An Explanation for Rapid Enzyme-Catalyzed Proton Abstraction from Carbon Acids: Importance of Late Transition States in Concerted Mechanisms

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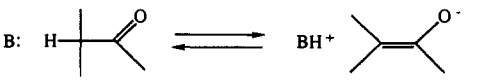
Abstract: The rates of enzyme-catalyzed abstraction of protons from carbons adjacent to carbonyl or carboxylic acid groups (α-protons of carbon acids) are rapid (k_{cat}s $\sim 10^1$–10^4 s^{-1}). We recently proposed that these rates can be understood if proton abstraction by an active site general basic catalyst is concerted with protonation of the carbonyl group by an active site general acidic (electrophilic) catalyst to generate an enol intermediate instead of an enolate anion (aldehyde, ketone, or thioester substrate) or dianion (carboxylic acid substrate): Gerlt, J. A.; Kozarich, J. W.; Kenyon, G. L.; Gassman, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9667. Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928. We now analyze concerted general acid–general base catalyzed enolization reactions in terms of Marcus formalism that partitions the activation energy barrier for the reaction, $\Delta G^{\ddagger}$, into (1) a thermodynamic component, ΔG°, associated with both the conversion of the keto tautomer of the carbon acid into its enol tautomer and the transfer of a proton from the general acidic catalyst to the general basic catalyst, and (2) an intrinsic kinetic component, $\Delta G^{\ddagger}_{int}$, the activation energy for the reaction in the absence of a thermodynamic barrier. We propose that in enzyme active sites both ΔG° and $\Delta G^{\ddagger}_{int}$ are reduced from the values that describe nonenzymatic reactions. The transition states for the enzymatic reactions are "late", i.e., the transition states resemble the enol tautomers of the substrate carbon acids, as suggested by the observation that the pK_as of the OH groups of the enols are similar to the pK_as of the uncharged active site general acidic catalysts. The late transition states in enzyme-catalyzed reactions can be explained best by reductions in $\Delta G^{\ddagger}_{int}$ from the values that describe nonenzymatic reactions. We propose that the reduction in $\Delta G^{\ddagger}_{int}$ is achieved by prepositioning the electrophilic catalyst adjacent to the carbonyl group of the substrate carbon acid, thereby negating development of a negative charge on the carbonyl oxygen as the α-proton is abstracted. We propose that the reduction in ΔG° is accomplished by stabilization of an enolic intermediate *via* the formation of a negatively charged, short, strong hydrogen bond between the anionic conjugate base of the active site general acidic catalyst and the OH group of the enol tautomer of the substrate. Such hydrogen bonds can be formed in the bulk solvent-excluded environments of enzyme active sites since the pK_as of the OH groups of the enol tautomers of the substrate carbon acids are similar to the pK_as of the active site general acidic catalysts. Taken together, the possible reductions in $\Delta G^{\ddagger}_{int}$ and ΔG° are quantitatively sufficient to explain the rapid rates of the enzyme-catalyzed reactions. We propose a "late transition state rule" that describes the requirements for concerted general acid–general base catalysis of enolization of carbon acids *in enzyme active sites*. This rule differs from the "libido rule" proposed by Jencks (Jencks, W. P. *J. Am. Chem. Soc.* **1972**, *94*, 4731) to describe the requirements for concerted general acid–general base catalysis of enolization of carbon acids *in nonenzymatic reactions*. The application of our rule to enzyme-catalyzed reactions involving successive enolization and reketonization reactions of carbon acids reveals that, while the ΔG° between bound substrate carbon acid and bound product carbon acid should approach 0, the ΔG° for formation of the bound enolic intermediate from either bound substrate carbon acid or bound product carbon acid must be endergonic. This description of the energetics of enzyme-catalyzed reactions differs in significant detail from that proposed by Albery and Knowles (Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631).

