The Enhancement of Enzymatic Rate Accelerations by Brønsted Acid−Base Catalysis[†]

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Water is an ideal solvent for many biological reactions because its high dielectric constant favors heterolytic bond cleavage and the formation of charged reactive intermediates, while the high acidity of its conjugate acid (hydronium ion) and basicity of its conjugate base (hydroxide ion) favor effective Brønsted catalysis. The functional replacement of hydronium and hydroxide ions by acidic and basic amino acid side chains at protein catalysts is essential for the observation of enzymatic rates that are comparable to those for nonenzymatic reactions in water. However, there is no simple requirement that the presence of such side chains result in large accelerations over specific-acid or -base-catalyzed reactions in water, because the rate accelerations from bimolecular Brønsted buffer catalysis in water are often small and sometimes undetectable (*1*).

The site-specific mutation of active-site residues that are implicated in Brønsted acid−base catalysis of enzymatic reactions often lead to large reductions in enzyme activity. Such results are difficult to interpret, because of uncertainties about whether the reduced activity is due to the loss of specific stabilizing Brønsted acid−base interactions at the transition state or to other changes in the local environment at the enzyme active site. Consequently, it is not generally known whether the stabilization of the transition state for reaction of an enzyme-bound substrate provided by Brønsted acid−base catalysis is relatively small, as is observed for bimolecular buffer catalysis in water, or whether such catalysis is enhanced at the enzyme active site.

Kirby and co-workers have observed enhanced Brønsted acid catalysis of reactions in which the products are stabilized relative to reactants by the formation of a strong intramolecular hydrogen bond that is partly developed in, and thus stabilizes, the transition state (*2*). Interactions at an enzyme-bound reactant and/or product which result in an increase in the overall thermodynamic driving force for proton transfer might also enhance Brønsted acid−base catalysis of enzymatic reactions. This article summarizes some of the experimental evidence for such enhanced Brønsted acid−base catalysis.
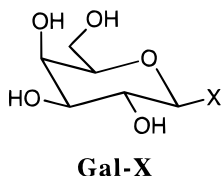
Gal-X

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Scheme 1
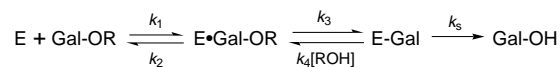
β-Galactosidase-Catalyzed Cleavage of Glycosides. β-Galactosidase catalyzes the hydrolysis of lactose and other β-D-galactopyranosyl derivatives (Gal-X) by a two-step mechanism through a covalent intermediate whose formation is rate determining for the reactions of alkyl β-D-galactopyranosides and 4-nitrophenyl β-D-galactopyranoside (Scheme 1) (*3*). This enzyme has a low specificity for the leaving group at the glycoside, so that structure−reactivity correlations for the cleavage of structurally homologous substrates are readily obtainable, and are interpretable in terms of changes in the "effective charge" at the leaving group on moving to the transition state (*4*).

The E461Q (E = Glu, Q = Gln) mutation at β-galactosidase abolishes the activity of this enzyme for cleavage of enzyme-bound 2,2,2-trifluoroethyl β-D-galactopyranoside (Gal-OTFE) (>500000-fold effect), a substrate with a relatively basic trifluoroethoxide leaving group ($pK_a = 12.4$), but results in only a ca. 10-fold reduction in the rate constant k_3 (Scheme 1) for cleavage of enzyme-bound 4-nitrophenyl β-D-galactopyranoside (Gal-OC$_6$H$_4$-4-NO$_2$), a substrate with a weakly basic 4-nitrophenoxide leaving group ($pK_a = 7.1$) (*5*). These results provide evidence that there is only a small difference between the transition-state interactions of the weakly basic 4-nitrophenoxide leaving group with a propionic acid and a propionamide side chain at position 461 of β-galactosidase, but a much larger difference in the transition-state interactions of the more basic trifluoroethoxide leaving group with these side chains. The 10-fold effect of the E461Q mutation on k_3 for cleavage of Gal-OC$_6$H$_4$-4-NO$_2$ sets an upper limit on the contribution of effects unrelated to Brønsted acid−base catalysis (e.g., changes in protein structure) to the observed effect of the E461Q mutation on the activity of β-galactosidase for cleavage of other substrates.

