

Rate-Limiting Steps, Low Barrier Hydrogen Bonds, Electrostatic Stabilization

The original version of this lecture was based on a group of mini-review articles published in *J. Biol. Chem.* in 1998. The introduction to these reviews (Neet, K.E., *J. Biol. Chem.* **273**:25527-8 [1998]) quotes his own 1968 review with Koshland (*Ann. Rev. Biochem.* **37**:357-410), "Until quantitative numbers for each factor can be assigned and until the products of these factors allow the enzymatic rate to be calculated from a model system, we will not have explained enzyme action. It is clear that the identification of these factors and their quantitative evaluation is one of the most important challenges of the next decade." He now says, "Three decades have now passed without complete quantitative evaluation." Indeed, it is clear from the reviews that it is not possible even to agree whether a given factor contributes significantly or only insignificantly to a given catalytic acceleration; the authors in effect argue quite heatedly about the significance of some factors. But I find that the ultimate conclusion of two of these three reviews is much the same as I suggested earlier, quite without knowledge of their high-powered thinking - that the significance of enzymes as structured catalysts is that they *position* various groups with catalytic effects, so that they can act in concert without paying the entropy penalty which would be necessary to assemble them in solution.

In the ensuing six years the situation has not been clarified, rather the arguments seem to have grown more bitter, as noted in the article from *Chemistry and Engineering News* (Feb. 23, 2004, pp. 35-9) which I am passing out.

The acceleration is represented by the **catalytic factor**, $\frac{\text{rate of catalyzed reaction}}{\text{rate of uncatalyzed reaction}}$, which however is not always measurable, because the uncatalyzed reaction may not be observable and because a 1st-order enzymatic reaction must be compared to 2nd-order catalysis by a small molecule. Nevertheless, factors of 10^8 to 10^{12} are often cited.

One of these suggested accelerating factors is the Low Barrier Hydrogen Bond, LBHB for short. This was long known by organic chemists, but was proposed as an accelerating factor separately by Gerlt, Cleland and Frey in 1993 and 1994, and is described by these authors (in order Cleland, Frey and Gerlt), *J. Biol. Chem.* **273**:25529-25532 (1998). A hydrogen bond you know is when a proton is between two electronegative atoms with some degree of bonding to both. In a 'normal' H-bond the negative atoms are at least 2.8 Å apart and the bonding is weak, 5 kcal/mole. (This still sounds fairly strong, but driving energy for catalysis is only available when an H-bond is formed where there was none before, and in water solution such a proton is always H-bonded to something.) H-bonding in water is weak because the pK_{as} of the groups formed if you transfer the H, H_3O^+ and OH^- , are very different, -1.7 and 15.7 when you take into account that water is 55 M.

The Low Barrier Hydrogen Bond is made possible by two factors: the negative atoms are closer together, <2.5 Å, and the pK_{as} of the groups involved are similar. The energy barrier for jumping between being closer to one negative atom and closer to the other decreases to less than the zero-point energy of the proton (**draw**), and it jumps back and forth; neutron diffraction, which can see protons, sees a blur in the middle of the bond. The strength of the bond increases to 15-20 kcal/mol, which means that a structure in which such a bond occurs is favored by that much over one without it. As the distance between the negative atoms decreases further, toward contact at 2.29 Å between their centers for O atoms, the two energy wells merge into one; this is only known in $[FHF]^-$ and $[HO-HOH]^-$. Formation of LBHBs is also favored by being in a non-polar environment, where water cannot compete to form other H-bonds and the dielectric constant is low. LBHBs are described as being largely covalent; 'normal' hydrogen bonds are largely ionic, H with a δ^+ attracted to O with δ^- .

What is the value of LBHBs for enzymic catalysis? They can stabilize transition states, which is now considered to be the broadest description of what enzymes do, and can stabilize useful intermediates of the reaction or make their formation possible.

Consider the suggested role in catalysis by chymotrypsin (see **Scheme I and Fig. 2**). There has been much argument over the position of the proton between his⁵⁷ and asp¹⁰². It has been shown to have an unusual chemical shift in nmr, 15 ppm at pH 9, 18 at pH 4 where the imidazole will be protonated. (This is also observed in the similar enzyme α -lytic protease, which has only one histidine, in the catalytic triad; the shift of the proton is coupled to the shift in ¹⁵N-imidazole.) The shift is 1 ppm less in D₂O at pD = 3.5, as expected for an LBHB.

