

## The Low Barrier Hydrogen Bond in Enzymatic Catalysis\*

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The proposal that low barrier (*i.e.* short, very strong) hydrogen bonds (LBHBs)<sup>1</sup> play a role in enzymatic catalysis was first put forth in 1993 and 1994 (1–4). The proposal was accepted by some but rejected by others (5–8). Initial rejection on theoretical grounds has been followed by increasing experimental support, and recent improvements in theory have been able to account for the experimental observations of LBHBs in enzymes (9–15). In this minireview we will explain the original proposal, summarize the experimental data from the past few years, and argue that LBHBs *do* play important roles in enzymatic reactions.

### Properties of Hydrogen Bonds

The strength of a hydrogen bond depends on its length and linearity, the nature of its microenvironment, and the degree to which the pK values of the conjugate acids of the heavy atoms sharing the proton are matched. In water, the hydrogen-bonded oxygens are separated by ~2.8 Å, and the  $\Delta H$  of formation is ~5 kcal mol<sup>-1</sup>. The hydrogen bonds in water are, however, weak because of the poor pK match between the participating oxygen atoms. Because the pKs of H<sub>3</sub>O<sup>+</sup> and H<sub>2</sub>O are -1.7 and 15.7, respectively, the proton in the structure H<sub>2</sub>O···H–OH is tightly associated with the OH<sup>-</sup> group as a water molecule.

In the gas phase, where the dielectric constant is low, hydrogen bonds between heteroatoms with matched pKs can be very short and strong, and experimental as well as calculated values of  $\Delta H$  of formation can approach 25 or 30 kcal mol<sup>-1</sup> (16, 17). Likewise, in crystals hydrogen bonds can be very strong. The O–O distance in the ion [H–O···H···O–H]<sup>-</sup> in a crystal of a chromium complex is only 2.29 Å (18, 19). In organic solvents, strong hydrogen bonds can also form, although the  $\Delta H$  of formation probably never exceeds 20 kcal mol<sup>-1</sup>. Recent calculations suggest that once the dielectric constant is at least 6 the strength of a strong hydrogen bond levels off at a level about half that in the gas phase (13, 17). Because the active site of an enzyme is no longer aqueous once it has closed around a substrate, the properties of hydrogen bonds in organic solvents are highly pertinent to enzymatic catalysis.

What happens energetically as hydrogen bonds become shortened can be seen in Fig. 1. Structure A represents the situation in water, where the hydrogen is firmly attached to either the left-hand or right-hand oxygen and is more loosely bonded to the other one, with an O–O distance of  $\geq 2.8$  Å. There is an energy barrier between the two possible positions of the hydrogen, with the zero point energy levels shown in Fig. 1. Such a hydrogen bond is essentially electrostatic, and the covalent O–H bond is the usual 0.9–1.0 Å in length.

As the overall O–O distance is shortened, the energy barrier drops until it reaches the zero point energy level at an O–O distance of ~2.5 Å (Fig. 1B); this is a LBHB. The  $\Delta H$  of formation has

increased to 15–20 kcal/mol, and the hydrogen can now move freely between the two oxygens. In crystals containing LBHBs, neutron diffraction shows the hydrogen diffusely distributed with its average position in the center (20). LBHBs are largely covalent (21). There are a number of possible structures between A and B in Fig. 1; however, the covalent O–H bond becomes longer and the overall covalent character of the hydrogen bond increases as the hydrogen bond becomes shorter and stronger (20). Further shortening leads to the limit of 2.29 Å and structure C in Fig. 1.