We have proposed that the more than 50000-fold larger effect of the E461Q mutation on the activity of β-galactosidase for cleavage of Gal-OTFE than of Gal-OC$_6$H$_4$-4-NO$_2$ corresponds to a greater than 6.4 kcal/mol stabilizing interaction between the acidic side chain of Glu-461 and the basic leaving group at Gal-OTFE that is lost upon replacement of the carboxylic acid side chain by an amide. This interaction presumably represents stabilization of the transition state for cleavage of Gal-OTFE by proton transfer from the acidic side chain of Glu-461 to the strongly basic trifluoroethoxide leaving group (*5*).
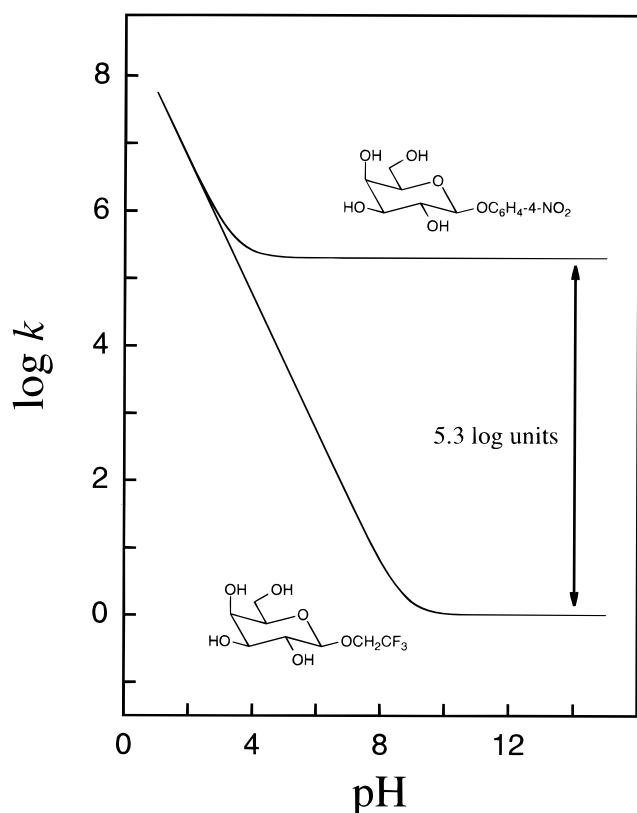
FIGURE 1: The pH dependence of the *relative* rate constants for the nonenzymatic spontaneous/acid-catalyzed hydrolyses of Gal-OC_6H_4-4-NO_2 and Gal-OTFE. These hypothetical pH-rate profiles were constructed from experimental data for the spontaneous/acid-catalyzed hydrolysis of 4-nitrophenyl β-D-glucopyranoside, as described in the text.

Figure 1 shows hypothetical pH-rate profiles for the nonenzymatic spontaneous/acid-catalyzed hydrolyses of Gal-OTFE and Gal-OC_6H_4-4-NO_2, which were constructed from experimental data for the spontaneous/acid-catalyzed hydrolysis of 4-nitrophenyl β-D-glucopyranoside (*6*) and (a) representative values of $\beta_{lg} = -1$ and $\beta_{lg} = 0$ (*7*), respectively, for the spontaneous (pH independent) and acid-catalyzed cleavage reactions of Gal-X; (b) pK_as of 7.1 for the aryl alcohol and 12.4 for the alkyl alcohol leaving groups; (c) an arbitrary unitary rate constant for the spontaneous cleavage of Gal-OTFE. These pH-rate profiles provide useful insight into the effect of E461 mutations on the cleavage of glycosides by β-galactosidase. Gal-OC_6H_4-4-NO_2 and Gal-OTFE exhibit similar chemical reactivities when they are bound to wild-type β-galactosidase at pH 8.6 ($k_3 = 140$ and 970 s^{-1}, respectively) (*7*) and when they are in water at pH <4 (Figure 1). This shows that the negative charge at the two leaving groups in the respective transition states is effectively neutralized by proton transfer, either from β-galactosidase or from weakly acidic aqueous solution. However, the E461Q mutation at β-galactosidase results in a large difference in the reactivities of enzyme-bound Gal-OC_6H_4-4-NO_2 ($k_3 = 10$ s^{-1}) and Gal-OTFE ($k_3 < 0.002$ s^{-1}) (*5*). The difference in the reactivities of these substrates when bound to the E461Q mutant enzyme is similar to the difference in their reactivities in basic aqueous solution (Figure 1). *In other words, the effect of an E461Q mutation on the relative reactivity of Gal-OC$_6$H$_4$-4-NO$_2$ and Gal-OTFE (>50000-fold increase) (5) toward cleavage catalyzed*

by β-galactosidase is similar to the change in the relative reactivity of these substrates on moving from weakly acidic to neutral aqueous solution.

