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Enzyme Reactivity from an Organic Perspective

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The Postulate

A prominent biochemist once remarked, "It does not matter how elegant an enzyme model you organic chemists construct, no biochemist will ever pay much attention to it."[1] This statement reflects an antireductionist sentiment with which I am, in general, sympathetic. After all, much of our own past work has been devoted to organic aggregates that are not describable as simple extrapolations of single-molecule chemistry.[2] How can I expect, therefore, biochemists to regard enzyme catalysis as a straightforward extension of organic catalysis? Yet this is exactly the position I plan to take. I will lay aside any antireductionist tendencies and assert that organic chemistry does in fact have a great deal to contribute to enzymology. Enzymes, as pointed out by Knowles,[3] are "not different, only better". And (as we shall see) organic chemistry has within its confines the ability to explain exactly why enzymes are "better". The principles involved are discussed using organic systems in the first half of the Account and enzymes in the second half.

If there is one attribute that chemists admire most about enzyme-catalyzed reactions, it is their speed. Enzymes are, typically, associated with 10^8–10^{10} accelerations. Naturally, the attainment of such velocities in an organic system need not, in and of itself, constitute a good "enzyme model". No one would say, for example, that the extremely fast Pt-catalyzed hydrogenation of olefins has any enzymological connection. At the other extreme, the chemical literature is so filled with $<10^2$ accelerations, all of them billed as "enzyme models", that the term has become a cliché. The problem is compounded by various claims that an enzyme rate has actually been surpassed. But on closer examination, one usually finds that the "model" utilized a highly reactive functionality (e.g., a *p*-nitrophenyl ester) when in nature the corresponding enzyme must operate on a relatively inert substrate (e.g., an amide).[4] As a result of these difficulties, I will avoid direct attempts to imitate or model. Instead, the focus will be on chemistry and how it reveals what an enzyme can or cannot do.

Many people, too numerous to specify, have pointed out the possible relationship between enzyme catalysis and intramolecularity.[5] The idea is attractive for two reasons: (a) Huge rate increases sometimes occur when an intermolecular reaction is converted into its intramolecular counterpart. (b) An enzyme reacting with its bound substrate resembles an intramolecular organic process. Thus, both enzyme–substrate complexes and intramolecular systems hold their reactive groups in proximity (the former via noncovalent forces, the latter via covalent bonds). This does not prove that enzymes and intramolecular systems achieve their accelerations by similar mechanisms, but it is quite natural to suspect that such is the case.

Decades ago Bender[6] and Bruice[7] argued that fast intramolecular rates are attributable to "proximity". They were absolutely correct. The problem, however, is that Bender and Bruice never defined "proximity", and the term remains undefined even today. Molecular proximity may signify 1 Å, 5 Å, or 10 Å; no one knows. In 1985 an attempt was made in this journal to place proximity on a somewhat more quantitative footing.[5] It was asserted that intramolecular reactions occur at enzyme-like rates when van der Waals contact distances (too small to accommodate intervening solvent) are imposed for finite times upon reactive groups. In other words, intramolecular reactivity was considered a function of distance and time; hence the name "spatiotemporal hypothesis". Spatiotemporality embodies, but also refines, the proximity concept. It may in fact be preferable to retain the word "proximity" as long as my definition in terms of distance is kept in mind. Thus, if two reactive groups are held such that a water molecule can situate itself between them, the groups are *not* in proximity. Since the water molecule is 3 Å in diameter, distances less than 3 Å are generally involved in the really fast intramolecular rates.

Page–Jencks theory[8] states that there is nothing remarkable about intramolecular reactions; their fast rates are merely an entropic consequence of converting a bimolecular reaction into a unimolecular reaction. Although the spatiotemporal hypothesis obviously incorporates entropy effects (i.e., there are entropic costs in retaining two atoms at contact geometries), distance and entropy are entirely different types of parameters. Distance is a simple, pure, and comprehensible quantity of the sort that Isaac Newton called a "fluent". Entropy is not a "fluent". Entropy is a hodgepodge mixture of changes in solvation, conformation, molecularity, etc. Activation entropies for reactions in water are such an entanglement of factors that they can be neither predicted nor rationalized. Little wonder that enzymologists are much more concerned with structure (e.g., distance relationships at active sites) than with thermodynamics (e.g., entropic changes during an enzyme reaction). The spatiotemporal hypothesis reflects the same bias.

Fredric M. Menger was born and educated in the United States. A favorite quote of his is found in a letter of Einstein to Pauli: "Sie haben also recht gehabt, Sie Spitzbube." Translated: "You were right after all, you rascal."

(1) Private communication.
(2) Menger, F. M. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1086.
(3) Knowles, J. R. *Nature* **1991**, *350*, 121.
(4) Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1987**, *109*, 3145.
(5) Menger, F. M. *Acc. Chem. Res.* **1985**, *18*, 128.
(6) Bender, M. L.; Chow, Y.-L.; Chloupek, F. *J. Am. Chem. Soc.* **1958**, *80*, 5380.
(7) Bruice, T. C. *Annu. Rev. Biochem.* **1976**, *45*, 331.
(8) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678. Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 219.

