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Analysis of Ground-State and Transition-State Effects in Enzyme Catalysis†

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ABSTRACT: "The entire and sole source of catalytic power is the stabilization of the transition state; reactant-state interactions are by nature inhibitory and only waste catalytic power". So reads a literature quote expressing the current view on enzyme catalysis proposed by Pauling over 40 years ago. Its validity is now examined by means of a "split-site" model in which an active site is subdivided into a region of binding and a region of reaction. Analysis of the resulting free energy levels clarifies several points of confusion regarding the nature of enzyme catalysis, including why enzyme/substrate complexes form if, indeed, they only "waste catalytic power". Circumstances are defined in which an evolving enzyme can both lower K_m (i.e., enhance substrate binding) and improve the forward catalytic rate while never meddling with the transition structure at the reactive site. It is argued that this process is most advantageously viewed as a substrate destabilization embodying "conserved" interactions at the binding region. Classical transition-state stabilization and an "anti-Pauling" effect are both capable of inducing rate accelerations. In certain circumstances, the latter can predominate as it does with many enzyme-like intramolecular reactions. Behavioral modes discussed herein are applicable to the chemistry of catalytic host/guest and abzyme systems.

M. I. Page listed no fewer than 21 published theories of enzyme catalysis including some with colorful names such as orbital steering, propinquity, vibrational activation, stereopopulation control, and group transfer hydration (Page, 1987). The recent trend, however, has been to adopt a simple and elegant idea originated over 40 years ago by Pauling (1946, 1948). In his view, an enzyme accelerates a reaction by binding strongly to a transition structure. This mode of catalysis, now known as "transition-state stabilization", will be deliberated in the present paper. For the moment, note simply

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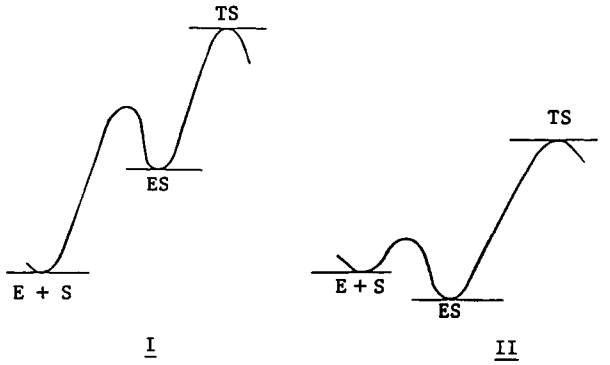


FIGURE 1: Free energy diagrams for enzyme-catalyzed reactions in which E = enzyme, S = substrate, ES = enzyme/substrate complex, and TS = transition state. Profile I: $[S] < K_m$. Profile II: $[S] > K_m$, where K_m is the Michaelis–Menten dissociation constant for ES. The important difference between profiles I and II lies in the relative energy level of (E + S) relative to other states. See Schowen (1978) for a detailed discussion of the two profiles.

that transition-state stabilization encompasses, rather than invalidates, the 21 theories of enzyme action. According to Schowen, all enzyme theories ultimately reduce to a single factor, namely, Pauling's transition-state binding (Schowen, 1978).

Transition-state stabilization is not without problems. For example, transition structures are bandied about so freely (often with bond lengths and angles having four significant figures) that one forgets their true nature: mental constructs with no finite existence. If this is too philosophical, then consider the practical problem of constructing and studying an actual enzyme/transition-structure complex. The best one can do is "model" a transition structure with a ground-state molecule having all its bonds intact. But in so doing, the essence of the transition structure (i.e., its partial bonds) is totally lost. The hope is that the enzyme/model complex will, nevertheless, simulate enzyme/transition-structure association. Success in such a venture is always complicated by the fact that, necessarily, the enzyme/model complex also resembles the enzyme/ground-state complex, and it is difficult to separate effects.