Overview

The mechanisms of a large number of enzyme-catalyzed reactions involve rapid heterolytic cleavage of C–H bonds. In almost all cases, these reactions involve abstraction of a proton from a carbon atom adjacent to a carbonyl,[1] carboxylic acid, or carboxylate anion group (α-proton of a carbon acid) by an active site general basic catalyst. The observed k_{cat}s for such enzymatic reactions are typically 10^1–10^4 s^{-1},[2,3] thereby requiring that the proton abstraction reactions are at least as rapid.[4] Accordingly, from transition-state theory, the activation energies ($\Delta G^{\ddagger}$s) for enzyme-catalyzed proton abstraction reactions range from 13 to 17 kcal/mol.

The α-protons of carbon acids are acidic relative to the protons in hydrocarbons by virtue of the resonance stabilization available to the enolate anion that results from proton abstraction by a general basic catalyst, B.



For example, the pK_as of α-protons of aldehydes and ketones are typically 18–20.[7] However, bioorganic chemists and mechanistic enzymologists have long realized that this acidifying effect of the carbonyl, carboxylic acid, or carboxylate anion group is not sufficient to explain the rapid rates of the enzyme-catalyzed reactions relative to those of nonenzymatic base-catalyzed reactions because the active site general basic catalyst is not sufficiently basic to provide a large enough concentration of an enolate anion intermediate to achieve the observed rates.[8] Despite this incomplete understanding of enzyme-catalyzed proton ab-

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(1) "Carbonyl group" refers to an aldehyde, ketone, or thioester functional group.

(2) Gerlt, J. A.; Kozarich, J. W.; Kenyon, G. L.; Gassman, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9667.

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straction reactions, mechanisms involving the formation of enolate anion intermediates have been widely published both in the chemical and biochemical literature and in modern textbooks of biochemistry.[9]

To solve this problem, we have proposed that the mechanisms of the enzyme-catalyzed reactions involve abstraction of the α-proton of a carbon acid by an active site general basic catalyst *concerted* with protonation of the carbonyl or carboxylic acid group by an active site general acidic (electrophilic) catalyst to generate an enol instead of an enolate anion as an intermediate.[2,10]

This concerted reaction can be viewed as an iterative process in which a small amount of proton transfer from the general acidic catalyst to the carbonyl group decreases the pK_a of the α-proton. As the α-proton is abstracted by the general basic catalyst, the pK_a of the conjugate acid of the carbonyl group increases, allowing additional proton transfer of the proton from the acidic catalyst to the carbonyl group. As this process is repeated, transfer of the α-proton to the general basic catalyst and transfer of the proton of the general acidic catalyst to the carbonyl group is achieved.

Depending upon the relative pK_as of the general acidic catalyst, HA^+, and the conjugate acid of the general basic catalyst, BH^+, this mechanism should be able to increase the concentration of an enolic intermediate significantly relative to the concentration of an enolate anion intermediate that can be obtained by general base catalysis alone. However, this mechanism would not initially appear to circumvent the additional problem that nonenzymatic reactions involving proton transfer to and from carbon are almost always slow.[11,12] In this article, we demonstrate that a concerted reaction with a transition state that resembles a *stabilized* enolic intermediate[16] can both solve the problem of slow proton-transfer rates and provide a *quantitative* insight into the rapid rates of enzyme-catalyzed enolization of carbon acids.

Our analysis and the resulting insight into the perceived problem of inherently slow proton abstraction from carbon is based on a Marcus *formalism* for describing the dependence of reaction rates on equilibrium constants.[13] Marcus formalism quantitatively partitions the activation energy barrier for a reaction, $\Delta G^{\ddagger}$, into (1) the thermodynamic barrier, $\Delta G°$, and (2) the intrinsic kinetic barrier, $\Delta G^{\ddagger}_{int}$, the hypothetical activation energy barrier in the absence of a thermodynamic barrier (i.e., when $\Delta G° = 0$). We propose that the rates of the enzyme-catalyzed enolization reactions of carbon acids can be explained only if both $\Delta G°$ and $\Delta G^{\ddagger}_{int}$ are reduced from the values that characterize nonenzymatic enolization of carbon acids.