The LBHB has other physicochemical properties in addition to its short heteroatom distance. Its proton NMR chemical shift is far downfield (17–21 ppm), and it can be observed in aqueous solution by application of appropriate water suppression pulse sequences when the exchange rate is slower than the spectrometer frequency. A second property is the low deuterium fractionation factor. Deuterium becomes enriched in more stiffly bonded positions, and the fractionation factor measures the degree of discrimination against deuterium in a given bond relative to the OH bonds in water. Because the covalent O–H distance increases from ~1.0 to ~1.2 Å as a weak hydrogen bond is converted into a LBHB, the bond order decreases and thus the discrimination against deuterium increases. Fractionation factors as low as 0.3 have been measured for symmetrical LBHBs such as that between *p*-nitrophenol and *p*-nitrophenolate ion in organic solvents (22). The fractionation factor of the hydrogen in a LBHB in a protein can be measured by integrating the low field proton NMR peak in mixed H<sub>2</sub>O/D<sub>2</sub>O mixtures. Other spectroscopic properties of LBHBs that have been used in studies of small molecules include perturbations of IR stretching frequency and differences in proton and deuterium NMR chemical shifts. For a review of the properties of hydrogen bonds, including LBHBs, see Ref. 23.

### The Role of LBHBs in Enzymatic Catalysis

Although the existence of LBHBs has been known to physical organic chemists for many years, the presence of LBHBs in proteins and the way in which they could play a role in enzymatic catalysis by conversion of a weak hydrogen bond in the initial enzyme-substrate complex into a LBHB in the transition state was only recently recognized (1–4). When a mechanism involves formation of an unstable intermediate, the transition state for forming it will closely resemble it, and the LBHB will also be found in the intermediate or in an enzyme-inhibitor complex that closely mimics it. Such mimics of metastable intermediates at enzymatic sites allow observation of LBHBs by spectroscopic and crystallographic methods (4, 24).

An enzyme converts a weak hydrogen bond into a strong one by changing the pK value of the substrate so that it is close to that of the enzymatic group to which it is hydrogen bonded. The pK values for protonating ketones are approximately -5, whereas the pK values of the corresponding enols or enediols are 10–14. If the enzymatic group hydrogen-bonded to the ketone is a neutral imidazole ring of a histidine (pK 14 for imidazolide formation), there will be a good pK match between the enolate and histidine, though not between the ketone and histidine. The increased strength of the LBHB between the enolate and neutral histidine can provide much of the energy needed to bring about enolization of the substrate (a Brønsted base is, of course, also required).

One criticism leveled at this proposal was that hydrogen bond strengths did not depend so heavily on pK matching (8, 25). However, the data of Shan and Herschlag (25, 26) show that the variation in  $\Delta G$  of formation with  $\Delta pK$  depends on the solvent. Although the slope of a plot of the log of the formation constant ( $K_{form}$ ) versus  $\Delta pK$  is only 0.05 in water, it is 0.73 in dimethyl sulfoxide for a series of salicylate monoanions and 0.65 in tetrahydrofuran for complexes between phenols of varying pK. Enzyme active sites are non-aqueous, and the effective dielectric constants resemble those in organic solvents rather than that in water. If we assume a slope

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<sup>1</sup> The abbreviation used is: LBHB, low barrier hydrogen bond.

of 0.7, this corresponds to a weakening of the hydrogen bond by a factor of 5 for each unit mismatch in  $pK$  values. If this change pertains over the full range of  $pK$  values, a change of  $-5$  to  $14$  in  $pK$  would provide over  $18$  kcal mol $^{-1}$  of energy, which corresponds to over  $13$  orders of magnitude in rate acceleration. Although the slope of  $\log(K_{\text{form}})$  versus  $\Delta pK$  probably does not remain constant as the  $\Delta pK$  increases, it is clear that considerable energy is potentially available to help catalyze enzymatic reactions.

### Acid-Base Catalysis

Enzymes catalyze the removal or addition of protons to substrates by employing their side chain functional groups as Brønsted acid or base catalysts. In the reaction catalyzed by lactate dehydrogenase, proton transfer from the OH group of lactate to His-195 accompanies hydride transfer to NAD. The  $pK$  of lactate is  $\sim 15$ , whereas that of pyruvate is approximately  $-5$  and the  $pK$  of the histidine imidazolium group is  $\sim 6$ . Therefore, the hydrogen bond between lactate and His-195 is weak, with a  $pK$  mismatch of  $\sim 9$  pH units. Likewise, the hydrogen bond between pyruvate and protonated His-195 has a mismatch of  $\sim 11$  units. During the reaction the  $pK$  of the lactate crosses that of His-195, and at that point the match in  $pK$  values should lead to strengthening of the hydrogen bond, accompanied by lowering of the activation energy for the reaction. The elimination of a  $pK$  mismatch of 9 units could potentially accelerate the reaction by 4.5 orders of magnitude, which is a typical contribution of general acid/base catalysis to the rate of an enzymatic reaction (the factor can reach  $10^5$  (27)).