Transition-state stabilization can be viewed in terms of the two free energy diagrams in Figure 1 (Schowen, 1978). Profile I represents the situation when the concentration of substrate S is low relative to the Michaelis–Menten dissociation constant, K_m. As seen, the ES free energy level lies above that of (E + S). Profile II represents the situation when the concentration of S is high compared to K_m, so that the enzyme exists mainly in the bound state. ES lies here below the (E + S) level. Both cases must be considered because one seldom knows whether or not the concentration of S exceeds K_m under actual biological conditions.

Since free energy levels will be jostled throughout the ensuing discussion, it is important that the relevant descriptors be properly defined. Stabilization of ES is equivalent to a tighter binding between E and S and, therefore, to a lower energy of the complex. In general, "lower free energy" will be used in preference to the other terms. By the same token, a "higher free energy" in ES will refer to a weaker association in which some destabilizing effect (e.g., compression or desolvation) raises the ES level.

One final note of clarification is necessary. The forward rate of an enzymatic reaction reflects the free energy difference between the highest and lowest points on the free energy profile. Thus, in profile I of Figure 1, one needs to consider the free energy difference between transition state TS and (E + S). In profile II, the critical difference is between TS and ES, a difference corresponding to k_{cat} in simple enzyme mechanisms (Zerner & Bender, 1964).

Table I: Effect of Free Energy Changes on Catalysis in Cases Where ES Is of Higher Energy Than (E + S) as in Figure 1, Graph I

case	(E + S)	ES	TS	ΔG_a	effect[a]
A	0	+5[b]	+20[b]	+20	
B	0	+4	+20	+20	none
C	0	+5	+19	+19	accel
D	0	+4	+19	+19	accel

[a] Case A is the standard to which the other cases are compared. ΔG_a = TS − (E + S). Where TS, ES, and (E + S) are the free energy levels of the transition state, complex, and reactants, respectively. [b] Positive values reflect the fact that ES and TS lie above (E + S).

Inspection of Figure 1 leads to curious conclusions. According to profile I in which ES is always above (E + S), a change in ES has absolutely no effect upon the rate. This is because the rate depends only on the free energy difference between TS and (E + S) and not on the exact pathway with which E plus S attain TS. According to profile II in which ES is always below (E + S), a lowering of ES slows down the rate by increasing the TS/ES differential. In other words, formation of an enzyme/substrate complex is "anticatalytic" (Schowen, 1978). The tighter the complex, the lower the ES free energy level, the more the enzymatic reaction is inhibited. In conclusion, therefore, formation of an ES complex either does nothing (profile I) or actually slows down the rate (profile II). It is this fact that gave rise to the so-called "fundamentalist position" on enzyme catalysis illustrated by a quote from Schowen: "The entire and sole source of catalytic power is the stabilization of the transition state; reactant-state interactions are by nature inhibitory and only waste catalytic power" (Schowen, 1978). The quote embodies current thinking on the energetics of enzyme catalysis (Hackney, 1990; Fersht, 1985).

All enzymes fashion a noncovalent complex prior to the bond-making and bond-breaking steps. Why is this true if ES formation is either irrelevant or harmful to the rate? The answer usually given to this question is brief, vague, and impossible to disprove. Enzyme/substrate complexes, according to fundamentalist dogma, must be connected to biological processes other than catalysis (e.g., regulation). In other words, a high forward flux is not the only selection criterion upon which molecular evolution has operated.

It is necessary immediately to forestall any suspicions that substrate selectivity, rather than rate, accounts for the ubiquitous existence of ES. Nonbonded interactions readily impart selectivity upon single-step reactions lacking any intervening prereaction association. One need look only at the Diels–Alder reaction to see how a simple bimolecular process, with no proven intermediate, manifests selectivity. Thus, regioselectivity and stereoselectivity are not predicated upon formation of an initial complex, and one must search elsewhere to justify ES. I will return to the ES dilemma shortly.