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Criticism of the spatiotemporal hypothesis, following its inception, centered around what seemed to be a general satisfaction with entropy as a rationale for intramolecularity. I thus feel obligated to relate here the story of Claude Shannon, inventor of the uncertainty function in communications engineering. Mathematician Jon Von Neumann suggested to Shannon that he call his new function "entropy". Von Neumann pointed out that "no one really knows what entropy is, so in a debate you will always have the advantage". Admittedly, entropy is fine for winning debates, but if one wishes to understand enzymes at the molecular level, entropy is best regarded as valid but not very helpful.

Energy is required to extrude solvent between two reactants and move them into a bonding distance. The source of the energy depends upon the particular system. In a bimolecular reaction, thermal energy does two things: (a) It desolvates the reactants and brings them into contact. (b) It promotes the resulting complex to the transition state. In contrast, an intramolecular reaction is accelerated by "covalent energy" (i.e., energy imparted to the molecule during its synthesis). In effect, an intramolecular system reacts rapidly because the van der Waals complex is present to begin with; its formation need not contribute to the overall energy consumption. Enzymes, as will be described in detail later, sacrifice binding energy in order to achieve the required desolvation and contact distance.

Intramolecularity and enzyme reactivity are being discussed here in terms of "distance". But at no time will it ever be claimed that distance is the *only* determinant of rate (any more than Arrhenius claimed that rate depends *only* on temperature). Spatiotemporal theory, or proximity theory if you will, merely states that rate is a sensitive inverse function of distance (all other parameters being held constant), and that enzymes take advantage of this fact to react rapidly.

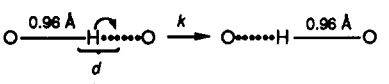
Having introduced the central theme of the Account, it remains to do three things: (a) to describe, very briefly, four of our investigations pertinent to the distance–time problem (including the design of two systems with astounding rates); (b) to develop the so-called "split-site" model of enzyme action; and (c) to apply our constructs to several enzymes using data gathered by others. Much of the paper, therefore, deals with enzymatic rather than organic behavior.

One final point by way of introduction. Richard Dawkins, a biologist, once wrote, "Explaining is a difficult art. You can explain something so that your reader understands the words, and you can explain something so that the reader feels it in the marrow of his bones. To do the latter, it sometimes isn't enough to lay the evidence before the reader in a dispassionate way." As may already be evident, I concur with Dawkins's sentiment.

The Evidence

What is the evidence? So reads the most important question in science. The present section summarizes four separate studies from our laboratory (two theoretical and two experimental) that provide support for the spatiotemporal concept.

1. Consider eq 1, in which a proton jumps from one oxygen to another. A question of obvious interest to us was how the proton-transfer rate varies with the "jump distance", d. Answering this question[9] required



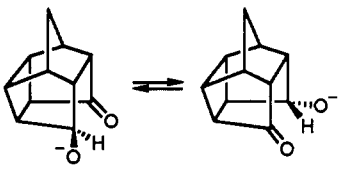
(1)

the analysis of two intersecting Morse potentials (not unlike the Marcus approach), but details here are unnecessary. Suffice it to state that the analysis led to eq 2, where E_a is the activation energy of the reaction; D_e is the O–H dissociation energy (102 kcal/mol); r_e is the equilibrium O–H distance (0.96 Å); and B is a constant (1.7 Å^{-1}). Although eq 2 is based on numerous

$$E_a = D_e[1 - \exp(-\beta r_e - \beta d/2)]^2 \qquad (2)$$

assumptions, the relationship nonetheless gives an "order-of-magnitude" approximation of how a proton transfer responds to changes in distance. It was found that reducing the O-to-O distance from 2.92 Å to 2.32 Å increases the rate by a factor of 2×10^7 (a number that, if anything, underestimates reality).[9] Reactivity is, no doubt, a sensitive function of distance, a point never stressed in "classical" proximity theory.[6,7]

2. AMPAC semiempirical MO computations on an intramolecular hydride transfer (eq 3)[10] gave an activation enthalpy of 18 kcal/mol. Yet the actual bond-