The pK_as of the uncharged active site general acidic (electrophilic) catalysts are approximately equal to those of the OH groups of the enol tautomers of the substrate carbon acids in several enzyme-catalyzed reactions for which detailed structural and mechanistic data are available.[17] On the basis of the matching of these pK_as, we propose that the transition states for enzyme-catalyzed enolization reactions resemble the enolic intermediates formed in our concerted mechanism. By applying Marcus formalism, we conclude that this proposal is correct and that the transition states are "late" on the reaction coordinate of the concerted reaction. The "late" transition states for the enzyme-catalyzed reactions contrast with the "intermediate" transition states for nonenzymatic enolization reactions. This difference in the structure of the transition states for enzymatic and nonenzymatic reactions is critical for rationalizing the rapid rates of the enzyme-catalyzed reactions and can be best explained by a smaller value for $\Delta G^{\ddagger}_{int}$ for the enzyme-catalyzed reactions.

We propose that there are two concepts that have important implications for the reduction in $\Delta G^{\ddagger}$ and a resulting understanding of the rates of the enzyme-catalyzed reactions relative to nonenzymatic reactions:

(1) A reduction in $\Delta G°$ will decrease $\Delta G^{\ddagger}$ for the reaction, thereby accelerating the rate of the concerted tautomerization of the keto tautomer of the substrate carbon acid to the enol tautomer. We propose that the *general method* for reducing $\Delta G°$ is differential hydrogen bonding of the conjugate base of the active site general acidic catalyst to the OH group of the enol intermediate relative to the hydrogen bonding of the active site general acidic catalyst to the carbonyl group of the keto tautomer of the substrate. *When removed from aqueous solvent*, short, strong hydrogen bonds are formed when a proton is shared by

(3) (a)The k_{cat} for triose phosphate isomerase is 8300 s^{-1} using glyceraldehyde 3-phosphate as the substrate and 600 s^{-1} using dihydroxacetone phosphate as the substrate: Blacklow, S. C.; Knowles, J. R. *Biochemistry* **1990**, *29*, 4099. (b) The k_{cat} for Δ^5-ketosteroid isomerase is 53 000 s^{-1}. (c) The k_{cat} for citrate synthase is 100 s^{-1}: Alter, G. M.; Casazza, J. P.; Zhi, W.; Nemeth, P.; Srere, P. A.; Evans, C. T. *Biochemistry* **1990**, *29*, 7557. (d) The k_{cat} for mandelate racemase is 700 s^{-1}: Powers, V. M.; Koo, C. W.; Kenyon, G. L.; Gerlt, J. A.; Kozarich, J. W. *Biochemistry* **1991**, *30*, 9255.

(4) For enzymes that catalyze only proton-transfer reactions, e.g., isomerases, racemases, and tautomerases, the rates of substrate binding and/or product dissociation could be less than those of the proton transfer reactions. Thus, the rates of proton abstraction are at least as rapid as the observed k_{cat}s. For example, the rate-determining step in the isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate catalyzed by triose phosphate isomerase is dissociation of glyceraldehyde 3-phosphate from the enzyme.[5] However, the rate-determining steps in the reaction catalyzed by mandelate racemase are the proton transfer reactions.[6]

(5) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5627.

(6) Koo, C.; Kallarakal, A.; Kenyon, G. L., Gerlt, J. A. Unpublished observations.

(7) (a) Kresge, A. J. *Pure Appl. Chem.* **1991**, *63*, 213. (b) Chiang, Y.; Kresge, A. J. *Science* **1991**, *253*, 395.

(8) Thibblin, A.; Jencks, W. P. *J. Am. Chem. Soc.* **1979**, *101*, 4963.

(9) Voet, D.; Voet, J. G. *Biochemistry*; Wiley and Sons: New York, 1990. Abeles, R. H.; Frey, P. A.; Jencks, W. P. *Biochemistry*; Jones and Bartlett: Boston, MA, 1992.

(10) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 5928.