In order to buttress the above conclusions, the free energy profiles in Figure 1 were analyzed in terms of a specific numerical examples. Thus, arbitrary values have been assigned to the free energy levels. Information derived from the numbers is generally valid because the information does not depend upon the initial choice of values. This approach is adopted because it is easier to grasp relationships using numbers than abstract symbols. Later on, when a more complicated model of ES will be invoked, the value of numerical examples will become particularly evident.

Table II: Effect of Free Energy Changes on Catalysis in Cases Where ES Is of Lower Energy Than (E + S)

case	(E + S)	ES	TS	ΔG_a	effect[a]
A	0	−5[b]	+15	+20	
B	0	−6	+15	+21	decel
C	0	−5	+14	+19	accel
D	0	−6	+14	+20	none

[a] Case A is the standard to which the other cases are compared. ΔG_a = TS − ES. [b] Negative values reflect the fact that ES lies below (E + S).

Table I lists free energy assignments for profile I in which ES is always more energetic than (E + S). A value of zero has been given to the reference point (E + S). As mentioned above, the activation energy, ΔG_a, equals the difference between TS and (E + S).

It is obvious from case B vs case A that lowering ES alone has no effect on ΔG_a and, thus, on the rate. In contrast, lowering TS alone (C vs A) reduces ΔG_a and thereby enhances the rate. One can lower both ES and TS, as in D, but the resulting acceleration is due solely to a transition-state effect (in line with fundamentalist thinking). Table I affirms that ES is kinetically irrelevant whenever its energy level is positive.

Table II lists free energy assignments for profile II in which ES is always less energetic than (E + S). The situation is completely different from that in Table I because now ΔG_a = TS − ES. Stabilization of ES (case B) slows down the reaction, whereas stabilization of TS (case C) accelerates it. If both ES and TS are lowered by the same amount (e.g., one energy unit as in D), then the rate remains unchanged. Clearly, the benefit of stabilizing TS is cancelled by an equivalent stabilization of ES. Of course, an enzymatic rate can be accelerated, despite an ES lowering, as long as the TS is stabilized to an even greater extent. Such possible permutations have not been incorporated into Table II but are self-evident from the four limiting cases.

There is nothing new in what has just been discussed except, perhaps, the phraseology. Tables I and II merely substantiate the currently popular line that enzymes operate solely by stabilizing transition states. (Hansen & Raines, 1990; Kraut, 1988; Lolis & Petsko, 1990; Rêtey, 1990; Wolfenden & Frick, 1987). Any attempt by an evolving enzyme to improve ES binding (i.e., lower K_m and the ES energy level) will only damage the catalytic cause.

Having espoused the validity (if not the obviousness) of fundamentalist notions, I will now proceed to demonstrate that they are, in fact, misleading. For example, as will be shown, an enzyme is capable of binding its substrate more tightly by a mechanism that leads directly to an improved catalysis. In other words, an enzyme can indeed have its binding and catalysis too. The object in elaborating the contrary position was not to delude the reader but, instead, to describe current thinking and to construct a framework from which an improved model can be launched.

The Split-Site Model. The ensuing discussion is based on the premise that substrates and cofactors can be subdivided into a binding site and a reactive site. Substrate association is describable, therefore, as the sum of interactions with the enzyme at the distinct binding and reactive sites (ES = ES_B + ES_R). Figure 2 depicts the concept, a concept that is hardly new. For example, in 1950 Bergmann, Wilson, and Nachmansohn proposed that acetylcholinesterase possesses (a) an anionic site for binding acetylcholine's quaternary ammonium group and (b) an esteratic site which brings about the actual ester hydrolysis (Bergmann et al., 1950). Note also that subdividing energies is a time-honored procedure. For example, molecular mechanics calculations give the total molecular energy as the sum of bond stretching, bond bending, torsional effects, etc., within a particular structure (Miwa & Machida, 1989). Treating ES_B and ES_R as independent entities seems like a minor assumption relative to the other uncertainties in enzymology.