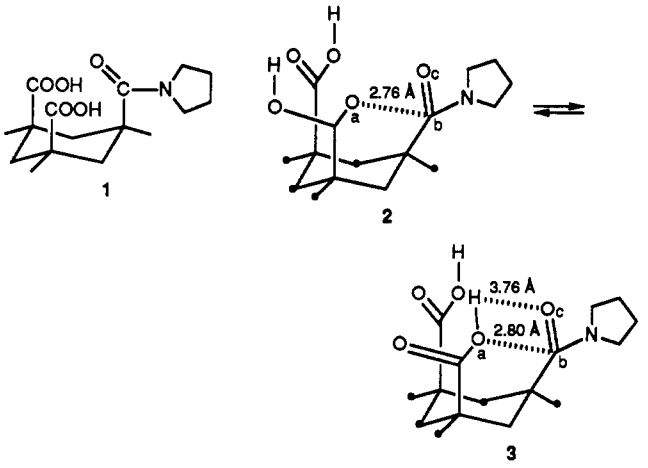

(3)

making/bond-breaking process begins only at a point 13 kcal/mol up the energy profile. What is going on? It is quite clear that a large portion of the energy requirement, namely 13 of the 18 kcal/mol, is used to distort the carbon framework so as to deliver the hydrogen to the recipient carbon. Once a C/H bond distance of 1.6 Å is reached, hydride transfer need surmount a mere 5 kcal/mol barrier to reach the transition state. The reaction is controlled far more by "delivery costs" than by changes in covalent bonds subsequent to delivery.

3. A theory must not only rationalize experimental results; it must predict them. If the spatiotemporal hypothesis has any merit, it should be possible to use it for designing new systems that react at enzyme-like rates. I now present two examples where exactly this was accomplished, the first of which focused on the problem of hydrolyzing unactivated amides. This is not an easy thing to do chemically. A typical procedure calls for a 10-h reflux in 8 N HCl. In contrast, chymotrypsin cleaves amides rapidly at neutral pH and ambient temperature. While searching for a way to emulate the enzyme rate, we had occasion to examine amide **1** via molecular mechanics calculations. The calculations told us that the molecule possesses two conformations (**2** and **3**), both having a carboxyl oxygen within van der Waals contact distance of the amide

(9) Menger, F. M. *Adv. Mol. Modeling* **1988**, *1*, 189.
(10) Sherrod, M. J.; Menger, F. M. *Tetrahedron Lett.* **1990**, *31*, 459.

carbonyl carbon. As seen from **3**, a carboxyl is poised for synchronous nucleophilic attack and proton transfer. On this basis, we predicted that the carboxyl was ideally disposed to cleave the amide at chymotrypsin-like rates, and accordingly, we proceeded to synthesize **1** for examination.[11]



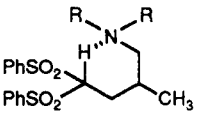
1 **2** **3**

Intramolecular-catalyzed cleavage of amide **1** under biological conditions (ambient temperature, neutral pH, and absence of alien transition metals) was found to occur with the fastest rate yet recorded for such a "model" reaction: $t_{1/2}$ = 8 min, corresponding to an effective molarity of at least 10^{12} M. Control experiments showed that only a single carboxyl is necessary for amide cleavage. The observed rate effect stems primarily from sustained proximity at van der Waals contact distances, distances too small to accommodate intervening water molecules.

The lesson here is important. If a proteolytic enzyme were to place one of its aspartate carboxyls adjacent to an amide substrate with the geometry portrayed in **2** or **3**, then little additional catalytic power would be necessary. It is tempting to conclude that one need never explain enzyme catalysis with some sort of esoteric mechanism.[12] Spatiotemporal effects, coupled to simple proton/metal transfers, seem sufficient for the task.

4. Enzymes are frequently called upon to remove a proton from nonacidic carbons (e.g., a ketone with pK_a = 20) using protein side chains that are not very basic (e.g., imidazole with pK_a = 7). We were anxious to test the idea that spatiotemporal effects might play a role in such dramatic "upstream" proton transfers taking place routinely at active sites. An opportunity to do so arose when we became aware of the X-ray analysis of amine **4** (R = CH_3). The X-ray data revealed an intramolecular CH···N hydrogen bond 2.34 Å in length (significantly less than the sum of the van der Waals radii, 2.75 Å). This striking result led us to predict a fast intramolecular general-base-catalyzed proton exchange between the amino group of **4** (R = H) and the methine proton, two entities shackled at a contact distance.[13]

Dynamic NMR methods were used to measure NH/CH exchange in **4** (R = H) in toluene-d_8. The reaction



4

is believed to occur via proton transfer to form an R_3C^-/H_3NR^+ ion pair, after which a different NH proton is returned to the carbon.

It was found that the intramolecular general-base-catalyzed proton interchange in 30 mM **4** was too fast to measure by NMR at −80 °C. On the other hand, the intermolecular counterpart (where the amine and carbon acid are separate molecules present at 30 mM each) was too slow to measure by NMR at 100 °C, a full 180 °C higher.

The pK_a's of the amine and carbon acid are 10 and 13.8, respectively. Of course, 3.8 grossly underestimates the thermodynamic barrier because the number is based on acidity in water, not toluene. Since ion-generating equilibria are often disfavored by 10^7–10^8 in aprotic solvents, the barrier to ion-pair formation in **9** should be more like 12 pK_a units. The extremely fast intramolecular proton interchange at −80 °C seems, in this light, all the more remarkable. No doubt a short CH/N distance lies at the source of the intramolecular reactivity, and enzymes might well achieve their uphill transfers in a similar manner, i.e., by imposing contact distances within hydrophobic pockets at the active site.