The reactivities of Gal-OC_6H_4-4-NO_2 and Gal-OTFE tend to become equal in water as the pH approaches 4. This is substantially lower than pH 8.6 at which nearly equal reactivities are observed for cleavage of these substrates when bound to β-galactosidase. This suggests that the pH at the active site of β-galactosidase during turnover at pH 8.6 is effectively less than 4. A change in "effective pH" at an enzyme active site is different from a change in pH in solution resulting from a change in hydrogen ion concentration (Figure 1). Changes in "effective pH" may be achieved through changes in the pK_a of catalytic residues at the enzyme active site, because pH can be defined by the pK_a of a Brønsted acid and the fraction of the acid which exists in the protonated form. I suggest that the low effective, or apparent, pH at the active site of wild-type β-galactosidase during turnover is the result of a downward perturbation in the pK_a of Glu-461 to a value below the pH of the solution and the absence of any equilibration of this now acidic proton at the enzyme with bulk solvent.

Perturbation of the pK_a of Glu-461: Evidence and Role in Catalysis. A large decrease in the pK_a of Glu-461 on proceeding from free enzyme to the covalent intermediate (E-Gal, Scheme 1) will result in a thermodynamically favorable loss of a proton from this residue at the intermediate complex, which will therefore increase the overall driving force for formation of the covalent intermediate. Simple Haldane relationships have been used to calculate the equilibrium constants and Gibbs free energy changes for transfer of the β-D-galactopyranosyl group from Gal-X to β-galactosidase (*8, 9*). These data show that transfer of the β-D-galactopyranosyl group to the enzyme is strongly favorable, and the following are consistent with the conclusion that galactosyl transfer is accompanied by a thermodynamically favorable deprotonation of the acidic side chain of Glu 461:

(1) The covalent acylal intermediate of the reaction catalyzed by β-galactosidase would be expected to show a high chemical potential relative to the substrate Gal-OTFE because (a) p$K_a \approx 4$ for the carboxylic acid side chain of Glu-537 is substantially lower than p$K_a = 12.4$ for the trifluoroethanol leaving group at Gal-OTFE, and it is known that there is a large decrease in the thermodynamic stability of alkyl β-D-galactopyranosides with decreasing basicity of the leaving group (*8*); (b) the "enzyme-bound" acylal is expected to be even less stable thermodynamically than the corresponding "free" acylal in water, because it is 10^8-fold more reactive toward hydrolysis (*8*). However, formation of the covalent acylal intermediate from the reaction of Gal-OTFE with wild-type β-galactosidase is in fact favorable by 3 kcal/mol (Figure 2A) (*8*), which suggests that galactosyl transfer is "driven" by a thermodynamically favorable proton transfer from the now strongly acidic side chain of Glu-461 (Figure 2A).