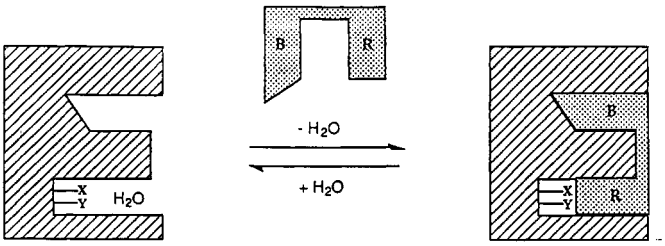


FIGURE 2: Split-site enzyme model in which an active site (large shaded area) is subdivided into a binding region and a reactive region. These regions associate with B and R of the substrate (dotted object above the arrows), respectively. Catalytic groups on the enzyme (X and Y) are brought into contact 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).

Before giving specific numerical examples for the split-site model (as was just done in Tables I and II for the "combined site" situation), it is necessary to establish certain principles of behavior. Most importantly perhaps, one should realize that the fates of ES_B and ES_R are totally different upon initiation of chemical reaction. As a first approximation, binding at ES_B is not changed as the transition structure is attained. In effect, ES_B binding is *conserved*. For example, a hydrogen bond at the ES binding site (and distant from the reactive site) persists in the TS. In contrast, a hydrogen bond at the reactive site is altered because chemical changes are taking place. ES_R interactions are *not* conserved.

A second reasonable stipulation will be imposed on the system. The ES_B level will always be lower than the ES_R level. The reasons are simple and in concert with current views of enzyme mechanisms. Attractive forces at ES_B (i.e., hydrogen bonding, electrostatics, hydrophobic bonding, and van der Waals association) all tend to stabilize ES. On the other hand, interactions at ES_R are regarded as destabilizing. As shown in Figure 2, the catalytic groups of the enzyme (X and Y) must be desolvated prior to bringing the reactive group of the substrate within "striking range". Solvent extrusion is only one of several possible energy-costing distortions that might be found at the ES_R site. For example, it also requires free energy to enforce a reactive conformation upon the substrate. In summary, ES_B is stabilizing, while ES_R is destabilizing, and the overall stability of ES depends on the sum of the two. I stress again that there is nothing new here. The preceding statements have been expressed in various forms by others in the past (Hackney, 1990; Hammes, 1964; Jencks, 1969). Yet the analysis given below, based on these statements, will clarify certain confusion in the area as well as establish generalities that have not been firmly fixed in literature.

Table III provides a numerical analysis of the split-site model under low substrate conditions. Understanding the table presupposes the following relationships (which have already been discussed and need no further justification): (a) ES = ES_B + ES_R; (b) TS = TS_B + TS_R; (c) ΔG_a = TS − (E + S). Recall also that the ES level dictates K_m (the lower the ES, the smaller the K_m). And, finally, it should be explained that TS_R was assigned a constant of 20 energy units under the simplifying assumption that we are dealing with the same transition structure in all cases. TS_R stabilization will be dealt with later.

Table III: Analysis of the Split-Site Model in Cases Where ES Is of Higher Free Energy Than (E + S) as in Figure 1, Graph I

case	(E + S)	ES_B[b]	ES_R	ES	TS_B	TS_R	TS	ΔG_a	effect[a]
A	0	+3	+7	+10	+3	+20	+23	+23	
B	0	+2	+8	+10	+2	+20	+22	+22	accel
C	0	+4	+6	+10	+4	+20	+24	+24	decel
D	0	+2	+7	+9	+2	+20	+22	+22	accel
E	0	+3	+6	+9	+3	+20	+23	+23	none

[a] Case A is the reference to which other cases are compared. [b] Values could have been made negative with no change in conclusions.