(11) Kresge, A. J. *Acc. Chem. Res.* **1975**, *8*, 354.

(12) Alternatively, using the terminology of a Marcus formalism[13] for describing the dependence of reaction rates on equilibrium constants (*vide infra*), for the concerted mechanism to be kinetically viable, an explanation must be provided for a reduction in the large intrinsic kinetic barrier ($\Delta G^{\ddagger}_{int}$) to the proton abstraction portion of the reaction, even though Marcus formalism might superficially appear to require that the intrinsic barrier should be identical for base-catalyzed reactions in solution and concerted general acid–general base catalyzed reactions in enzyme active sites.

(13) Marcus *theory*[14] was developed to describe the dependence of the rates of electron-transfer reactions on their equilibrium constants. The analogous quantitative partitioning of the activation energy barrier into thermodynamic and intrinsic kinetic barriers using the equation for an inverted parabola (Marcus *formalism*) has been applied to a variety of nonenzymatic proton, hydride, and methyl-transfer reactions.[15] In this article, we use this formalism to analyze quanitatively the rates of enzyme-catalyzed proton-transfer reactions.

(14) (a) Cohen, A. O.; Marcus, R. A. *J. Phys. Chem.* **1968**, *72*, 4249. (b) Marcus, R. A. *J. Am. Chem. Soc.* **1969**, *91*, 7224. (c) Albery, W. J. *Annu. Rev. Phys. Chem.* **1980**, *31*, 227.

(15) Bernasconi, C. F. *Adv. Phys. Org. Chem.* **1992**, *27*. 119.

(16) We purposely use the term "enolic intermediate" (rather than the terms "carbanion", "enolate," or "enol") to describe the structure of the intermediate in enzyme-catalyzed reactions so that we can avoid explicitly specifying the extent of proton transfer from the active site general acidic catalyst to the oxygen atom of the intermediate. As described in this article, we propose that the intermediate in enzyme-catalyzed reactions is neither an enolate anion nor a neutral enol but a species in which the proton from the general acidic catalyst is *partially* transferred to the carbonyl oxygen of the substrate keto tautomer of the carbon acid, i.e., an enolic intermediate.

(17) The analysis in this article uses values for pK_as for general acids, conjugate acids of general bases, and enol tautomers of carbon acids that have been measured in aqueous solution. While we realize that the pK_as of acids can be very dependent upon the medium, with neutral acids having higher pK_as and cationic acids having lower pK_as in solvents of lower dielectric constant than water, to the best of our knowledge no unequivocal measurements are available for the pK_as of the functional groups *within* the active sites of enzymes. While under limited circumstances the pK_as for active site groups may be either directly measured (e.g., the pK_a of the ϵ-ammonium group of the active site lysine in acetoacetate decarboxylase)[18] or inferred from the dependence of kinetic parameters on pH,[18,19] the values so determined reflect the solution conditions required for protonation or deprotonation of these groups and may not necessarily reflect the ionization behavior of the functional group *in* the active site.

(18) Schmidt, D. J.; Westheimer, F. H. *Biochemistry* **1971**, *10*, 1249. Frey, P. A.; Kokesh, F. C.; Westheimer, F. H. *J. Am. Chem. Soc.* **1971**, *93*, 7266. Kokesh, F. C.; Westheimer, F. H. *J. Am. Chem. Soc.* **1971**, *93*, 7270.

(19) Bruice, T. C.; Schmir, G. L. *J. Am. Chem. Soc.* **1959**, *81*, 4552. Knowles, J. R. *Crit. Rev. Biochem.* **1976**, *4*, 165.

two heteroatom bases whose conjugate acids have equal pK_as.[20,21] As noted previously, the pK_as of the OH group of the enol tautomer of the substrate carbon acid and the uncharged active site general acidic catalyst are approximately matched in several enzyme active sites. The absolute strengths of these hydrogen bonds can exceed 20 kcal/mol when the hydrogen bonded system is charged.[20,23,24] This differential hydrogen bonding permits a significant reduction in the $\Delta G°$ for formation of the enolic intermediate. Thus, we propose that the intermediate in the enzyme-catalyzed reactions is neither an enol nor an enolate anion but an anionic strongly hydrogen bonded complex of the enol and the conjugate base of the active site general acidic catalyst (or, equivalently, the enolate and the active site general acidic catalyst), i.e., an enolic intermediate.[16]