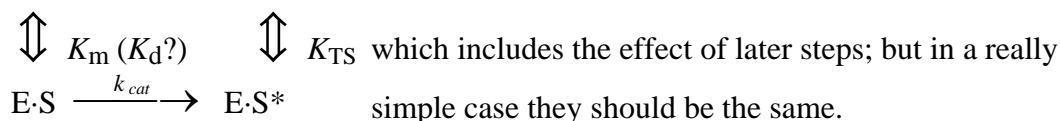
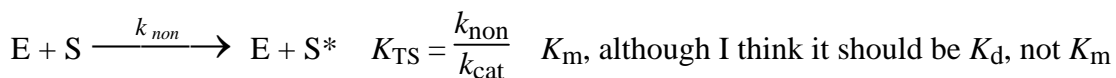
But pH 4 is not where chymotrypsin works. An LBHB is formed at this pH because the imidazole is protonated, and its pK_a and that of asp¹⁰² are similar, 7.0 and 4.8, probably more similar in the nonpolar enzymic environment which will lower the pK_a of an imidazole and raise that of a carboxyl. At neutral pH an LBHB would not be formed, even though it would relieve a 'compression stress' between two rather closely placed negative atoms, because it would require pulling a proton away from neutral imidazole, pK_a = 14. But when a substrate binds, ser¹⁹⁵ moves close enough to the other side of the imidazole that what was a weak, distorted H-bond becomes a hand-off of the proton, protonating the imidazole so that the LBHB can now be formed; this favors formation of ser-O⁻, pK_a normally 14, which attacks the substrate to form the tetrahedral intermediate. The imidazolium charge is just about half transferred to the aspartate, as expected in a LBHB.

Stable tetrahedral intermediates can be made with peptidyltrifluoromethyl ketones (structure II of scheme I). In these the chemical shift of the imidazole proton is 18.9 ppm, indicating a good LBHB, and the pK_a of the imidazolium is 12.1, indicating that this form is stabilized by 7.3 kcal/ mole compared to substrate-free chymotrypsin. The imidazole in effect is a much stronger base, facilitating proton removal from ser¹⁹⁵ and thus its attack on the substrate to form the tetrahedral intermediate.

It is then considered that the departure of the leaving group (HOR or H₂NR) from the tetrahedral intermediate, the 4th structure in Fig. 2, is facilitated by formation of a LBHB between the imidazole and it, with eventual transfer of the H; but much less is said about this.

The review goes on to argue for low barrier hydrogen bonds in the mechanisms of ketosteroid isomerase, triose phosphate isomerase and citrate synthase; but I see some problems with the application of the concept in these cases, so I won't spend time on them.

Cannon and Benkovic (*J. Biol. Chem.* **273**:26257-260 [1998]) argue that an important part of enzyme catalytic power comes from the substitution of the nonpolar environment of the enzyme active site for the polar environment of the substrate in water. They set up a simple thermodynamic cycle comparing the transformation of a reactant in solution (rate constant k_{non}) through its transition state to the transformation via the enzyme-bound transition state (rate constant k_{cat}). With this cycle one can calculate the theoretical dissociation constant K_{TS} of the enzyme-bound transition state to free enzyme E and free transition state S*:



Either the transition state binds much better to the enzyme than the ground state of the substrate - the usual explanation of transition state stabilization, which may also include small adjustments of the enzyme - or the free energy of interaction between S* and the solvent is worse than between S* and the enzyme, which could be stated, "the solvent binds the ground state better". In the first case, acceleration is due to better binding of the transition state, low K_{TS} should be associated with a high k_{cat} ; in the second case, acceleration is due, conceptually, to removal of the transition state from a poor solvation environment that impedes reaction, and k_{non} for the uncatalyzed reaction would be low. The plot of k_{cat}

and k_{non} vs. K_{TS} shows a good negative correlation of $\log k_{\text{non}}$ with $\log K_{\text{TS}}$, but a poor correlation (high scatter, low slope) of $\log k_{\text{cat}}$ with K_{TS} , which the authors take as confirmation of their idea.. Also, some simple chemical reactions (the example is $\text{Cl}^- + \text{CH}_3\text{Br} \rightarrow \text{CH}_3\text{Cl} + \text{Br}^-$) are faster in organic solvents than in water, which is a more structured, high dielectric medium. As I have already mentioned, ionic forces are stronger in a low dielectric medium. This may be why in many cases enzymes wrap themselves around the substrates, to ensure exclusion of water from a low-dielectric medium.

They conclude, "Enzymes are pre-organized for reaction" - I think this is the same concept that I stated as escaping the payment of an entropy penalty for the organization of catalytic groups in position. A variety of this concept has been described by Bruice as "near-attack conformations": enzymes favor reactions by binding particular conformations of substrates which are close to the transition state, rather than the commonest conformation found in solution. This is argued particularly for chorismate mutase, a key enzyme in biosynthesis of tyrosine and phenylalanine, in which an enolpyruvate side chain O-linked to a six-membered ring switches to C-linkage to a different position on the ring, a rather dramatic reaction; the enzyme binds a conformation of chorismate with the CH_2 right over the C atom of the ring with which it reacts. Fine for this reaction, but most reactions are not so dramatic and do not offer such a possibility of binding a favorable conformation. And in this reaction as in the lysozyme reaction, it is hard to say whether the enzyme selects a pre-existing rare conformation or bends the substrate from the common conformation to the rare one as it binds and makes all available interactions. The end result is the same, structurally and energetically: stabilizing the reactive conformation is an important step toward stabilizing the transition state.