Table IV: Analysis of the Split-Site Model in Cases Where ES Is of Lower Free Energy Than (E + S) as in Figure 1, Graph II

case	(E + S)	ES_B	ES_R	ES	TS_B	TS_R	TS	ΔG_a	effect[a]
A	0	−7	+3	−4	−7	+20	+13	+17	
B	0	−8	+4	−4	−8	+20	+12	+16	accel
C	0	−6	+2	−4	−6	+20	+14	+18	decel
D	0	−8	+3	−5	−8	+20	+12	+17	none
E	0	−7	+4	−3	−7	+20	+13	+16	accel
F	0	−9	+4	−5	−9	+20	+11	+16	accel

[a] Case A is the reference to which other cases are compared. ΔG_a = TS − ES.

Table III immediately attracts interest because the "effect" column shows that by tinkering with ES_B and ES_R one can accelerate, decelerate, or do nothing to the catalytic rate. A far cry from fundamentalist theory that ES is irrelevant! The most important conclusions, along with the particular case that proves the point, are listed below.

(1) Case B. At constant K_m, stabilization of the binding site (and an equivalent destabilization of the reactive site) accelerates the reaction. This is because the effect at ES_B is conserved in the transition structure.

(2) Case C. At constant K_m, destabilization of the binding site (and an equivalent stabilization of the reactive site) decelerates the reaction for the same basic reason as in case B.

(3) Case D. Stabilization (or destabilization) of the binding site accelerates (or decelerates) the reaction independent of perturbations experienced by the reactive site.

(4) Case E. Stabilization (or destabilization) of the reactive site at constant binding energy does nothing to the rate.

Two crucial points arise from this analysis. First, an attractive interaction at the binding site accelerates a reaction no matter how far away the interaction is located from the on-going chemistry. Thus, a hydrogen bond to ring A of an enzyme-bound steroid will necessarily facilitate a reaction at ring D within the Michaelis–Menten complex. It now becomes clear why cofactors and coenzymes (e.g., thiamine and coenzyme A) possess so much peripheral organic baggage. Each attractive ground-state interaction lowers the activation energy by an amount exactly equal to the attractive energy.

The second major point has to do with semantics which, inevitably, enter into the enzyme catalysis problem. Suppose an enzyme mutates so as to transform an alanine into a serine. Suppose further that the mutation benefits the enzyme kinetically by forming a new hydrogen bond to the substrate bound to the active site. The hydrogen bond may happen to be nowhere near the site of chemical action, but (as I have just proved) a rate acceleration must occur 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 subtleties created at the reacting center. One could also argue, however, for a transition-state effect because the new hydrogen bond ultimately operates by lowering the total transition structure energy. The truth is that both positions are acceptable. Confusion and quibbling has come about from the fact that changes in the ground state translate into an identical effect in the transition state by means of conserved perturbations.

We now arrive, finally, at the most intriguing aspect of the split-site model, namely, its ability to clarify misleading assertions voiced by the fundamentalists. Consider profile II of Figure 1 where the substrate concentration is high relative to K_m. This is another way of expressing conditions where ES is always lower than (E + S). Table IV shows the effect upon rate of various shifts in energy levels. ES and TS are still the sum of the parts, as in Table III, but now ΔG_a = TS − ES. The binding site of the complex, ES_B, replete with attractive forces, has been given negative values. And since the reactive site ES_R may be characterized by desolvation, strain, etc., it has been assigned positive values. The sum of the two, ES, is always negative, however, in accordance with profile II. Restricting ES_B to large negative values, and ES_R to small positive values, was mechanistically reasonable and convenient for maintaining a manageably compact table. But one could have just as readily selected other sets of numbers without changing the overall conclusions. These are listed below case-by-case (all relative to the reference, case A).

(1) Case B. At constant K_m, lowering ES_B and raising ES_R accelerates the rate.

(2) Case C. At constant K_m, raising ES_B and lowering ES_R decelerates the rate.

(3) Case D. Lowering (or raising) ES_B at constant ES_R does nothing to the rate.

