ARTICLE

Low-Barrier Hydrogen Bonds and Enzymic Catalysis

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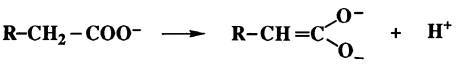
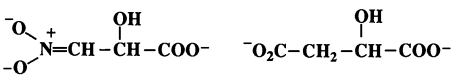
Formation of a short (less than 2.5 angstroms), very strong, low-barrier hydrogen bond in the transition state, or in an enzyme-intermediate complex, can be an important contribution to enzymic catalysis. Formation of such a bond can supply 10 to 20 kilocalories per mole and thus facilitate difficult reactions such as enolization of carboxylate groups. Because low-barrier hydrogen bonds form only when the pK_a's (negative logarithm of the acid constant) of the oxygens or nitrogens sharing the hydrogen are similar, a weak hydrogen bond in the enzyme-substrate complex in which the pK_a's do not match can become a strong, low-barrier one if the pK_a's become matched in the transition state or enzyme-intermediate complex. Several examples of enzymatic reactions that appear to use this principle are presented.

Enzymic catalysis is commonly attributed to tighter binding between the enzyme and its reactants in the transition state than in the initial enzyme-substrate complex. For a stepwise mechanism with an intermediate structurally similar to the most energetic transition state, this intermediate must be more tightly bound than the original substrate. These statements do not, however, explain how an enzyme can bind an intermediate or a transition state much more tightly than the substrate. Enzymologists have long wondered, for example, how enzymes find it so easy to remove protons from carbons next to carboxylate groups to give "carbanion" intermediates, which appear to be aci-carboxylates:



For example, in the reaction catalyzed by fumarase, the 3*R* proton from malate (whose pK_a is more than 30) is transferred to a base with a pK_a of 5.7 in free enzyme, and the process comes to equilibrium between pH 6 and 8 (*1*). This base is thought to be a carboxylic acid because of the temperature coefficient of its pK_a, and because its apparent pK_a is elevated by 0.7 or 1.3 pH units in the presence of malate or fumarate (*2*). Thus, the pK_a of the 3*R* proton is lowered by more than 23 pH units, equivalent to about 31 kcal mol^{-1}. All of this energy is unlikely to come from a better steric fit of the intermediate than of the substrate, although the aci-nitro dianion of 3-nitrolactate (an analog of the putative intermediate) binds to fumarase 900 times more tightly than it does to malate (*3*)



K_i = 27 nM

K_m = 24 μM

where K_i is the inhibition constant and K_m is the Michaelis constant.

Ionized nitro analogs of putative intermediates in other elimination reactions show similar binding ratios, with the binding of the nitro analog of isocitrate to aconitase being 72,000 times tighter than that of isocitrate (*4*). These effects account for at most five orders of magnitude and leave 18 orders of magnitude unexplained (corresponding to 25 kcal mol^{-1}). Part of the difference may come from the double negative charge of the aci-carboxylate, whereas the aci-nitronate has only a single charge; however, this does not account for all of the energy difference.

It has become apparent to us and others (*5*, *6*) that the missing energy comes from the formation of very strong hydrogen bonds to the intermediate while the corresponding hydrogen bonds to the substrate are weak. We attribute the strengthening of the hydrogen bond to two factors. (i) The donor-acceptor distance is reduced, and any competing water is squeezed out by the tight fit of the transition state (and any intermediates that closely resemble it) in the enzyme. (ii) The proton affinities of the two heteroatoms bridged by the hydrogen bond are brought to near equality.