(2) A reduction in $\Delta G^{\ddagger}_{int}$ will decrease $\Delta G^{\ddagger}$ for the reaction, thereby accelerating the rate of the concerted tautomerization of the keto tautomer of the substrate carbon acid to the enol tautomer. That enzyme active sites can decrease $\Delta G^{\ddagger}_{int}$ from the values that characterize nonenzymatic reactions is supported by the observation that the values of $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton from either a protonated enol ether[25,26] or an iminium ion[27] are significantly smaller (representative value 3.6 kcal/mol; *vide infra*) than $\Delta G^{\ddagger}_{int}$ for base-catalyzed enolate anion formation in solution (representative value 12 kcal/mol; *vide infra*).[28-30] Partially protonated enol ethers and iminium ions are conceptual models for transition states in which the negative charge that develops on the carbonyl group as the α-proton is abstracted can be prestabilized without the requirement for solvent reorganization. As a result of the reduction in $\Delta G^{\ddagger}_{int}$, the transition states for the enolization reactions are late on the reaction coordinate, so the rates of the reactions are determined primarily by the $\Delta G°$ for formation of the enolic intermediate.

In the following sections, we develop the concepts summarized in this overview and demonstrate that the rapid rates of enzyme-catalyzed abstraction of the α-protons of carbon acids *can be quantitatively explained* through a full understanding of the origins and implications of a late transition state for proton abstraction.

Importance of Electrophilic Catalysis in Enzyme-Catalyzed Reactions

Our proposal[2,10] that the rapid rates of the enzyme-catalyzed reactions can be understood by concerted general acid–general base catalysis[31] originated with our observation that the pK_a of the α-proton of a carbon acid can be reduced by as much as 15 units by preequilibrium protonation of the carbonyl group.[2] As examples, the pK_as of the α-protons of aldehydes and ketones are reduced from 18–20 to about 4 by protonation of the carbonyl group, and the pK_a of the α-proton of mandelic acid is reduced from 22 to approximately 7 by protonation of the carboxylic acid group.[2] We reasoned that the impressive kinetic advantage provided by this reduction in the pK_a of the α-proton would be important in concerted general acid–general base catalyzed enolization reactions if the transition state resembled the enol. We noted that the transition state for concerted general acid–general base catalyzed formation of an enol is, in fact, expected to resemble the enol, since enols are unstable relative to their keto tautomers.[10] However, as described in the present article, the endergonicity of the keto–enol tautomerization reaction alone is insufficient to explain both the existence of transition states that resemble the enolic intermediate *and* the rapid rates of the enzyme-catalyzed reactions.

The enolic intermediate ketonizes in a second step to form a product that depends on the nature of the enzyme-catalyzed reaction: protonation in the reactions catalyzed by isomerases, racemases, and tautomerases; elimination of a substituent β to a carbonyl, carboxylic acid, or carboxylate anion group in the reactions catalyzed by dehydratases and lactonizing enzymes; carboxylation in the reactions catalyzed by biotin-dependent carboxylases; and aldol and Claisen condensations in the reactions catalyzed by aldolases and enzymes involved in citrate and fatty acid metabolism. Thus, enzymes must be able to catalyze both enolization of the carbon acid substrate and subsequent reketonization of the enolic intermediate.[34] As described in this article, the requirement that these enzymes catalyze successive enolization–ketonization reactions *via* an enolic intermediate allows the formulation of new principles governing concerted general acid–general base catalysis in enzyme active sites.