(4) Case E. Raising ES_R at constant ES_B accelerates the rate.

(5) Case F. Lowering ES_B by an amount greater than that which ES_R is raised accelerates the reaction.

Numerous comments concerning this group of conclusions are necessary. Case B shows that one can keep the ES level constant and yet accelerate the rate by tightening the association to the binding sites. This "drags" down TS via a conserved effect, and the rate increases. Similarly, raising ES_B and lowering ES_R by the same amount (case C), thereby maintaining a constant K_m, reduces the reaction rate.

Cases D and E demonstrate the opposite behavior from that seen in Table III. The rate is now independent of ES_B if ES_R is kept constant. This is because lowering ES_B, for example, also lowers TS by an equal amount, and the deleterious effect on the former cancels the beneficial effect of the latter. Raising ES_R at constant ES_B, which did nothing in Table III, now accelerates the reaction (case E) because ES is also raised. The difference between TS and ES, equal to ΔG_a, is therefore lessened.

Case F is worth particular mention because it reveals the misleading nature of the previously quoted fundamentalist dictum that "reactant-state interactions are by nature inhibitory and only waste catalytic power" (Schowen, 1978). Case F describes an example of ES_B being lowered more than the amount that ES_R is raised. As a consequence, ES is lowered (which is tantamount to a tighter overall binding and smaller K_m). Nevertheless, the rate increases. What is the source of the apparent contradiction to current beliefs that stabilizing ES necessarily impedes the rate? The answer lies in the fact that the larger reduction of ES_R is conserved in TS while the smaller elevation of ES_B is not. Therefore, TS is reduced more than ES, and the rate increases. In terms of specific numbers, ES in case F is lowered only from −4 to −5, while TS is lowered from +13 to +11. The net effect is that ΔG_a is lowered from +17 to +16 to the benefit of the rate.

The fundamentalist response to the above analysis would be, no doubt, "But in case F, one is not just lowering ES, one is also tinkering with TS". It is here where we run again into semantics. Owing to the rule of conserved energies, an adjustment of ES_B necessarily leads to a modification of TS. They are inseparable. One can call this a ground-state effect or a transition-state effect; it makes no difference. The important point, however, is that the fundamentalists mislead everybody by claiming that tighter binding in ES is always harmful. Case F says differently.

Clarification of the fundamentalist misstatements is so important that it merits another paragraph. Under saturation conditions, stabilization of ES is claimed to be invariably harmful. As shown, this is true only when ES_R is stabilized. Generally, however, an enzyme stabilizes ES via the much larger binding site replete with multiple contact points. But lowering of this ES_B has absolutely no effect on the rate. And an enzyme could readily lower ES by stabilizing ES_B while destabilizing ES_R to a lesser degree. In such a case, lowering ES actually speeds up the reaction.

Implications. Chemists often prefer chemistry to numbers. The question therefore arises as to how, given the principles already illustrated in terms of numbers, an enzyme might be structurally modified to improve its function. In the interest of space, the question will be answered with only a single example, case B of Table IV, but this should suffice to drive home the general idea.

Consider an organism that would, in the course of evolution (Benner, 1989), attain survival advantage if a particular digestive peptidase improved its catalytic ability. However, owing to certain other constraints, it is beneficial not to alter (up or down) the effectiveness of substrate/enzyme binding. In other words, k_{cat} should be larger, and K_m should remain constant. How can this be accomplished?