(2) Studies of the β-galactosidase-catalyzed cleavage of Gal-N_3 at pH 8.6 show that the covalent intermediate for the wild-type enzyme lies in a free-energy well, of uncertain depth relative to the free enzyme plus Gal-N_3 (Figure 2B) (*9*). By contrast, the covalent intermediate for the reaction catalyzed by the E461G (G = Gly) mutant enzyme lies 2
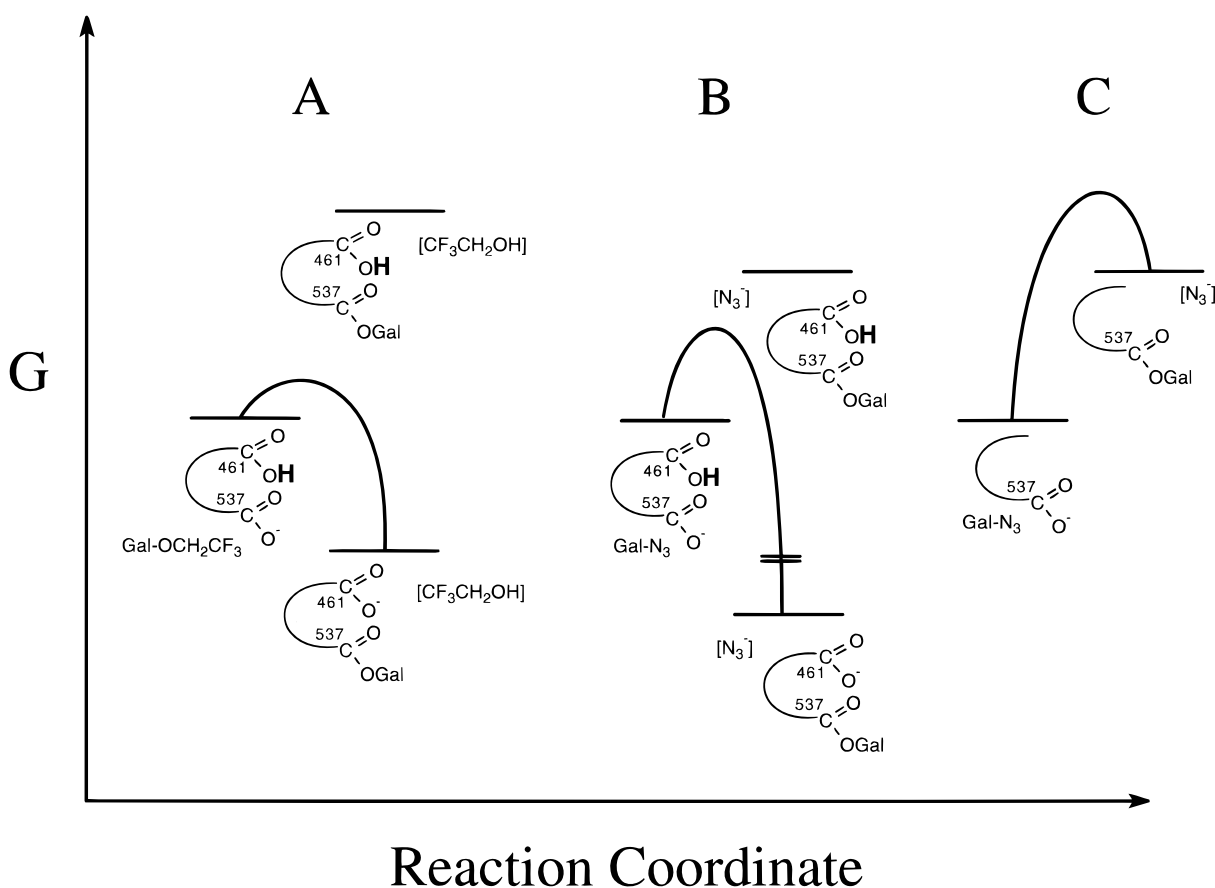
Reaction Coordinate

FIGURE 2: Free energy profiles for the reactions of β-D-galactopyranosyl derivatives with wild-type and E461G mutant β-galactosidases at pH 8.6 to give the covalent galactosyl-enzyme intermediates. The square brackets around the leaving groups indicate that they are formed as pH-dependent mixtures of their anionic and neutral forms. (A) Reaction of wild-type enzyme with Gal-OTFE. The difference in the Gibbs free energies of the reactants and products was calculated from $K_{eq} = 160$, which is the ratio of the second-order rate constants for reaction in the forward and reverse directions (*8*). The difference in the Gibbs free energies of the covalent intermediates containing the protonated and ionized forms of Glu-461 is not known, but it is assumed to be the same as that estimated for Figure 2B. (B) Reaction of wild-type enzyme with Gal-N$_3$. The difference in the Gibbs free energies of the reactants and products was calculated from $K_{eq} \geq 230$, which is the ratio of the second-order rate constants for reaction in the forward and reverse directions (*9*). The rate constant for reaction of the covalent intermediate with N$_3^-$ is an upper limit (*9*). The Figure was drawn for a hypothetical case in which the covalent intermediate containing the protonated form of Glu-461 lies at the same Gibbs free energy relative to reactants as does the covalent intermediate for the E461G mutant enzyme (Figure 2C), so that the entire difference in the Gibbs free energy changes for the reaction of Gal-N$_3$ with wild-type and E461G β-galactosidases results from a favorable proton transfer from Glu-461 at the galactosylated wild-type enzyme. (C) Reaction of E461G mutant enzyme with Gal-N$_3$. The difference in the Gibbs free energies of the reactants and products was calculated from $K_{eq} = 0.029$, which is the ratio of the second-order rate constants for reaction in the forward and reverse directions (*9*).