Enzymes

Spatiotemporal theory is best applied to enzyme systems using what I have previously called the "split-site" model.[14] A substrate is divided into a binding portion B and a reactive portion R (Figure 1). Interaction at the active site is then treated as the sum of the component interactions at the separate and distinct regions. The construct is hardly new. In 1950 it was proposed that acetylcholinesterase possesses (a) an anionic site for binding acetylcholine's quaternary ammonium group and (b) an esteratic site that brings about actual ester hydrolysis. A long and detailed analysis of the split-site model is given elsewhere.[14] Space restrictions here limit its description to the bare essentials.

The fates of the binding portion (ES_B) and the reactive proton (ES_R) of the enzyme–substrate complex ES are totally different when an enzyme reaction is initiated at the active site. As a first approximation, binding at ES_B remains constant as the ground state transforms into the transition state. ES_B binding is said to be *conserved*. In contrast, interactions at the reactive site are altered during catalysis because substrate functionality is being chemically modified.

It is also assumed that interactions at ES_B are stabilizing, whereas ES_R interactions are, overall, destabilizing. Thus, attractive forces at ES_B (i.e., hydrogen bonding, electrostatics, hydrophobic bonding and van der Waals association) all tend to stabilize ES and to promote its formation. On the other hand, as shown in Figure 1, the enzyme's catalytic groups at ES_R, namely, X and Y, must desolvate in order to achieve proper contact with the substrate. Solvent extrusion is only one of several possible energy-costing "tensions"

(11) Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1988**, *110*, 6794. Carboxyl-catalyzed amide cleavage has been known for many years. See: Kirby, A. J.; McDonald, R. S.; Smith, C. R. *J. Chem. Soc., Perkin Trans. 2* **1974**, 1495.

(12) Page, M. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; Chapter 1.

(13) Menger, F. M.; Gabrielson, K. *J. Am. Chem. Soc.* **1992**, *114*, 3574.

(14) Menger, F. M. *Biochemistry* **1992**, *31*, 5368.

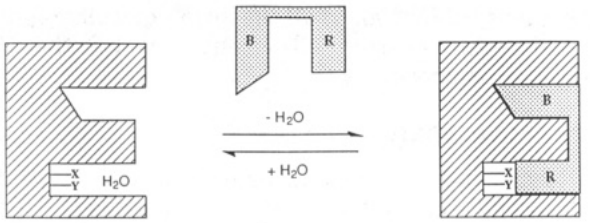


Figure 1. The split-site enzyme model in which an active site (large shaded area) is subdivided into a binding region and a reactive region. These regions associate with B and R of the substrate (dotted object above the arrows), respectively. Catalytic groups on the enzyme (X and Y) are brought into contact distances with the labile group of the substrate when the enzyme–substrate complex is formed. It is assumed that the total ES free energy equals the sum of the parts (i.e., $ES = ES_R + ES_B$).

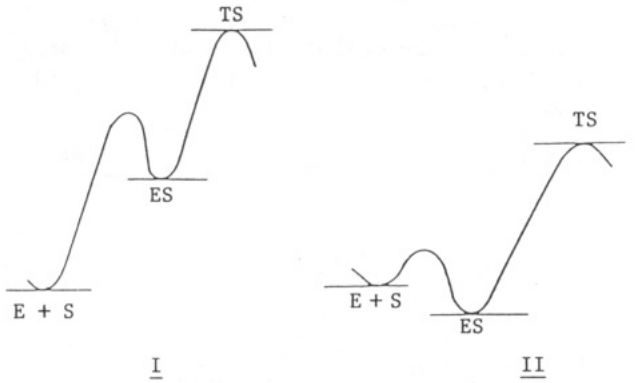


Figure 2. Free energy diagrams for enzyme-catalyzed reactions in which E = enzyme, S = substrate, ES = enzyme–substrate complex, and TS = transition state. Profile I: $[S] < K_m$. Profile II: $[S] > K_m$. K_m is the Michaelis–Menten dissociation constant. The important difference between profiles I and II lies in the relative energy levels of E + S and ES.

that might be created at the ES_R site. Energy input at ES_R would also be required, for example, to enforce a reactive conformation upon the substrate atoms.

Enzyme reactions can be viewed in terms of the two free energy diagrams in Figure 2. Both cases must be considered because the concentration of substrate S relative to the Michaelis–Menten constant K_m (a ratio that determines the relevant profile) is often unknown under actual biological conditions. In the ensuing discussion, numerical free energy values will be assigned to the various species. This approach is adopted because it is easier to grasp relationships using numbers rather than abstract symbols.