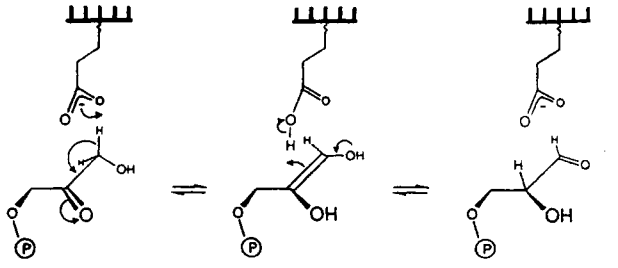
Our prehistorical peptidase might, conceivably, reach its kinetic goal by stabilizing TS. This could happen by an alanine-to-aspartate mutation in the ES_R portion of the active site. Hydrogen-bonding to the peptide's labile carbonyl by the carboxyl could, no doubt, stabilize the tetrahedral intermediate and the transition state leading to it. Of course, the same hydrogen bond would also stabilize ES at the reactive site. To the extent that the latter occurs, the catalysis at higher substrate levels would be diminished. Catalysis comes about, of course, only when TS_R is stabilized more than ES_R. Such a situation would pertain, for example, if the carbonyl in the transition structure bore a larger negative charge than in the ground state. But owing to the fact that the transition structure always resembles the ground state, TS_R stabilization is almost always counteracted, to some extent, by an ES_R stabilization. There is, however, a more serious problem than a partially compromised catalytic effectiveness. K_m would be decreased as a result of the additional substrate binding (see Table IV). Since the stipulation was to keep K_m constant, and thus not negate the survival value of the Ala-to-Asp mutation, the enzyme would require a second mutation to destabilize ES back to its original value. The second mutation would have to appear, in this evolutionary scenario, before the first one was eliminated from the population owing to the less viable K_m.

A second avenue for improved catalysis at constant K_m is open to the evolving enzyme. Mutational modification can again create a new hydrogen bond, but this time the hydrogen bond is distant to the reactive site (e.g., at a carbonyl of a peptide linkage adjacent to the labile one). If this enhanced enzyme/substrate attraction helps "tuck in" the substrate, destabilization would arise from a closer contact distance between the labile carbonyl and an active site nucleophile (e.g., a serine hydroxyl). Extrusion of water between the peptide carbonyl and the serine could also contribute to the destabilization. Overall, the K_m could remain approximately constant because a favorable interaction at ES_B has paid the "energy bill" for compression at ES_R. And, of course, k_{cat} would increase because stabilization at ES_B is conserved at TS_B. The above is merely a verbal expression of case B, Table IV.

Call the above a "transition-state effect" if you will, but bear in mind that the driving force, a hydrogen bond to a substrate carbonyl distant from the point of reaction, has nothing directly to do with the partial bonding and other accoutrements of the transition structure. For this reason, I much prefer to confine "transition-state stabilization" to cases in which there is preferential stabilization directly at the reactive site itself. Otherwise, it is far less confusing and, perhaps, conceptually more correct, to employ "ground-state destabilization".

No doubt each enzyme has its own recipe for catalysis. The key point, however, is that the Pauling idea of transition-state binding is only partly correct. 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 enforced distances and desolvation at the reactive site is also a direct repository of catalytic potential. To accelerate a reaction by this mechanism, an enzyme need only "tighten its grip" upon a substrate by means of an additional attractive contact somewhere in the molecule.

Triosephosphate Isomerase. Recent work on triosephosphate isomerase (Pompliano et al., 1990) illustrates the above construct. Salient facts are now listed: (a) Triosephosphate isomerase catalyzes the conversion of D-glyceraldehyde 3-phosphate into dihydroxyacetone phosphate (eq 1) with a $k_{cat} = 430\ s^{-1}$ and $K_m = 0.97$ mM. (b) The enzyme



(1)

possesses a 10-residue mobile "loop" that interacts directly, by means of one or two hydrogen bonds, to 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. (d) Molecular mechanics calculations on the mutant enzyme show that the substrate phosphate is exposed and the substrate, as a whole, is less tightly and rigidly bound.