Strong hydrogen bonds, which Cleland (*7*) has called "low-barrier hydrogen bonds," can have energies of formation in the gas phase as high as 31 kcal mol^{-1}, whereas ordinary hydrogen bonds of the type between water molecules are relatively weak (5 kcal mol^{-1}, or even weaker, in the gas phase). The O–O distance for weak hydrogen bonds is 2.8 to 3.0 Å, whereas that for strong hydrogen bonds is <2.5 Å [table 4 of (*8*)]. In a weak hydrogen bond the hydrogen is attached to one or the other of the oxygens by a covalent bond, whereas the interaction with the other oxygen is largely electrostatic. When the pK_a's of the two oxygens are similar, the hydrogen can be attached to either one, and there is an energy barrier for transfer between the two oxygens (Fig. 1A). As the two oxygens become closer to each other in a hydrogen bond, the barrier between the two hydrogen positions becomes lower and eventually is low enough that the zero point energy level is at or above the barrier (Fig. 1B). At this point, which corresponds to an O–O distance of ~2.5 Å, the hydrogen can freely move in the space between the two oxygens, and its bonding to both oxygens becomes essentially covalent (*9*). A further shortening of the O–O distance would result in a single well hydrogen bond (Fig. 1C), but such hydrogen bonds are only thought to exist in the $[FHF]^-$ and $[HO\text{-}HOH]^-$ ions, where the F–F and O–O distances are 2.26 Å [table 7 of (*8*)] and 2.29 A (*10*), respectively.

The requirements for forming low-barrier hydrogen bonds appear to be the absence of a hydrogen-bonding solvent such as water and similar pK_a's of the two heteroatoms involved in the bond (*11*). The strongest bonds form when the two heteroatoms are the same (oxygen or nitrogen), but N··H··O bonds can be the low-barrier type, although probably not as strong as O··H··O bonds (*11*). The bond strength seems well correlated with the distance between the heteroatoms, with the shortest bonds being the strongest (*9*).

From the above discussion it becomes clear how low-barrier hydrogen bonds can play a role in enzymic catalysis. There only has to be a hydrogen bond between the substrate and enzyme when the substrate is initially bound, which is weak because the pK_a's of the substrate and enzymic group do not match, but where the pK_a of the intermediate or transition state or both will match that of the enzymic group. Thus, the hydrogen bond goes from being a low-energy one in the enzyme-substrate com-

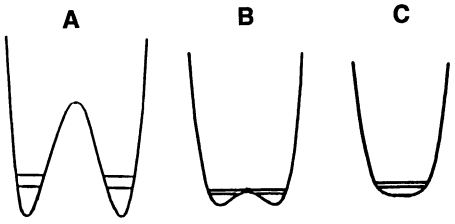


Fig. 1. Potential functions for (**A**) a double-well hydrogen bond, (**B**) a low-barrier hydrogen bond, and (**C**) a single-well hydrogen bond (*35*). The horizontal lines represent lowest energy levels for hydrogen (upper) and deuterium (lower).

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plex to being a high-energy low-barrier one in the enzyme-intermediate complex or the transition state, and the energy of formation of the low-barrier hydrogen bond (from 10 to 20 kcal mol^{-1}) becomes available to help facilitate the reaction. Because the environment of an enzyme active site should resemble solution in a nonprotic solvent rather than water, there would be no hindrance to the formation of a low-barrier hydrogen bond, provided that the pK_a's are properly matched. Some actual examples for which an x-ray structure is available and the chemistry of the enzymatic reaction is well enough understood that we can draw definite conclusions are presented below.

During the reaction catalyzed by ketosteroid isomerase, a dienol or dienolate intermediate is formed from a substrate or product that is a ketone (Fig. 2). The proton is removed by Asp^{38} during formation of the intermediate, and Tyr^{14} is hydrogen-bonded to the carbonyl oxygen of the substrate before the reaction begins (*12*). In such a hydrogen bond the pK_a of Tyr^{14} is 11.6 (*13*), whereas that of the ketone is negative. But the pK_a of the dienol intermediate [10 in water (*14*), but probably higher on the enzyme] should match that of Tyr^{14}, so that the hydrogen bond becomes a low-barrier one, thus producing the energy to bring about the enolization. Note that it would not be advantageous for the enzyme to transfer the proton from the tyrosine to the dienolate, because this would require a drop in the pK_a of tyrosine or an increase in the pK_a of the dienolate (either one an energy-requiring process) without gaining the energy of formation of the low-barrier hydrogen bond.

