figure 2. Free energy changes associated with an enzyme reaction in which the active site opens to permit substrate access and product egress, but closes to permit maximal contact with the altered substrate in the transition state. The free energies of ES and EP have been normalized. The free energy of activation for reaction of ES is the sum of the free energy of activation for reaction of S, plus the free energy of distortion of the enzyme to its configuration in the transition state, plus the free energy of formation of attractive interactions between the distorted substrate and enzyme in the transition state

complex, however, would seem to be as arbitrary as to choose any other point along the rising limb of the free energy diagram that describes the progress of the enzyme-substrate complex toward the transition state.

The openness of the active site to substrate access, as implied by the occurrence of these reactions at nearly diffusion-controlled rates, is of interest in connection with the recurring observation that enzyme active sites tend to surround the altered substrate in the transition state (or its analogue) so completely that it becomes completely inaccessible to solvent, before relaxing to release the product (Fig. 2). That scenario, which maximizes the fleeting binding contacts between the enzyme and the substrate in the transition state, is expected to benefit catalysis if the cost in free energy of closing the enzyme, relative to its open, native structure, is relatively minor compared with the benefit from improved contacts in the transition state. Later, the enzyme presumably returns to its open state to release the weakly bound product.[11,49] When two substrates of similar inherent reactivity are compared, the 'better' substrate is simply the one for which the enzyme exerts a greater attraction in the transition state. That specificity may be reflected in K_m (the case considered in Fischer's lock-and-key hypothesis) or in k_{cat}, depending on the extent to which bonding differences between the two substrates in the transition state are already present in their respective Michaelis complexes.[50]

HOW HIGH AN AFFINITY IS ACTUALLY PRESENT IN THE TRANSITION STATE?

The very large apparent second-order rate constants (k_{cat}/K_m) shown in Fig. 1 seem especially remarkable in view of the rates at which some of these same reactions have been found to take place spontaneously in water in the absence of an enzyme (k_{non}), shown in Fig. 1. The hydrolysis of some glycosides and of phosphate ester dianions, and the decarboxylation of amino acids and of OMP, occur slowly on a geological, or even a cosmological, time scale. In contast, the spontaneous hydration of carbonic acid and the *cis–trans* isomerization of peptide bonds occur rapidly even in the absence of an enzyme.

Figure 2 compares some of the values of k_{cat}/K_m in Table 1, collapsed to to the same logarithmic scale as the values of k_{non}, and permits comparison of their numerical ranges. A few reactions that are known to involve covalently bound enzyme derivatives as intermediates have been omitted from this comparison. These cases require special treatment because there is a difference in mechanism between the enzymatic and non-enzymatic reactions.[39] As Lienhard has suggested, an equilibrium constant for 'transition state interchange' can be calculated, between an enzyme nucleophile and a model nucleophile, can still be calculated from the second-order rate constants for the two reaction.[51] According to Scheme 1, the ratio $k_{non}/(k_{cat}/K_m)$ provides an inverse measure of the affinity which must be developed in the transition state to explain the catalysis produced by the enzyme. Because of the extreme variations in the rate of biological reactions in the absence of enzymes (Fig. 1), some reactions are very much more difficult to catalyze than others. The range of transition-state affinities is correspondingly enormous, and sub-yoctomolar (10^{-24}) affinities are achieved in several cases.

It is tempting to suppose that there must be some simple explanation for the extraordinary affinities that can be inferred from the values in Fig. 1. It is sometimes suggested, for example, that covalent bonds may be developed in the transition state. However, closer inspection reveals that covalent bonding is unlikely to provide any recurring basis for unusual catalytic proficiency. There is no sign that orotidine 5′-phosphate decarboxylase, for example, acts by forming a covalent intermediate, nor has any model reaction been discovered in which an amino acid side-chain, or its analogue, has been found to participate covalently in the decarboxylation of orotic acid derivatives.[52] Moreover, many instances are known in which both covalent and non-covalent mechanisms are found in enzymes (aldolases, kinases, proteases, glycosidases, phosphate monoesterases) that catalyze essentially the same reaction. The reactions catalyzed by glycosidases and phosphatases are conspicuously difficult, but covalent and non-covalent mechanisms appear to be about equally common among enzymes catalyzing these two types of reaction. Conversely, one of the least

proficient enzymes (carbonic anhydrase) uses divalent cations in much the same way as do enzymes of medium (cytidine deaminase) and very high proficiency (fructose 1,6-bisphosphatase). Hence there is no obvious dichotomy of mechanisms among enzymes, whereby covalent intermediates show a greater tendency to intervene in the more difficult reactions.

Cofactors such as PLP, TPP, biotin, corrinoids and the redox cofactors are often employed in difficult reactions, but their presence changes the nature of the reaction to such an extent that it differs fundamentally from that of the reaction in the absence of the cofactor. Each of these cofactors can really be considered a second substrate. If one considers these as two-substrate reactions, one can simply compare their rate constants on and off the enzyme, and several reactions of this kind are currently under investigation.[53,54]

Perhaps it may not be necessary to invoke extraordinary forces of attraction to explain examples of very high catalytic proficiency. In adenosine deaminase,[55] mandelate racemase,[56] orotidine 5′-phosphate decarboxylase[20] and triosephosphate isomerase,[57]mutations of either the enzyme or the substrate indicate that polar interactions (including H-bonds) show great departures from additivity in free energy.[58] The effects of 'cutting' either the enzyme or the substrate in two and then comparing the transition state binding affinities of the whole with those of the pieces reveal connectivity effects whose magnitudes match and even exceed the the values roughly estimated by Page and Jencks.[59,60] So great is this synergism that very high levels of transition-state binding affinity may be within reach, using ordinary non-covalent forces of attraction.

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