kcal/mol higher in energy than the free enzyme plus Gal-N$_3$ (Figure 2C), and the experimental data show that the E461G mutation results in a greater than 5.3 kcal/mol destabilization of the covalent galactosyl−enzyme intermediate relative to reactants (*9*). These data provide evidence that the galactosylated wild-type enzyme is strongly stabilized relative to the free enzyme plus Gal-N$_3$ because formation of the covalent galactosyl−enzyme intermediate is accompanied by thermodynamically favorable deprotonation of the propionic acid side chain of Glu-461. This requires that the pK_a of Glu-461 at the free enzyme be high (pK_a > pH), so that this residue exists in the protonated form which is catalytically active for substrate cleavage, but that transfer of a β-D-galactopyranosyl group to the enzyme results in a large decrease in the pK_a of Glu-461 (pK_a < pH), so that this residue now exists in the ionized form which is catalytically active for transfer of the β-galactopyranosyl group from the enzyme to water (k_s, Scheme 1).

In summary, I propose that transfer of the β-D-galactopyranosyl group from Gal-X to β-galactosidase is favored thermodynamically (Figure 2) as a result of interactions

between the enzyme and the catalytic side chain of Glu-461 that develop at the covalent intermediate and which result in stabilization of the carboxylate form of Glu-461 relative to the carboxylic acid form. This stabilization would be partly, or entirely, the direct consequence of the chemical mechanism for glycosyl transfer. A relatively high pK_a is expected for Glu-461 at the free enzyme because deprotonation of this residue results in unfavorable electrostatic interactions of the resulting anion with the carboxylate ion side chain of Glu-537, the neighboring active-site nucleophile (*10, 11*). Deprotonation of Glu-461 will thus be more favorable at the covalent intermediate, in which the negative charge at Glu-537 has been neutralized by the transfer of a β-D-galactopyranosyl group. Direct titration of the carboxylic acid residues of the 20 kDa xylanase from *Bacillus circulans* has shown that the pK_a of Glu-172, the putative general acid−base catalyst, depends on the state of protonation and covalent modification of Glu-78, the acceptor of the glucosyl group from substrate (*12*).

A change in the pK_a of a Brønsted acid or base that is "shielded" from reaction with solvent will result in an

Scheme 2
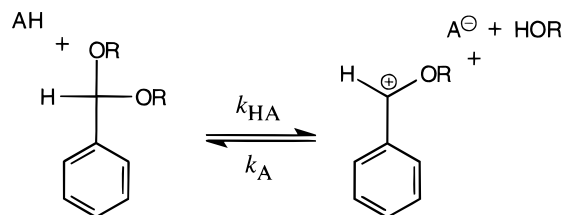
Scheme 3
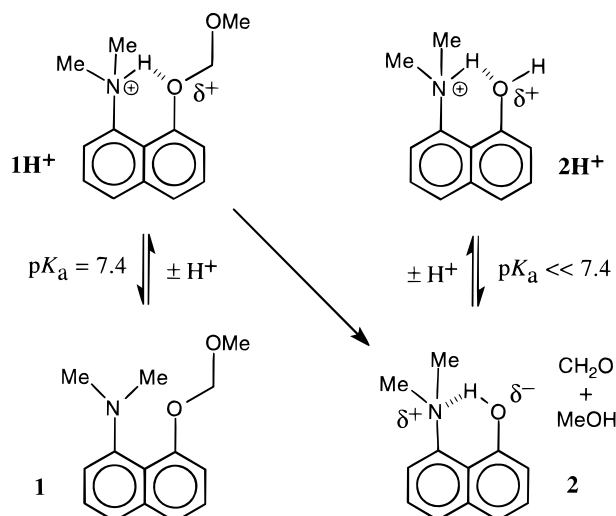
increase in the catalytic reactivity of this residue, with the net rate acceleration depending upon ΔpK_a for the catalytic residue and the value of the Brønsted coefficient α or β for catalysis. Values of α for the general-acid-catalyzed cleavage of benzaldehyde dialkyl acetals (Scheme 2) generally lie between 0.9 and 1.0 (*13*). Therefore, it is expected that at least 90% (>4.8 kcal/mol) of the greater than 5.3 kcal/mol increase in the thermodynamic driving force for proton transfer from Glu-461 that occurs upon transfer of a β-D-galactopyranosyl group to the enzyme (*9*) will be expressed in the transition state for cleavage of Gal-OTFE (Figure 2A). This would account for most, and possibly all, of the observed >6.4 kcal/mol stabilization of the transition state for enzyme-catalyzed cleavage of Gal-OTFE resulting from general acid catalysis by Glu-461 (*5*).