Case I. ES Lies above E + S. Assume the following free energy assignments when an "old" enzyme is converted into a "new" enzyme:

	old	new
E + S	0	0
ES_B	−3	−4
ES_R	+13	+13
ES	+10	+9
TS_B	−3	−4
TS_R	+20	+20
TS	+17	+16
$\Delta G^{\ddagger}$	+17	+16
effect		accel

A few remarks on the old enzyme will clarify the table. Enzyme plus substrate is the starting point, so that E + S = 0. Binding at the ES_B portion of ES is stabilizing ($ES_B = -3$), while the ES_R portion is destabilizing ($ES_R = +13$). Overall, the enzyme–substrate complex binds with the sum of the two ($ES = ES_B + ES_R = +10$). As already mentioned, the binding-site energy is conserved in the transition state ($ES_B = TS_B = -3$). The transition-state configuration at the reactive site is assigned an arbitrary but costly number ($TS_R = +20$). Overall, TS = +17. Since the activation free energy corresponds to TS − (E + S), $\Delta G^{\ddagger} = +17$.

The "new" enzyme is similar to the "old" except that the binding site interaction has been strengthened ($ES_B = -4$). This could occur, for example, via addition of a hydrogen bond at some point remote from the reactive site. Such a change generates a $\Delta G^{\ddagger}$ of +16, indicating that the new enzyme is more efficient by 1 energy unit.

The above analysis leads to an important conclusion: An attractive interaction at the binding site accelerates the enzyme reaction no matter how far away the interaction is located from the ongoing chemistry. For example, a hydrogen bond at ring A of an enzyme-bound steroid will necessarily facilitate a reaction at ring D.

Suppose an enzyme mutates so as to transform an inert alanine into a proton-donating serine. Suppose further that the mutation creates a new hydrogen bond to the substrate at the active site nowhere near the point of chemical reaction. As just proved, a rate acceleration occurs nonetheless. Is this a "ground-state effect" or a "transition-state effect"? In one sense, it is a ground-state effect because the enzyme achieves its rate improvement simply by binding more tightly to the ground state via a hydrogen bond. The enzyme has been improved with total disregard for the partial bonding and other accoutrements of the reacting center. One could also argue, however, for a transition-state effect because the new hydrogen bond operates formally by lowering the transition energy. The truth is that both positions are acceptable. The indecisiveness comes about from the fact that changes in the ground state translate into an identical effect in the transition state by means of a conserved perturbation.

Case II. E + S Lies above ES. Consider profile II in Figure 2 where the substrate concentration is high relative to K_m (another way of expressing the condition that ES is lower than E + S). Once again, energy values are assigned to old and new enzymes.

	old	new
E + S	0	0
ES_B	−7	−8
ES_R	+3	+4
ES	−4	−4
TS_B	−7	−8
TS_R	+20	+20
TS	+13	+12
$\Delta G^{\ddagger}$	+17	+16
effect		accel

In the old enzyme, noncovalent forces at site B stabilize the enzyme substrate complex ($ES_B = -7$). Desolvation and contact distances imposed at the reactive site are destabilizing ($ES_R = +3$). The net effect is a stabilized complex (ES = −4). The activation energy $\Delta G^{\ddagger}$ (corresponding to the difference between the high point, TS, and the low point, ES, on the energy profile) equals +17.

The new enzyme, with an ES_B of −8, displays a 1-unit stabilization at the binding site as might arise, for example, by an additional hydrogen bond. Simultaneously, enhanced spatiotemporal compression at ES_R

destabilizes this site by 1 energy unit. As a direct result, the activation energy is lowered from $\Delta G^{\ddagger} = +17$ to $\Delta G^{\ddagger} = +16$. In effect, noncovalent association at B pays the energy bill at R for the enhanced desolvation/contact whereby the reaction is accelerated. Jencks in his discussion of the "Circe effect" arrived at the same conclusion.[8]

In the above scenario, the energy costs at ES_R are fully compensated by the attractive energy at ES_B. ES = −4 with both the old and new enzymes, and K_m remains unaltered. But if it happened that ES_B were stabilized more than ES_R is destabilized, then *both* the reaction rate and the enzyme–substrate association would be improved.

A Hypothetical Example

Consider an organism that would, in the course of evolution, attain survival advantage if a particular digestive peptidase improved its catalytic ability. However, owing to certain concentration constraints, assume that it would be beneficial not to alter (up or down) the effectiveness of enzyme–substrate binding. How could this be accomplished?