### Serine Proteases

A clear example of a LBHB in enzymatic catalysis is shown by chymotrypsin. The low field proton seen at  $\delta$  18.3 ppm for the proton between His-57 and Asp-102 in acidic solutions of chymotrypsin (28) has been assigned as a LBHB (4). Chemical models in nonaqueous media confirm the low field NMR signal, as well as reveal characteristic Fourier transfer IR features (29). The LBHB-containing structure **I** (Scheme I) displays the low field proton signal as well as a very short N–O distance of  $<2.6$  Å between the heteroatoms. The van der Waals contact distance for nitrogen and oxygen is 2.7 Å. The LBHB partially depolarizes the ion pair between His-57 and Asp-102, so that in structures **I** and **II** (Scheme I),  $1 > y \geq 0.5$ . The LBHB-containing complex **I** is related to the transition state or tetrahedral intermediate for the formation of acyl chymotrypsin in that His-57 is protonated; however, the peptidyl group and tetrahedral carbon are absent, so that **I** is an imperfect analog observed only at low temperatures and pH. Much better mimics are the tetrahedral complexes of chymotrypsin with peptidyl trifluoromethylketones (**II**). These are excellent analogs of tetrahedral intermediates and display the LBHB at  $\delta_H$  18.6–18.9 ppm, which varies with the structure of the peptidyl group (24, 30).

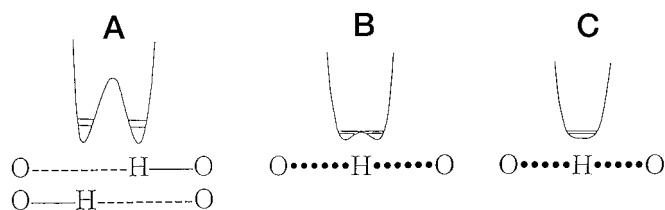
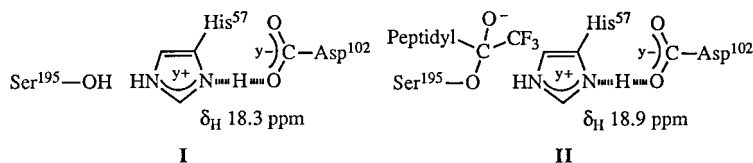


FIG. 1. Energy diagrams for hydrogen bonds between groups of equal  $pK$ . **A**, weak hydrogen bond with O–O distance of 2.8 Å; the two positions of the hydrogen are shown. **B**, low barrier hydrogen bond of length 2.55 Å; the hydrogen is diffusely distributed, with average position in the center. **C**, single-well hydrogen bond with length 2.29 Å. The upper and lower horizontal lines are zero point energy levels for hydrogen and deuterium, and the curves define the energetic barrier to changes in bond length. The distances are to scale.



SCHEME I

The low field proton is observed at pH 4–12 and above 30 °C. The N–O distance for the LBHB is 2.5 Å when the peptidyl group is *N*-Ac-Leu-Phe and 2.6 Å when it is *N*-Ac-Phe (31). In a tetrahedral intermediate,  $CF_3$  would be replaced by the leaving group. The LBHB is postulated to stabilize the tetrahedral intermediate and lower the activation energy for its formation (4).