The authors make a pursuasive case that loop/phosphate interaction impedes a wasteful liberation of an unstable reaction intermediate. This feature in no way precludes the likelihood that the tight grip of the loop on the phosphate also accelerates k_{cat} in accordance with the split-site model. In chemical terms, the loop/phosphate association at the binding site (distant though it may be from the reactive site) could help enforce a contact distance between the carboxylate and enolizeable proton. This might include an energetically costly, but kinetically beneficial, desolvation of the carboxylate. By destabilizing the substrate in such a manner, proton flow from the weak carbon acid to the carboxylate is facilitated.

The enzyme can also be analyzed in more quantitative terms. Assume that the loop/phosphate binding is worth 8.4 kcal/mol corresponding to two hydrogen bonds. Assume that the free energy of the reactive site (ES_R) is thereby elevated 7.0 kcal/mol owing to the resulting compressive and desolvation forces in the ground state. The reaction would then be accelerated by 10^5, relative to a mutant lacking the loop, although the enzyme–substrate binding would be improved only 10-fold (i.e., 8.4 − 7.0 = 1.4 kcal/mol = 10×). These are the exact numbers found with triosephosphate isomerase. And "transition-state stabilization" was never mentioned.

Final Remarks. No paper on substrate destabilization, at least no paper written by this particular author, can finish without mentioning the so-called "spatiotemporal hypothesis" (Menger, 1985). Fast intramolecular and enzymatic reactions were rationalized here in terms of short contact distances between functional groups. Reaction between A and B was viewed, therefore, as a two-step process: (1) movement of A and B to van der Waals contact distances with concomitant extrusion of solvent and between them and (2) formation of the actual A–B bond. Since the energy requirements for the first step can often exceed those of the second, a high rate enhancement is possible when contact distances are imposed at an active site. Enzymatic activity and geometric disposition in the *ground state* are believed to be intimately related.

Support for the distance concept included our design of an enzyme model in which a single well-positioned carboxyl group, adjacent to an aliphatic amide, cleaved the amide at pH 6–7 at 22 °C in minutes (Menger & Ladika, 1988)! Sustained proximity at van der Waals contact distances gave rise to an enzyme-like rate acceleration. Support for the distance concept also came from theoretical studies in which it was shown that a major portion of the energy requirements for a hydride transfer entails hydride relocation to a critical distance from a recipient carbonyl; once this distance is attained, bond breakage and cleavage is extremely fast (Sherrod & Menger, 1990). We also derived a Marcus-like equation showing that a few tenths of an angstrom decrease in distance can enhance proton transfer rates by several orders of magnitude (Menger, 1988).

In fast intramolecular reactions, covalent bonds impose contact distances between functionalities. In enzymatic reactions, noncovalent forces serve the same purpose. Hydrogen-bonding, electrostatic attraction, hydrophobic association, etc., at various locations up and down a substrate or cofactor create a tight contact at the reactive site. Solvent is extruded from the site, and a fast reaction ensues. This substrate destabilization does not, of course, preclude classical Pauling transition-state stabilization. Both mechanisms must be incorporated into any complete picture of enzyme catalysis.

X-ray pictures of enzyme/substrate/cofactor complexes are appearing in increasing numbers (Bystroff et al., 1990; Karpusas et al., 1991). How might one recognize "anti-Pauling" behavior (i.e., substrate destabilization) in such structures? A full response to this question merits another paper, but to answer it briefly one would have to mention two features that are particularly revealing: (a) multiple contacts between enzyme and substrate at points on the substrate distinct from the actual reactive site and (b) van der Waals distances between the catalytic groups of the enzyme and the reactive group of the substrate (the distances being too small to permit intervening solvent). Examples of the latter could include an enzyme's imidazole ring perched on a substrate's enolizable proton, or NADH's mobile hydrogen perched on a substrates's carbonyl carbon, or an enzyme's carboxyl perched on the double bond of an unsaturated substrate. In all these examples, conserved attractions at ES_B serve to activate the substrate at ES_R. As already argued in detail, it is preferable to regard this activation mechanism as substrate destabilization, rather than transition-state stabilization, although the two are inseparably connected.

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