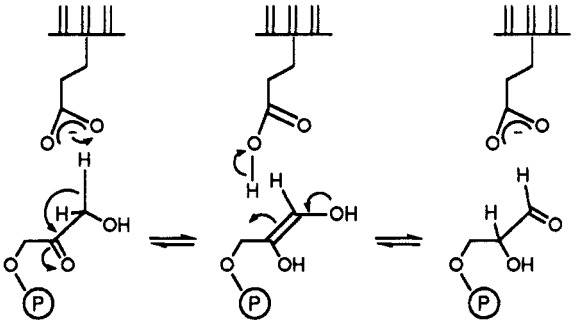
Our prehistoric peptidase might, conceivably, reach its kinetic goal by stabilizing TS. This could happen by an Ala-to-Asp mutation in the ES_R portion of the active site. Hydrogen bonding to the substrate's labile carbonyl by the Asp carboxyl could, no doubt, stabilize the tetrahedral intermediate and the transition state leading to it. Of course, the same hydrogen bond would also stabilize the substrate carbonyl within the ES complex. To the extent that this occurs, the catalysis would be diminished at higher substrate concentrations. There is, however, a more serious problem than a partially compromised catalytic effectiveness. K_m would decrease as a result of the additional substrate binding. Since the stipulation was to keep K_m constant, and thereby not negate the survival value of the Ala-to-Asp mutation, we must search elsewhere for a catalytic mechanism.

A second avenue for improved catalysis is open to the enzyme. Mutational modification can again create a new hydrogen bond, but this time the hydrogen bond is distant from the reactive site (e.g., at the carbonyl of a peptide linkage adjacent to the labile one). If this enhanced enzyme–substrate attraction helped enforce a contact distance between the labile carbonyl of the substrate and a nucleophile of the enzyme (e.g., a serine hydroxyl), then the reaction would be accelerated. And the K_m would remain approximately constant if the hydrogen bond balanced the energy requirements for attaining a closer contact distance.

Note that a hydrogen bond far from the point of reaction can accelerate the reaction although there is no direct interconnection with the transition structure. I prefer to call such a catalytic mechanism "ground-state destabilization" or an "anti-Pauling effect". The term "transition-state stabilization" or a "Pauling effect" should be reserved for cases in which there are selective interactions directly at the transition-state region. Transition-state stabilization is indeed a source of catalysis, but not the only one, and perhaps not even the most important contributor with many enzymes. Destabilization of the substrate via desolvation and enforced distances at the reactive site is also a repository of catalytic potential. If all the intramolecular organic chemistry cited earlier tells us anything, it is that this potential is enormous.

Triosephosphate Isomerase[15]

Recent work on triosephosphate isomerase illustrates clearly and quantitatively the above construct. Salient facts are now listed: (a) Triosephosphate isomerase catalyzes the conversion of D-glyceraldehye 3-phosphate into dihydroxyacetone phosphate (eq 4) with $k_{cat} = 430$ s^{-1} and $K_m = 0.97$ mM. (b) The enzyme possesses a 10-residue mobile "loop" that interacts, by means of one or two hydrogen bonds, with the phosphate of the substrate. No residue in the loop is directly involved with the actual enolization chemistry. (c) A mutant enzyme, lacking four residues in the loop, has a k_{cat} nearly 10^5 times lower than that of the wild-type; K_m is 8.5 times greater, indicating only a small impairment of binding.



(4)

The tight grip of the loop on the phosphate can accelerate k_{cat} in accordance with the split-site model. In other words, loop–phosphate association in the wild-type enzyme helps impose contact distances between the carboxylate and enolizable proton. This might include an energetically costly, but kinetically beneficial, desolvation of the carboxylate. Proton flow from the weak carbon acid to the carboxylate is facilitated as it is in chemical models (e.g., compound 4).

The enzyme can also be analyzed quantitatively (recognizing that 1.4 kcal/mol corresponds to a factor of 10 in k or K). Assume that loop–phosphate binding in wild-type enzyme is worth 8.4 kcal/mol owing to two strong hydrogen bonds. Assume further that the free energy of the reactive site (ES_R) is thereby elevated 7.0 kcal/mol owing to desolvation and contact elements. The reaction would then be accelerated by $10^{7.0/1.4} = 10^5$ relative to the mutant lacking the complete loop. But 1.4 kcal/mol of the 8.4 kcal/mol has not been used for kinetic purposes. This extra energy expresses itself as a 10-fold-stronger substrate binding to wild-type enzyme relative to the mutant. Everything fits well without "transition-state stabilization" ever being mentioned.

Chymotrypsin[16]

Serine proteases hydrolyze amides and esters by a three-step mechanism: substrate binding (K_m); acylation of serine (k_2); and deacylation (i.e., hydrolysis of the acyl enzyme intermediate, k_3). Classic experiments

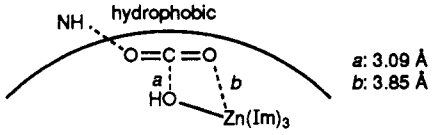
(15) Pompliano, D. L.; Peyman, A.; Knowles, J. R. *Biochemistry* **1990**, *29*, 3186.
(16) Hedstrom, L.; Szilagyi, L.; Rutter, W. J. *Science* **1992**, *255*, 1249.

of Bender have shown that deacylation is rate-determining with simple amino acid esters. Acylation, on the other hand, is rate-determining for amino acid amides. It is not generally recognized, however, that deacylation can become rate-determining for large oligopeptide amide substrates.[17] In spatiotemporal terms, an oligopeptide has an extended binding site that helps position its labile amide group into closer contact with the enzyme's serine. Acylation is accelerated via an "anti-Pauling" effect, and deacylation is relegated, by default, to the slow step.