Stabilization by the LBHB in **I** and **II** should increase the basicity of His-57. This is observed for **II**, in which the apparent value of  $pK$  at 5 °C is 12.1 when the peptidyl group is *N*-Ac-Leu-Phe and 10.6 when it is *N*-Ac-Phe (24). The difference in  $pK$  between 12.1 and the value of 6.8 for glycyl histidine indicates  $\sim 7.3$  kcal/mol of stabilization by the LBHB (24). However, the apparent value of  $pK$  for His-57 in free chymotrypsin, **I**, is in the normal range, 7.5 at 3 °C (32). These facts can be understood by considering the differences between **I** and **II** and the properties of His-57 and Asp-102. **I** is a poor mimic of the transition state in that it lacks the peptidyl group and tetrahedral carbon bonded to Ser-195. Their presence leads to the increased basicity of His-57 in **II**. Therefore, the binding of the peptidyl group and tetrahedral carbon alters the interactions of His-57 and Asp-102 by promoting the formation of the LBHB. Conformational compression of His-57 and Asp-102 upon binding the peptidyl group and approaching the transition state would facilitate LBHB formation and increase the basicity of His-57 (24). Increased basicity for His-57 will enhance its reactivity as a Brønsted base in removing the proton from Ser-195 and lower the energy of the transition state for forming the tetrahedral intermediate (Fig. 2). Low field protons in trypsin, subtilisin, and  $\alpha$ -lytic protease confirm the generality of LBHBs in serine proteases (33, 34).

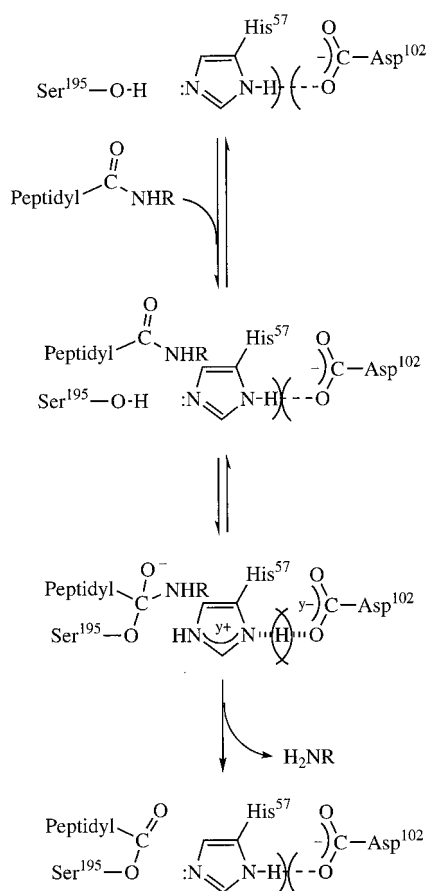
Studies of boronate complexes of chymotrypsin and other serine proteases are compatible with the compression and low barrier hydrogen-bonding mechanism. Borate, phenylboronate, and peptidylboronates form tetrahedral complexes with serine proteases through covalent bonding to Ser-195. Based on studies of the chymotrypsin-phenylboronate adduct, it was postulated that expulsion of the leaving group from the tetrahedral intermediate through general acid catalysis might be facilitated by low barrier hydrogen bonding between  $HN^{\epsilon 2}$  of His-57 and the departing amine (35, 36).

The importance of LBHB formation as a means of increasing the basicity of His-57 is underscored by the discovery of a class of serine proteases, such as the *Escherichia coli* leader peptidase, that use lysine in place of His-57 and Asp-102. The  $\epsilon$ -amino group of lysine is a much stronger base than the imidazole ring of histidine; however, the amino group is effective only at high pH. Thus, the *E. coli* leader peptidase is optimally active at pH 9–10 (37). LBHB formation in chymotrypsin allows optimal activity at pH 7, with high basicity on the part of His-57, despite the fact that histidine is normally a much weaker base ( $pK = 6$ ) than lysine ( $pK = 10.5$ ).