In the reaction catalyzed by triosephosphate (triose-P) isomerase, Glu^{165} removes a proton from glyceraldehyde-3-P or dihydroxyacetone-P to give an enediolate intermediate (Fig. 3). The group hydrogen bonding to the carbonyl oxygen of the substrate is a neutral histidine (*15*). This discovery was a surprise, but the reason for this arrangement becomes obvious if one considers the necessity to match the pK_a's of the enediol and the histidine to form a low-barrier hydrogen bond to the intermediate. The pK_a of the enediol will be ~14, which matches the pK_a of neutral imidazole but not the pK_a of protonated imidazole (~6); both pK_a's are probably decreased by the local environment on the enzyme (*15*). Furthermore, when His^{95} is mutated to glutamine, the enzyme adopts a different mode of acid-base catalysis in which Glu^{165} catalyzes all proton transfers, and the reaction slows down by a factor of 380 with glyceraldehyde-3-P as substrate (*16*). In this reaction there are two enediolate intermediates, one with the low-barrier hydrogen

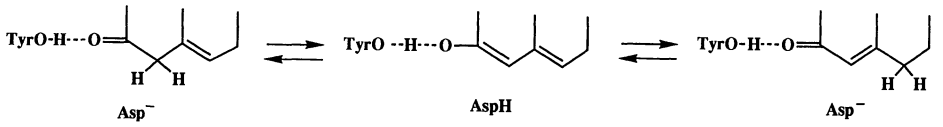
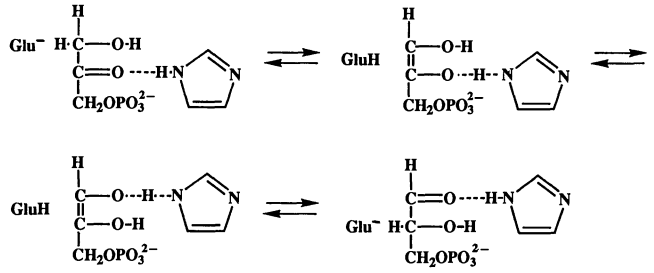


Fig. 2. Mechanism of ketosteroid isomerase.

Fig. 3. Mechanism of triosephosphate isomerase.



bond to the oxygen at C-1, and the other with a hydrogen bond to the oxygen at C-2.

Citrate synthase is thought to enolize acetyl coenzyme A (acetyl-CoA) and then catalyze the attack of the enolate on oxalacetate (Fig. 4). Specifically, Asp^{375} acts as the base to remove the proton from the methyl group of acetyl-CoA, and the carbonyl oxygen is hydrogen-bonded to His^{274} in its neutral form (*17*). As with triose-P isomerase, in the enolized acetyl-CoA complex, a low-barrier hydrogen bond can form between the carbonyl oxygen and the imidazole ring, both of which should have pK_a's of ~14 at this point. Formation of this bond provides energy for the enolization. Attack of the enolate intermediate on the carbonyl carbon of oxalacetate is assisted by hydrogen bonds from the carbonyl oxygen of oxalacetate to His^{320} and Arg^{329}. However, which residue provides the proton during the formation of the citryl-CoA intermediate is not clear; it may come ultimately from Asp^{375}, in that this group would have to lose its proton before acting as a general base during the hydrolysis step. Hydrolysis of citryl-CoA by addition of water may again involve a low-barrier hydrogen bond to His^{274}, as the pK_a of the tetrahedral intermediate would be close to that of His^{274}.