Description of Reaction Coordinates Using Marcus Formalism

Our analysis of the abstraction of the α-protons of carbon acids is based on a Marcus formalism.[13] The Marcus formalism we are using describes a reaction coordinate as an inverted parabola (Figure 1, both profiles), with the relative free energies of the reactants and products ($\Delta G°$) and of the transition state ($\Delta G^{\ddagger}$) being related by two independent parameters, $\Delta G°$ and $\Delta G^{\ddagger}_{int}$:[35,36]

$$G = -4\Delta G^{\ddagger}_{int}(x - 0.5)^2 + \Delta G°(x - 0.5) \qquad (1)$$

where G is the free energy at any point x on the reaction coordinate ranging from reactant ($x = 0$) to product ($x = 1$). The equation for an inverted parabola requires that $\Delta G°/4 \leq \Delta G^{\ddagger}_{int} \leq 4\Delta G°$.

(20) For pertinent articles and reviews, see: (a) Emsley, J. *Chem. Soc. Rev.* **1980**, *9*, 91. (b) Kreevoy, M. M.; Liang, T. M. *J. Am. Chem. Soc.* **1980**, *102*, 3315. (c) Emsley, J. *Struct. Bonding (Berlin)* **1984**, *57*, 147. (d) Hibbert, F.; Emsley, J. *Adv. Phys. Org. Chem.* **1990**, *26*, 255. (e) Perrin, C. L.; Thoburn, J. D. *J. Am. Chem. Soc.* **1992**, *114*, 8559.

(21) A variety of terms have been used to describe these hydrogen bonds, including "very strong hydrogen bonds"[20d] and "low barrier hydrogen bonds".[22] While these hydrogen bonds are "very strong" and this strength can be associated with the fact that the potential energy barrier for proton transfer between the donor and acceptor is less than the zero point energy of the hydrogen bond (hence, the term "low barrier"), we use "short, strong hydrogen bond" to indicate that an important structural consequence of this type of hydrogen bond is an unusually short distance (*vide infra*) between the donor and acceptor. We do not imply that *imposing* a short bond distance results in a very strong hydrogen bond.

(22) Cleland, W. W. *Biochemistry* **1992**, *31*, 317.

(23) (a) Kolthoff, I. M.; Chantooni, M. K. *J. Am. Chem. Soc.* **1969**, *91*, 4621. (b) Pawlak, Z.; Magonski, J.; Strohbusch, F. *J. Chem. Soc., Faraday Trans. 1* **1985**, *81*, 2021.

(24) Chantooni, M. K.; Kolthoff, I. M. *J. Phys. Chem.* **1975**, *79*, 1176.

(25) (a) Chwang, W. K.; Eliason, R.; Kresge, A. J. *J. Am. Chem. Soc.* **1977**, *99*, 805. (b) Kresge, A. J.; Sagatys, D. S.; Chen, H. L. *J. Am. Chem. Soc.* **1977**, *99*, 7228.

(26) Toullec, J. *J. Chem. Soc., Perkin Trans. 2* **1989**, 167.

(27) Stivers, J. T.; Washabaugh, M. W. *Bioorg. Chem.* **1991**, *19*, 369; **1992**, *20*, 155.

(28) (a) Albery, W. J.; Gelles, J. S. *J. Chem. Soc., Faraday Trans. 1* **1982**, *78*, 1569. (b) Albery, W. J. *J. Chem. Soc., Faraday Trans. 1* **1982**, *78*, 1579.

(29) Guthrie, J. P. *Can. J. Chem.* **1979**, *57*, 1177.

(30) Guthrie, J. P. *J. Am. Chem. Soc.* **1991**, *113*, 7249.

(31) Although concerted general acid–general base catalysis had been proposed for the mechanisms of the reactions catalyzed by Δ^5-ketosteroid isomerase[32] and citrate synthase,[33] the kinetic advantage provided by the general acid component was not quantitated so the kinetic viability of the concerted mechanism remained uncertain until our clarification of the effect of electrophilic catalysis on the pK_a of the α-proton of a carbon acid.[2]

(32) Xue, L.; Talalay, P.; Mildvan, A. S. *Biochemistry* **1990**, *29*, 7491.