### Ketosteroid Isomerase

This enzyme interconverts non-conjugated and conjugated ketosteroids via a dienolate intermediate. Asp-38 removes a proton from C-4 of a  $\Delta^5$  ketosteroid to give the dienolate and puts the proton back on C-6 to give a  $\Delta^4$  product. The keto group is hydrogen-bonded to Tyr-14, and the Y14F and D38N isomerases show rate reductions of 4.7 and 5.6 orders of magnitude, respectively; the double mutant is  $\sim 10$  orders of magnitude slower than wild type enzyme (38). Because the  $pK$  of the dienol intermediate in solution is 10 (39), (close to that for tyrosine), it was proposed that a LBHB formed between the intermediate and Tyr-14 during the reaction and provided the energy for the enolization (1, 3).

A steroid aromatic in the A ring (and thus containing a phenolic hydroxyl in place of the ketone) bound at least 1000-fold tighter to the D38N variant than to wild type isomerase. In D38N the neutral Asn-38 mimics the protonated Asp-38 in the intermediate enzyme-



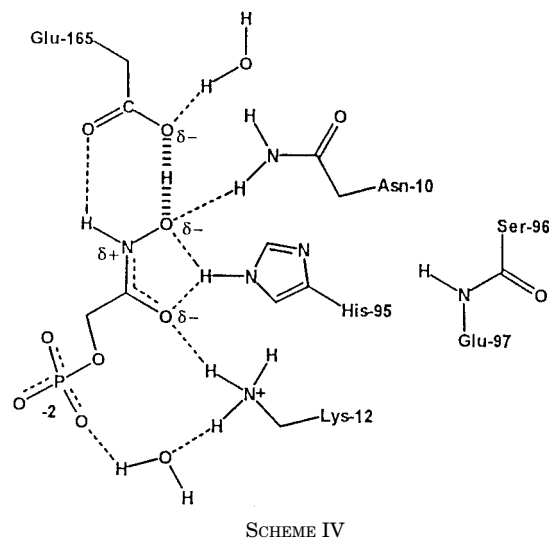
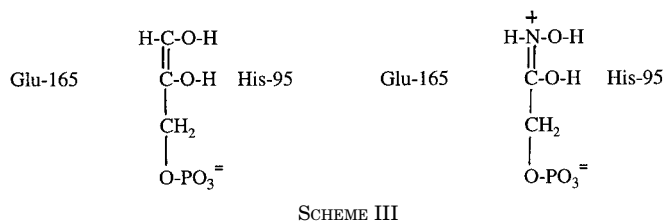
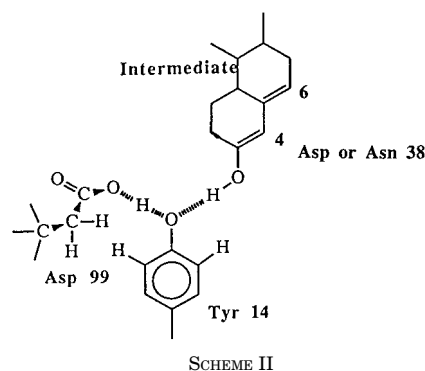
**FIG. 2. The role of the low barrier hydrogen bond in the acylation of chymotrypsin.** Upon binding a specific substrate, the active site undergoes a compression that brings His-57 and Asp-102 close together. The required energy is provided by the binding energy derived from specific enzyme-substrate contacts. Because the  $\Delta pK$  between neutral His-57 and Asp-102 is  $>10$  units, the hydrogen bond remains weak and cannot relieve the strain of compression. Protonation of N<sup>e2</sup> allows LBHB formation between His-57 and Asp-102 because the pK values are much more closely matched. The short LBHB relieves the strain of compression. This effect makes it easier to protonate N<sup>e2</sup>, resulting in a higher pK value and increased reactivity as a Brønsted base for deprotonating Ser-195 in the transition state.

dienolate complex (38). In this complex, proton NMR peaks were seen at 18.15 and 11.6 ppm. The proton at 18.15 displayed a deuterium fractionation factor of 0.34, and the strength of the hydrogen bond was estimated to be 7.1 kcal/mol greater than one between the inhibitor and water. This increase in the hydrogen bond strength corresponds to 5.2 orders of magnitude rate acceleration and closely matches the 4.7 orders of magnitude decrease in rate in the Y14F mutant.