Model Reactions. Bimolecular Brønsted acid catalysis of the cleavage of benzaldehyde dialkyl acetals (Scheme 2) results in only small rate accelerations over the specific-acid-catalyzed reaction (*13*). Much larger rate accelerations are observed when the catalysis of acetal cleavage is accompanied by the formation of a strong intramolecular hydrogen bond in the product, because this stabilizing interaction also develops in the transition state (*2*). The high reactivity of N-protonated 8-(dimethylamino)-1-methoxymethoxynaphthalene (1H$^+$) toward hydrolysis at neutral pH (Scheme 3) is a particularly dramatic example of such intramolecular catalysis (*14*). There are at least two similarities between the mechanisms that are proposed to explain the large transition-state stabilization which results from intramolecular general acid catalysis of acetal cleavage at 1H$^+$ (*2*) and from the interactions between Gal-OTFE and the carboxylic acid side chain of Glu-461 at β-galactosidase:

(1) For both Gal-OTFE and 1H$^+$, cleavage of the acetal linkage is proposed to result in stabilization of the product

Scheme 4
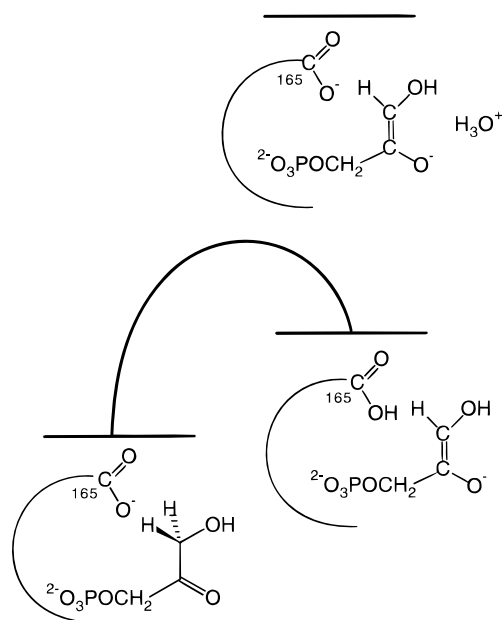
of the reaction, which is partly expressed as stabilization of the reaction transition state. In the case of β-galactosidase, this stabilization is due, at least in part, to a reduction in unfavorable electrostatic interactions resulting from the neutralization of negative charge at Glu-537 by galactosyl transfer; while the product of the reaction of 1H$^+$, 2, is stabilized directly by the formation of a strong intramolecular hydrogen bond (Scheme 3) (*14*).

(2) The tertiary ammonium cation will undergo a large increase in acidity on conversion of 1H$^+$ [$pK_a = 7.4$ (*14*)] to 2H$^+$, due to the stabilization of 2 by a strong intramolecular hydrogen bond (Scheme 3) (*14*). This hydrogen bond is largely responsible for the 5.6 unit higher pK_a of 2 ($pK_a = 14.9$) than of 1-naphthol ($pK_a = 9.3$) (*15*). Evidence for a decrease in the pK_a of the carboxylic acid side chain of Glu-461 upon transfer of a β-D-galactosyl group to β-galactosidase is given above.

Other Reactions. There is evidence that Brønsted acid−base catalysis is enhanced for other enzymatic reactions by interactions at an enzyme-bound reactant and/or product which result in a net increase in the overall thermodynamic driving force for proton transfer.

It is generally recognized that the large rate accelerations observed for enzymatic catalysis of deprotonation at α-carbonyl carbons are primarily the result of specific stabilization of the enzyme-bound enolate intermediate relative to the keto reactant. However, there is no general agreement about the origin of this stabilization (*16, 17*).