A recent x-ray study of citrate synthase with carboxyl or amide analogs of acetyl-CoA bound in the active site shows short (2.4 to 2.5 Å) hydrogen bonds between Asp^{375} and a carboxyl oxygen or amide nitrogen (*18*). The hydrogen bonds to His^{274} are 2.7 to 2.8 Å. These inhibitors appear to be bound more tightly than acetyl-CoA as the result of formation of low-barrier hydrogen bonds to Asp^{375}. Contrast this mode of binding with that during the catalytic reaction, where Asp^{375} removes a proton from the CH_3 group of acetyl-CoA, and the putative low-barrier hydrogen bond is the one to His^{274}. The difference results from altered pK_a's of the bound molecules.

Orotidylate decarboxylase catalyzes the decarboxylation of orotidylate by means of a zwitterionic intermediate (Fig. 5). The

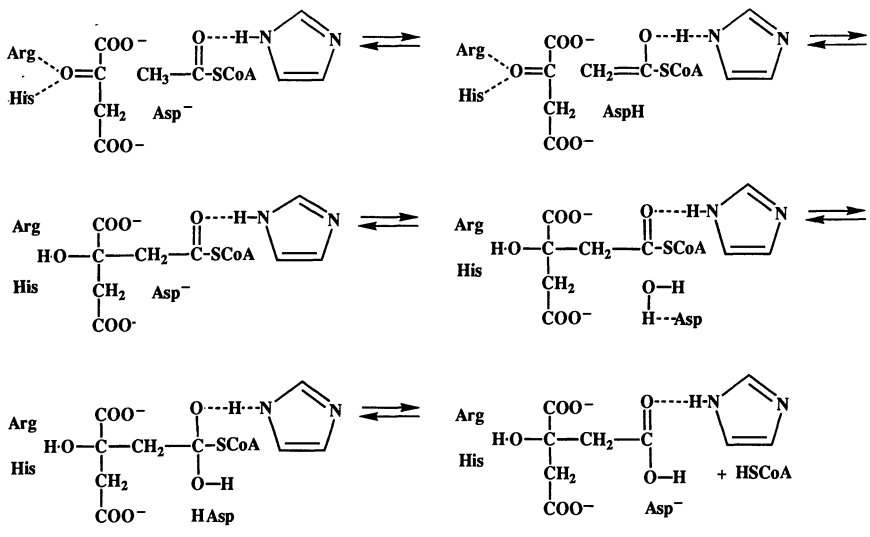


Fig. 4. Mechanism of citrate synthase.

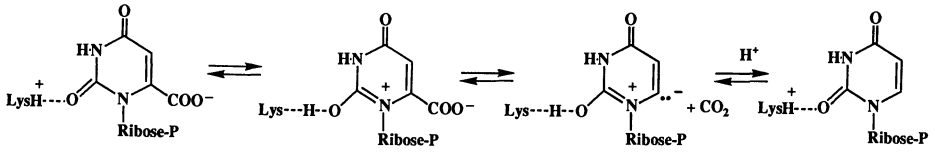
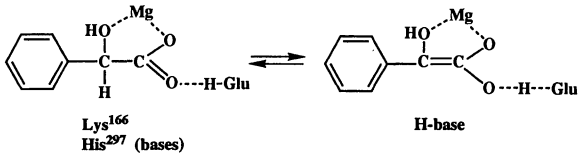


Fig. 5. Mechanism of orotidylate decarboxylase.

Fig. 6. Mechanism of mandelate racemase.



Lys[93] is an essential group and is thought to hydrogen bond to the carbonyl oxygen at C-2 (*19*, *20*). The electronic structure of the substrate is polarized toward the enolate at C-2 by the formation of a low-barrier hydrogen bond to the enolate oxygen because the pK_a's should then be matched. The positive charge at N-1 then permits decarboxylation to give the ylid structure at N-1 and C-6. Protonation of C-6 returns C-2 to the keto form. Once again, there is more energy to be gained by sharing the proton in a low-barrier hydrogen bond than by fully transferring it.