(33) Karpusas, M.; Branchaud, B.; Remington, S. J. *Biochemistry* **1990**, *29*, 2213.

(34) In our previous papers, we pointed out that proton transfer to the carbonyl group of a carbon acid can reduce the pK_a of the α-proton by as much as 15 pK_a units[2] and that enzymes were likely to utilize concerted proton transfer to the carbonyl group to achieve the observed reactions rates.[10] While in these papers we discussed the formation of *enol* intermediates in which proton transfer from an active site general acid catalyst to the carbonyl group was "complete", in this article we point out that *partial* proton transfer from the acid catalyst to the carbonyl group is sufficient to explain the observed rates of the enzyme-catalyzed reactions so that these reactions are likely to involve *enolic*[16] rather than *enol* intermediates.

(35) The algebraic treatment of Marcus formalism given here is based on that presented in a recent review: Grunwald, E. *Prog. Phys. Org. Chem.* **1990**, *17*, 55.

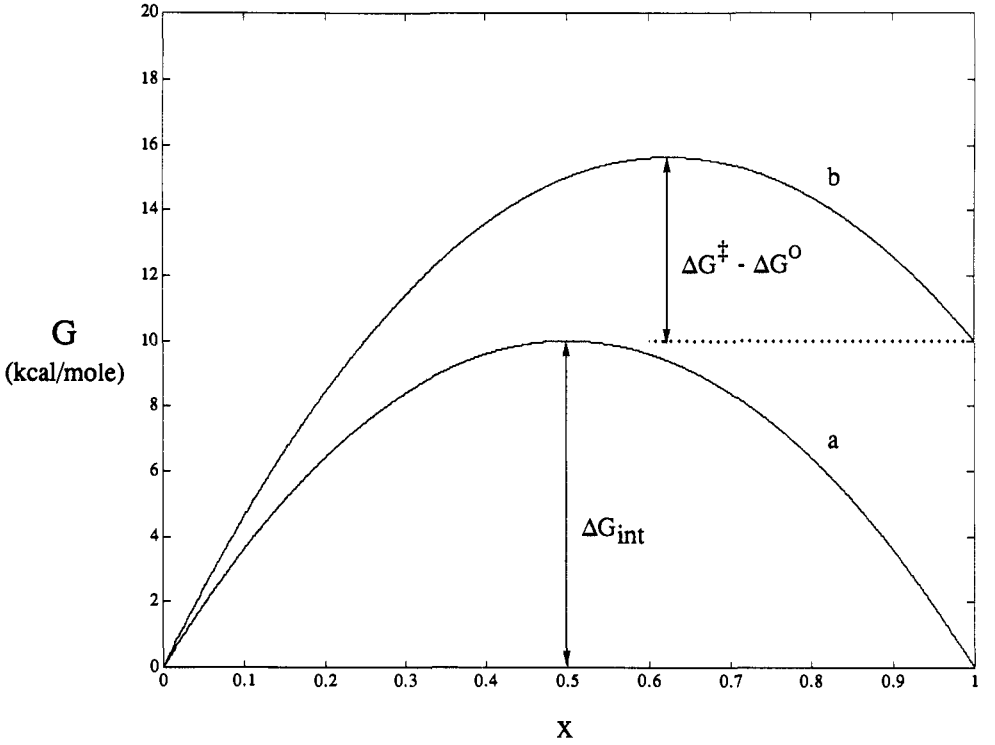


Figure 1. Reaction coordinates described by the equation for an inverted parabola (eq 1 in the text). In this and the following figures which describe the dependence of G on the position of the reaction coordinate, x, the value of G is normalized so that $G = 0$ when $x = 0$. This normalization is accomplished by adding the value of G when $x = 0$ ($\Delta G^{\ddagger}_{int} + \Delta G°/2$) to the value of G calculated with eq 1. When $\Delta G° > 0$, $\Delta G^{\ddagger} - \Delta G° < \Delta G^{\ddagger}_{int}$ (curve a); when $\Delta G° = 0$, $\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}$ (curve b). The reaction coordinates illustrated were calculated assuming (a) $\Delta G° = 10$ kcal/mol and $\Delta G^{\ddagger}_{int} = 10$ kcal/mol and (b) $\Delta G° = 0$ kcal/mol and $\Delta G^{\ddagger}_{int} = 10$ kcal/mol. For curve a, $\Delta G^{\ddagger} - \Delta G° = 5.63$ kcal/mol; for curve b, $\Delta G^{\ddagger} - \Delta G° = 10$ kcal/mol. The reaction coordinates plotted in this and the following figures were calculated and plotted with Macintosh MATLAB 3.5, The Mathworks, Inc., Natick, MA.

The position of the transition state on the reaction coordinate, $x^{\ddagger}$, occurs at the maximum of the function described by eq 1:

$$x^{\ddagger} = 0.5 + \Delta G°/8\Delta G^{\ddagger}_{int} \quad (2)$$

From eqs 1 and 2

$$\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}(1 + \Delta G°/4\Delta G^{\ddagger}_{int})^2 \quad (3)$$