The numbers support the above contention. *N*-Acetylphenylalaninamide is associated with the following parameters: K_m = 0.02 M; k_2 = 0.43 s^{-1}; and k_3 = 60 s^{-1}. An amide of succinyl-Ala-Ala-Pro-Phe has these parameters: $K_m = 5.2 \times 10^{-5}$ M; k_2 = 160 s^{-1}; and k_3 = 52 s^{-1}. Extended binding with the oligopeptide greatly enhances k_2 without changing k_3, as one would expect from a spatiotemporal effect on the acylation step. One surmises that the K_m for the oligopeptide would be at least a factor of 100 smaller than actually observed were it not for the fact that part of the binding energy is used to destabilize the ground state prior to acylation.[12]

Carbonic Anhydrase[18]

Carbonic anhydrase catalyzes the equilibration between CO_2 and HCO_3^- with an astounding efficiency. This might seem to present a problem for spatiotemporal theory because CO_2 appears to lack the "handles" by which an enzyme could enforce contact distances. Recent molecular dynamics calculations tell us otherwise. CO_2 is a nonpolar molecule that resides in a hydrophobic pocket of the enzyme (**5**). One of the CO_2



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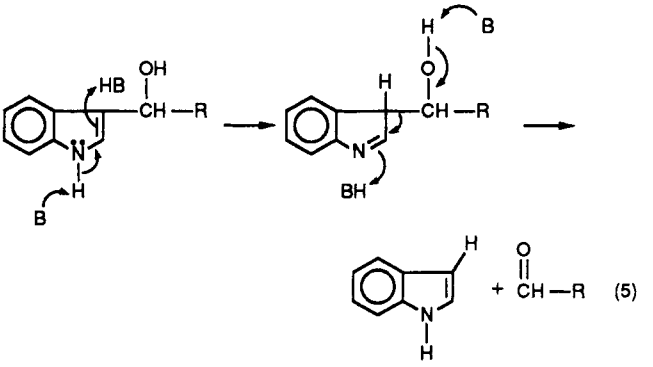
oxygens is hydrogen-bonded to a peptide NH, the other to zinc. The nucleophilic hydroxyl is held only 3.09 Å away from the reactive CO_2 carbon. Thus, at least three separate attractive forces impose a contact distance (somewhat greater than the sum of the van der Waals radii) upon the reactive atoms. Nature took ample advantage of "distance effects" when designing this active site.

Tryptophan Synthase[19]

Tryptophan synthase cleaves indoleglycerol phosphate into indole and glyceraldehyde 3-phosphate (eq 5). Glutamate-49 of the enzyme's α subunit is an essential residue since 19 mutant proteins substituted at position-49 are inactive. When glutamate-49 was mutated to glycine, the enzyme was inactive as expected. But the interesting observation was that the mutant binds a substrate analog (indole-3-propanol phosphate) 10-fold better than does the wild-type (4.1 μM vs 40



μM, respectively). How can this be rationalized? The simplest explanation is that the glutamate carboxylate is forced into close contact (perhaps van der Waals contact) with the substrate, thereby destabilizing the ground state. Recall that such contacts require an energy-consuming desolvation of the carboxylate. When the carboxylate was removed, the nonbonded "tension" was relieved, so that substrate binding actually improved despite the loss of enzyme activity.

Thermolysin[20]

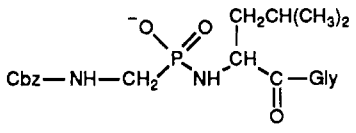
The Kurz equation (eq 6)[21] is crucially important to those interested in enzyme mechanism. It shows that the rate constant for an enzyme-catalyzed reaction (k_e)

$$k_e/k_u \cong K_S/K_T \qquad (6)$$

relative to the uncatalyzed reaction (k_u) is approximated by the ratio of substrate dissociation constant (K_S) to transition-state dissociation constant (K_T). In other words, if one observes a 10^{10} enzyme catalysis, then the transition state should bind 10^{10} better than the substrate.

Equation 6 provides the theoretical backdrop for the "transition-state analog" approach. The idea is to design molecules that "resemble" the transition state and, thereby, create inhibitors with huge binding constants. The success of such a venture can be judged with transition-state analogs of thermolysin.

Phosphonamidate peptides, such as **6**, simulate the tetrahedral intermediate of a peptide hydrolysis.[20] The compounds act as inhibitors of thermolysin with K_i values ranging from 10^{-6} to 10^{-8} M. These numbers



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seem reasonably impressive. But are they really? If one assumes a modest substrate dissociation constant K_s of 10^{-5} M and a typical peptidase catalysis of 10^{10}, then the Kurz equation tells us that the transition-state dissociation constant K_T should equal 10^{-15} M. The transition-state analogs are, therefore, deficient by a factor of 10^7–10^9! Although the best of the analogs binds 10^3 better than the substrate, it lies 10^7 away from what should be happening if it were a "perfect" transition-state analog.