Like the other enzymes discussed above that enolize their substrates, mandelate racemase also forms an aci-carboxylate or enolized intermediate (Fig. 6). The bases that remove the proton from C-2 and put it back on the other face of the aci-carboxylate intermediate are Lys[166] and His[297] (*21*). A Mg^{2+} ion is coordinated between the oxygens at C-1 and C-2, and this helps stabilize the intermediate. The major stabilization, however, probably comes from a low-barrier hydrogen bond between the other oxygen at C-1 of the intermediate and Glu[317]. The pK_a of mandelic acid is 3.4, whereas that of the aci-carboxylate in this case is estimated to be 6.6, which is probably a good match to that of Glu[317]. Gerlt and Gassman (*5*, *6*) have discussed this case at length and have come to the same conclusions.

The crystal structure of aconitase held many surprises (*22*), but the mechanism is now well understood as the result of the collaborative efforts of several groups (Fig. 7). The enzyme contains a 4Fe-4S center, and in the free enzyme the exposed Fe is four-coordinate, being coordinated to hydroxide in addition to the three sulfurs (*23*). When isocitrate or citrate bind, this Fe becomes octahedral, and both the OH group of the substrate and one of its carboxyl groups become coordinated. At this point Ser[642], which sits in an oxyanion hole with its oxygen hydrogen bonded to Arg[644] and a main chain amide (*22*), donates a proton to the Fe-coordinated OH group to turn it into water. This Fe-coordinated water is hydrogen-bonded to the carboxyl group that is next to the carbon from which proton removal occurs during the reaction. The alkoxide of Ser[642] acts as the base to remove this proton, producing an aci-carboxylate whose pK_a presumably now matches that of the Fe-coordinated water. This hydrogen bond becomes a low-barrier one, providing the energy to drive the enolization. In particular, this hydrogen bond is 2.7 Å in the enzyme-isocitrate structure and 2.5 Å in the structure with the nitro analog of isocitrate bound as an aci-nitronate (*22*). After decomposition of the aci-carboxylate intermediate by transfer of the substrate OH group to Fe (assisted by protonation from His[101]), one is left with *cis*-aconitate coordinated through one of its carboxyl groups, and two water molecules on the Fe. There are two ways that *cis*-aconitate can bind with one or the other of its vinyl carboxylates coordinated to the Fe; one mode of binding produces citrate upon hydration, and the other produces isocitrate.

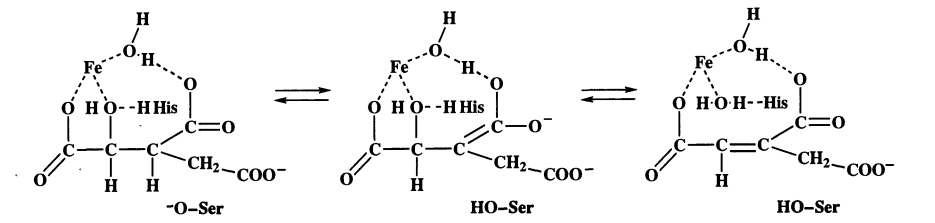


Fig. 7. Mechanism of aconitase.

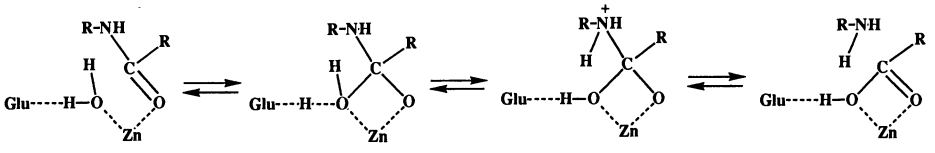


Fig. 8. Mechanism of thermolysin and carboxypeptidase.