Further work has shown, however, that the 18.15 ppm proton is actually between Tyr-14 and Asp-99, and it is the 11.6 ppm proton that is between Tyr-14 and the phenolic inhibitor (40). Thus the structure of the enzyme-inhibitor complex is as shown in Scheme II. In this structure the proton between Asp-99 and Tyr-14 is presumably equidistant from the oxygens of both groups, whereas the proton between Tyr-14 and the intermediate or intermediate analog is mainly bonded to the latter (its fractionation factor is 0.97 (40)). This means that this proton is transferred from Tyr-14 to the substrate during the enolization and thus that there presumably is a LBHB between these groups in the transition state.

### Triose-phosphate Isomerase

This enzyme isomerizes dihydroxyacetone phosphate to glyceraldehyde 3-phosphate via an enediol(ate) intermediate. It was proposed that when Glu-165 acted as a general base to remove a proton from the substrate, the resulting enediolate could form a LBHB with His-95, which hydrogen bonds to the carbonyl oxygen of the substrate (1, 3). The pK of His-95 would be 14 in solution (it is not protonated on the enzyme (41)), which is close to the expected



pK of an enediol. The close pK match and ideal geometry would strengthen the hydrogen bond (weak in the initial enzyme-substrate complex), and thus the liberated energy would be available to speed up the enolization.

Phosphoglycolohydroxamate is a tightly bound inhibitor that is thought to mimic the enediol(ate) intermediate (Scheme III). Recent NMR studies (38, 40, 42) have shown that the actual structure of the bound hydroxamate is as shown in Scheme IV, and a LBHB is postulated between Glu-165 and the hydroxamate oxygen (42). The proton in this bond has a chemical shift of 14.9 ppm (6 ppm downfield from the position of the analogous proton in aceto-hydroxamic acid in dimethyl sulfoxide) and a deuterium fractionation factor of 0.38. The N- $\epsilon$  proton of His-95 forms a hydrogen bond with the carbonyl oxygen of the inhibitor with a chemical shift of 13.5 ppm (0.4 ppm downfield from its position in free enzyme) and a fractionation factor of 0.71. It is thus a strong but not low barrier hydrogen bond. The NH proton of the inhibitor is hydrogen-bonded to Glu-165 with a chemical shift of 10.9 ppm.

Mildvan and co-workers (38, 40, 42) suggest that Glu-165 plays a role in moving protons to and from the oxygens of the intermediate, as well as in initially removing a proton from substrate, a mechanism similar to that postulated for the H95Q mutant by Knowles and co-workers (43). However, the pKs of the hydroxamate (9.5) and the enediol intermediate ( $\sim 14$ ) are quite different. The LBHB forms between Glu-165 and the hydroxamate oxygen of

the inhibitor as the result of a favorable  $pK$  match between these groups after His-95 polarizes the carbonyl oxygen and lowers the  $pK$  of the inhibitor, probably to 7 or below. In the enediol intermediate, however, the best  $pK$  match will be between His-95 and either oxygen of the enediol. The fractionation factor of the proton between the inhibitor and His-95 is 0.7, showing increased hydrogen bond strength over a weak hydrogen bond. With a further improvement in  $pK$  match of perhaps 7  $pK$  units, the LBHB may well form with His-95, as originally proposed (1, 3).