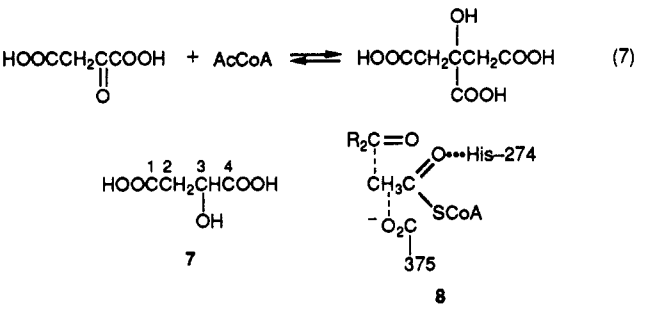
(17) Christensen, U.; Ipsen, H. H. *Biochem. Biophys. Acta* **1979**, *569*, 177.
(18) Merz, K. M., Jr. *J. Am. Chem. Soc.* **1991**, *113*, 406.
(19) Miles, E. W.; McPhie, P.; Yutani, K. *J. Biol. Chem.* **1988**, *263*, 8611. I thank Dr. Robert S. Phillips for pointing out this example.
(20) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618.
(21) Kurz, J. L. *J. Am. Chem. Soc.* **1963**, *85*, 987.

The usual rationale for badly underperforming transition-state analogs is that they are, after all, not actual transition states but only models of them. This is certainly a valid point, and one can wave off much disappointment with it. But there is another possibility, namely, that transition-state binding is not the only, or even the predominate, mechanism for catalysis. Ground-state destabilization can contribute to catalysis, and to the extent that it does, discrepancies from Kurz expectations will occur.

Citrate Synthase[22]

As a last example, I will discuss an enzyme that catalyzes an ester enolization. This reaction was selected because of the difficulties in enolizing an ester in the laboratory. A powerful base, such as lithium diisopropylamide with a pK_a of 33, is required. How does an enzyme, limited to weak acids and bases in water, manage the task? A 1.9-Å-resolution X-ray structure of citrate synthase complexes shows that spatiotemporal effects are critical.

Citrate synthase catalyzes the reversible condensation of acetyl-CoA with oxalacetate (eq 7). Enolization of the acetyl group is the rate-limiting step in the overall reaction. An X-ray structure was obtained from a ternary complex between enzyme, acetyl-CoA, and malate. Malate (7) is an unreactive analog of oxalacetate. It was found that His-274 hydrogen bonds to the acetyl-CoA carbonyl. The enolizable methyl group of acetyl-CoA engages in a tight van der Waals contact (approximately 2.9 Å) with both the C3 of malate and a carboxyl oxygen of Asp-375. The Asp-375 deprotonates the methyl group while the His-274 protonates the carbonyl to form a neutral enol. The enolic carbon can then attack the oxalacetate carbonyl (8). Everything is perfectly aligned. Distances are too close to permit even a water molecule to intervene between the reactive groups. Since the carboxyl has been desolvated, the system at the reactive site (ES_R) has been destabilized by attractive forces at the binding site (ES_B). The effects of ES_R and ES_B might more or less cancel, leaving the overall ES level unchanged. But, in a "case II" situation, the rate can increase dramatically nonetheless because the perturbation at ES_B is conserved. Model work from ourselves (e.g., 4) and many others[23] points to the reasonableness of this picture.



Final Remark

I began this Account with remarks on organic "models" and will end it similarly. What would it take to design a truly efficient enzyme "model" (i.e., one that operates on a realistic and unactivated substrate, such as an aliphatic amide, with a 10^8 acceleration)? Spatiotemporal theory proclaims that an organic host or catalyst must noncovalently bind a substrate such that the reactive functional groups come into van der Waals contact. This implies a precision in noncovalent association that has not yet been realized in the laboratory. If the noncovalent binding is too loose, then solvent will intervene between the functionalities, and the catalysis will be to a large extent ruined. If complexation holds the functionalities rigidly but "out of reach", then the "model" could turn out to be an inhibitor rather than a catalyst. In order to better fine-tune our models, one needs to have a deeper understanding of the relationship between rate and geometry.[24] How does "rate vs distance" depend upon the choice of reaction, the solvent, or the angle of attack? How might noncovalent forces (which are far less directional than covalent bonds) be controlled to achieve a desired interatomic distance? Once answers to such questions are available, it will be possible to design enzyme models that might please even the antireductionists.

This work was supported by the National Science Foundation.

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(24) For studies along these lines, see: Kemp, D. S.; Carey, R. I.; Dewan, J. C.; Galakatos, N. G.; Kerkman, D.; Leung, S.-L. *J. Org. Chem.* **1989**, *54*, 1589.