Thermolysin and carboxypeptidase hydrolyze amides or esters by using Zn^{2+} to polarize the carbonyl oxygen, and a glutamate residue (270 in carboxypeptidase; 143 in thermolysin) to act as a general base during attack of Zn-coordinated water on the carbonyl carbon (Fig. 8). Transfer of a proton to the leaving group permits decomposition of the bidently coordinated tetrahedral intermediate to the final products. The tetrahedral intermediate has been mimicked by various phosphonates, one of which binds with a dissociation constant of 10 fM (*24*). In x-ray studies these inhibitors are bidentate ligands of Zn, and the hydrogen bond between the catalytic glutamate and one coordinated oxygen shows a distance in three structures of each enzyme with different inhibitors of 2.2 to 2.5 Å (*25*–*28*). These short distances suggest that in the tetrahedral intermediate this is a low-barrier hydrogen bond, thus providing the energy to drive the formation of the intermediate.

Evidence that serine proteases have low-barrier hydrogen bonds between the catalytic aspartate and histidine in the active site has now been presented by Frey *et al.* (*29*), who conclude that the strength of this bond increases as the tetrahedral intermediate forms. These data should settle the previous arguments about whether the aspartate or the histidine is protonated in the tetrahedral intermediate—in fact, they share the proton.

In our discussion above we have described enzymatic reactions with true intermediates where it seems likely that low-barrier hydrogen bonds would occur between the intermediate and the enzyme. The stabilization of the intermediate is maintained in the transition state, which resembles the intermediate, so catalysis is enhanced. Also, protons are transferred in a number of reactions in which a low-barrier hydrogen bond may occur in the transition state, but not in the ground state or any intermediate. For example, a number of dehydrogenases catalyze oxidation of alcohols, hemiacetals, or thiohemiacetals involving hydride transfer from substrates to a pyridine nucleotide. In most cases proton transfer from the OH group to a

catalytic base [histidine for lactate (*30*), malate (*31*), and glyceraldehyde-3-P dehydrogenases (*32*); a carboxyl group for glucose-6-P dehydrogenase (*33*)] is concerted with hydride transfer. During the reaction the pK_a of the OH group goes from 13 to 15 to a negative value as this oxygen becomes part of a carbonyl group, whereas the pK_a of the catalytic base is within a few pH units of 7. If the transition state occurs at the point where the pK_a of the OH group is equal to that of the catalytic base, the hydrogen bond between them can transiently become a low-barrier one and thus provide considerable energy for stabilizing the transition state. Whether this actually happens is difficult to establish, but it is certainly an attractive way to help catalyze a reaction!

The examples given above are based on comparisons of x-ray structures and known chemistry of the enzymatic reactions. Can experimental evidence other than short distances in x-ray structures be found for the existence of low-barrier hydrogen bonds in enzymes? The most promising tool is nuclear magnetic resonance (NMR), because the protons in low-barrier hydrogen bonds show chemical shifts of 17 to 20 parts per million (ppm). This approach has been used by Frey *et al.* (*29*) to show the presence of low-barrier hydrogen bonds in serine proteases. Low-field proton peaks have been seen for aspartate aminotransferase, and the lowest field one (at 17.8 ppm in the presence of a substrate analog) has been assigned to the proton between the ring nitrogen of pyridoxal and Asp^{222} (*34*).

Once low field proton signals are seen in the NMR spectrum, the fractionation factor of the hydrogen can be used to show that the hydrogen bond is a low-barrier one. The fractionation factor is the equilibrium isotope effect for exchange of deuterium from this position into water, and values of 0.3 (representing threefold discrimination against deuterium in the hydrogen bond) have been seen for model compounds in organic solvents (*35*). Fractionation factors should be measurable by integrating low field proton NMR peaks in mixtures of H_2O and D_2O after hydrogen exchange has come to equilibrium.

In summary, it appears that the low-barrier hydrogen bond may play a very important role in stabilizing intermediates in enzymatic reactions and in lowering the energy of transition states. These bonds can be identified by their short interatomic distances, their low fractionation factors, and in favorable cases by physical methods such as infrared, NMR, or neutron diffraction. Crystallographers and enzymologists who study mechanisms need to be familiar with the properties of these bonds, which are described in the recent review by Hibbert and Emsley (*8*).

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