Warshel (*J. Biol. Chem.* **273**:27035-8 (1998) generally attacks the concept of ground state destabilization (making the bound substrate less stable as a way of lessening the ΔG^* to reach the transition state). (A specific attack on this concept in the case of orotidine-5-phosphate decarboxylase, the last enzyme in formation of uridine monophosphate, is Warshel, Strabjl, Villa & Florián, *Biochemistry* **39**:14728-38 [2000].) He points out that ground state destabilization will not reduce $k_{\text{cat}}/K_{\text{m}}$. Mutations of enzymes which affect activity by reducing ground state destabilization are not found, only mutations which affect transition state stabilization or binding of parts of the substrate distant from the catalytic site. He shoots down the desolvation hypothesis - actual enzyme active sites, he says are polar; polar transition states would be *less* stable in a nonpolar environment (but this is not exactly what Benkovic proposes; rather, polar *interactions* are *more* powerful in a less polar environment). He also minimizes the effects of reduction of binding entropy and 'orbital steering', attacks low barrier hydrogen bonds (saying they are really ionic hydrogen bonds, or they wouldn't stabilize transition states; and the pK_{a} s of groups involved are *not* similar as predicted by Cleland's theory), and dismisses 'dynamic effects' and nuclear tunnelling effects.

He believes that electrostatic effects are paramount, and analyzes them by computer simulation, but only when one can look at a real enzyme; he focuses on changes in energetics between the reaction in solvent and in the enzyme active site, using what he calls the Empirical Valence Bond method.

Electrostatic effects are hard to study directly in aqueous solution; the real evidence comes from the effects of site-specific mutations. Initial surveys by computer simulation suggested that electrostatic interactions of the transition state with the enzyme were no better than it would have with water. But for a reaction in water the dipolar water molecules must be properly arranged around the transition state, half the energy gained from interaction between water dipoles and substrate charges is spent in ordering the water molecules. In enzyme active sites, however, all polar groups that interact with the transition state are already aimed in the right direction, so that the *net* energy gain by such interactions is greater. He sums this up by saying "the catalytic energy is not stored in the enzyme-substrate interaction but in the enzyme itself". It seems to me that this is equivalent to what I suggested earlier (and wrote long before this paper), that the significance of enzymes is that they pay the entropy penalty of folding to

precise ordered conformations *once*, but can use this precise arrangement of groups interacting with the transition state over and over again with multiple substrate molecules.

Another supposed mechanism is quantum mechanical tunneling, proposed to contribute to many reactions where either a proton or a hydride ion, H^+ or H^- , is being moved, which includes all sorts of dehydrogenase reactions. This has been pushed particularly by Judith Klinman at UC Berkeley. She has built up experimental evidence for this, mainly in the form of otherwise unexpected kinetic isotope effects, and others have supported this by computer simulations. However, opponents have argued that the tunneling can happen also in non-enzyme-catalyzed reactions, so it doesn't offer that much acceleration. As I said, I never took quantum mechanics, so I can't really talk about this, but you can see in our metaphor of crossing a mountain pass that tunneling underneath it could greatly decrease the activation energy. However, in cases where the effect has been measured, the acceleration due to it is not large, 1.5 to 780-fold.

A very recent review which touches on these concepts and more is by Garcia-Viloca, Gao, Karplus and Truhlar, *Science* **303**:186-195 (2004). They emphasize the power of computer simulations to separate out the role of particular factors in catalysis. One example is orotidine-phosphate decarboxylase, a very efficient enzyme. They express the difference in the free energy barrier between the uncatalyzed and enzyme-catalyzed reactions as $\Delta G_1 + \Delta G_2$, where ΔG_1 is the change in interaction energy of the *substrate* from a water environment to enzyme bound and ΔG_2 is the change in free energy of the environment, i.e. of the enzyme. They find that $\Delta G_1 =$ only -2 kcal/mole while $\Delta G_2 = -20$ kcal/mole, i.e. the enzyme is strained by binding the substrate, but this strain is relieved by the substrate going to the transition state.

They state the basic rate equation as $k(T) = \gamma(T)(k_B T/h)(C^0)^{1-n} e^{(-\Delta G^*/RT)}$, where the T in parentheses just specifies that it is the equation for a particular temperature – an expression more consistent with what I gave you before would be $k_T = \gamma_T \left(\frac{\kappa T}{h} \right) [C^0]^{1-n} e^{(-\Delta G^*/RT)}$, where C^0 is the standard state concentration of the reacting compound, n is the order of the reaction, T is the absolute temperature, and $\Delta G^*/RT$ is the free energy of activation. What they really want to talk about is the term γ_T , which they call a generalized transmission coefficient, which seems to be a fancy name for a fudge factor reconciling theory and reality. This can contain: a term Γ_T which takes into account “dynamic recrossing”, which seems to refer to molecules which get over the top of the pass but lose their way and wander back again, a term κ_T which is not Boltzmann's constant but a correction for tunneling effects, and a term g_T which “arises from deviations of the equilibrium in phase space”, which I don't understand and they admit has been so little studied that they can't tell whether it plays a role – certainly not a large role.

The first term, Γ_T , is less than or equal to 1, i.e. it may reduce the rate, but not much; the tunneling factor can only increase it; and the g_T term could be larger than or smaller than 1, i.e. could increase or decrease the rate. The net effects of all these thus seem to be small unless tunneling is more significant than it appears to be in most reactions.