### Citrate Synthase

This enzyme enolizes acetyl-CoA and catalyzes attack of the enol(ate) on oxalacetate to give citryl-CoA, which is then hydrolyzed. Asp-375 removes the proton from acetyl-CoA, whereas His-274 (presumed to be neutral) is hydrogen-bonded to the carbonyl oxygen (44). In the enolized intermediate the close  $pK$  match between the enol and His-274 should permit a LBHB to form, thus producing the energy for enolization (1, 3). An x-ray study of citrate synthase with bound carboxyl or amide analogs of acetyl-CoA showed a very short (2.4–2.5 Å) hydrogen bond between the carboxyl or amide group (which corresponds to the methyl carbon of acetyl-CoA) and Asp-375 (45). Although the dissociation constant of the amide was pH-independent, that of the carboxyl inhibitor decreased as the pH was lowered, showing that the carboxyl must be protonated, with the proton presumably between Asp-375 and the carboxyl of the inhibitor. Because the difference in dissociation constants between the carboxyl and amide inhibitors is only a factor of 20 at pH 6, the authors concluded that the short hydrogen bonds did not differ in strength by more than this factor, despite the apparent differences in degree of  $pK$  mismatch in the two cases. However, the carboxyl inhibitor binds 4 orders of magnitude tighter than acetyl-CoA, and thus the LBHB (now shown to have a chemical shift of 20 ppm (46)) is contributing at least this much to binding.

As in the triose-phosphate isomerase case, the LBHB formed with a tightly bound inhibitor is not to the histidine, where it presumably will be in the normal catalytic mechanism but to the carboxylate base. In each case this results from the  $pK$ s of the inhibitor being lower than that of the putative intermediate and the better  $pK$  match to the carboxyl group.

### Mandelate Racemase

Mandelate racemase catalyzes the interconversion of the enantiomers of mandelate via an enol(ate) intermediate. Lys-166 and His-297 are the (*S*)- and (*R*)-specific bases, respectively, that abstract the  $\alpha$ -proton to generate the intermediate. This reaction and understanding the physical basis for stabilization of the intermediate provided the basis for the early statements of the proposal that LBHBs are important in enzymatic catalysis (1, 2); the  $pK$  of the  $\alpha$ -proton of the enantiomers of mandelate is  $\geq 29$ , whereas the  $pK$ s of the conjugate acids of the bases that receive the proton are  $\sim 6$ , yet  $k_{\text{cat}}$  is  $\sim 500 \text{ s}^{-1}$ . Thus, kinetic competence of the intermediate requires that it be stabilized. The x-ray structure of a complex with (*S*)-atrolactate ((*S*)- $\alpha$ -methylmandelate) reveals that the carboxylate group of the bound inhibitor is both coordinated to an essential  $\text{Mg}^{2+}$  and hydrogen-bonded to Glu-317 (as evidenced by the 2.7-Å O–O distance) (47).

As the substrate is converted to the enolate anion, the charge densities on the carboxylate oxygens increase (from  $-0.5$  to  $-1$  per oxygen atom). Although this increase undoubtedly will increase the strength of the electrostatic interaction with the  $\text{Mg}^{2+}$ , the hydrogen bond strength to Glu-317 is also expected to increase; the  $pK$  of the conjugate acid of a carboxylic acid group (or charge-neutralized carboxylate anion) is approximately  $-8$ , whereas the  $pK$  of the mandelic acid enol is  $\sim 7$ . Thus, as the enol(ate) intermediate is formed, the  $pK$  of the hydrogen-bonded carboxylate oxygen will increase toward that of Glu-317. The  $k_{\text{cat}}$  of the E317Q racemase is decreased by a factor of  $\sim 10^5$ , suggesting that the increased strength of the hydrogen bond between the enolate anion and Glu-317 relative to that involving the bound substrate contributes at least 7 kcal/mol to the stabilization of the intermediate (48). Because the kinetic evidence suggests that a transiently stable intermediate is also formed in the reaction catalyzed by E317Q and a hydrogen bond is observed between Gln-317 and (*S*)-atrolactate,

these observations support the proposal that significant increases in hydrogen bond strength are possible when the  $pK$ s of the active site donors and substrate/intermediate acceptors become more closely matched.

In conclusion, LBHBs appear to play a role in a number of enzymatic reactions, including the serine proteases and certain enzymes that enolize their substrates. In contrast, enzymes such as enolase use metal ions as electrostatic catalysts (49). Any enzymatic reaction in which proton transfer from a general acid or to a general base occurs likely may involve a LBHB. This does not include all enzymes, and other catalytic factors are clearly involved. We believe that LBHBs can provide at least 5 orders of magnitude in rate acceleration, with other factors providing the rest of the catalysis.

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