Scheme 4 illustrates how specific stabilization of the enediolate phosphate intermediate of the reaction catalyzed by triosephosphate isomerase could result from a large increase in the pK_a of Glu-165 (the base which abstracts a proton from substrate) on proceeding from the enzyme−substrate to the enzyme−intermediate complex. In this example, there is a change in the pK_a of Glu-165 from pK_a < pH at the enzyme−substrate complex, to pK_a > pH at the enzyme−intermediate complex, so that formation of the enediolate phosphate intermediate is accompanied by a thermodynamically favorable protonation of the carboxylate

side chain of Glu-165. It is proposed that the intrinsic binding energy (*18*) of the phosphate group and other portions of the carbon acid substrate is utilized to generate an enediolate intermediate in which there is a strong destabilizing electrostatic interaction between the anionic intermediate and the carboxylate side chain of Glu-165. The result of utilization of intrinsic binding energy in this way would be to increase the pK_a of the carboxylate side chain of Glu-165, and hence the rate of deprotonation of the enzyme-bound substrate by this side chain.

The enediolate phosphate intermediate analogue phospho-glycolate has been shown to bind specifically to a form of triosephosphate isomerase from rabbit muscle which contains one more proton than the free enzyme, and $pK_a = 6.7$ has been determined for the residue which undergoes protonation upon binding of this inhibitor (*19*). This is higher than pK_a = 3.9 estimated for Glu-165 from analysis of the pH-rate profile for inactivation of the structurally similar enzyme from yeast by 3-chloroacetol sulfate (*20*). These data are consistent with the conclusion that binding of phosphogly-colate induces a 2.8 unit increase in the pK_a of Glu-165 at triosephosphate isomerase, and a substantially larger upward perturbation of the pK_a of this residue upon binding of the true enediol(ate) phosphate intermediate is conceivable. Similarly, the X-ray crystal structure of a complex between triosephosphate isomerase and the enediolate phosphate intermediate analogue phosphoglycolohydroxamic acid shows that Glu-165 is protonated in the inhibitor complex (*21*), and NMR studies at pH 7.5 are also consistent with the conclusion that this residue is protonated ($pK_a >$ pH = 7.5) and forms a low-barrier hydrogen bond to O1 of the bound inhibitor (*22*).

It has been proposed that the pK_a of His-57 increases during the course of conversion of substrates bound to chymotrypsin to their tetrahedral adducts with Ser-195 (*23, 24*). This proposal is supported by the observation that the pK_a of 12 for the His-57-Asp-102 diad in the tetrahedral complex of chymotrypsin with the peptidyl trifluoromethyl ketone *N*-acetyl-L-Leu-DL-Phe-CF$_3$ is 5.2 units higher than the pK_a of 6.8 for the imidazolium ion of free glycylhistidine in water (*25*). The high basicity of the imidazole side chain of His-57 in the covalent intermediate is postulated to result from (1) destabilizing steric interactions between the pro-tonated form of His-57 and Asp-102 in the Michaelis complex; (2) the relief of the unfavorable interactions between these residues at the tetrahedral adduct, as a result of formation of a low-barrier hydrogen bond between His-57 and Asp-102 which accompanies proton transfer from Ser-195 to His-57 (*25*). This proposed increase in the pK_a of His-57 would have the effect of promoting general base catalysis of addition of the hydroxyl group of Ser-195 to the enzyme-bound peptide substrate.

Concluding Remark. There are still relatively few data to support the notion presented here that enzymatic Brønsted acid−base catalysis must be enhanced compared to its counterpart in water if it is to make a significant contribution to the observed rate accelerations for enzymatic reactions. One purpose of this article is to prompt enzymologists to carefully examine X-ray crystallographic and other structural data for protein catalysts, to identify changes in the local environment of essential acidic or basic side chains which might result in a perturbation of the pK_a of the functional group during the catalytic cycle that would favor effective Brønsted catalysis. Such changing interactions would prob-ably be restricted to those normally implicated in enzymatic catalysis and would include the formation or cleavage of a strong hydrogen bond to the catalytic side chain, as well as changes in the electrostatic or steric interactions of the acidic or basic side chain with the enzyme-bound substrate or other groups at the enzyme active site.

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