Equation 3 partitions $\Delta G^{\ddagger}$ into contributions from $\Delta G°$ and a second energy that describes the difference between $\Delta G^{\ddagger}$ and $\Delta G°$ and is associated with $\Delta G^{\ddagger}_{int}$. The quantity $\Delta G^{\ddagger} - \Delta G°$ is $\leq \Delta G^{\ddagger}_{int}$ (Figure 1, profile b); only if $\Delta G° = 0$ does $\Delta G^{\ddagger} - \Delta G° = \Delta G^{\ddagger}_{int}$ (the definition of $\Delta G^{\ddagger}_{int}$; Figure 1, profile a). When $\Delta G°$ can be varied (e.g., by varying the pK_a of a general acidic or general basic catalyst) and the observed dependence of $\Delta G^{\ddagger}$ on $\Delta G°$ obeys eq 3, $\Delta G^{\ddagger}_{int}$ can be determined by the observed dependence of $\Delta G^{\ddagger}$ on $\Delta G°$.

Equation 3 predicts the Brønsted relationship, with decreasing values of β (the slope of a plot of $\Delta G^{\ddagger}$ *vs* $\Delta G°$) resulting from decreasing values for $\Delta G°$ and/or increasing values for $\Delta G^{\ddagger}_{int}$:

$$\Delta\Delta G^{\ddagger}/\Delta\Delta G° = \beta = 0.5 + \Delta G°/8\Delta G^{\ddagger}_{int} \quad (4)$$

From eq 3, $\beta = x^{\ddagger}$ (compare eqs 2 and 4).

Marcus formalism assumes that $\Delta G°$ and $\Delta G^{\ddagger}_{int}$ are independent energetic parameters that together allow description of the reaction coordinate. However, $\Delta G°$ and $\Delta G^{\ddagger}_{int}$ are not completely independent in the active sites of enzymes that catalyze the concerted general acid–general base catalyzed abstraction of the α-protons of carbon acids: the structural features that are responsible for reductions in $\Delta G^{\ddagger}_{int}$ also produce reductions in $\Delta G°$ (*vide infra*).

Values of $\Delta G^{\ddagger}_{int}$ for Nonenzymatic Reactions: Kinetics of Proton Abstraction from Carbon and Normal Acids

The values for $\Delta G^{\ddagger}_{int}$ that are available for nonenzymatic reactions that involve abstraction of α-protons of carbon acids as the rate-determining step are 13 kcal/mol (enolization of acetone),[28b] 12.1 kcal/mol (enolization of aldehydes and ketones),[29] and 10.7 kcal/mol (aldol condensation reactions).[30] Although somewhat different assumptions were used in estimating the values for the work terms[36,37] in these reactions, we assume that the average of these values, 12 kcal/mol, is a useful estimate of the value for $\Delta G^{\ddagger}_{int}$ for a hypothetical nonenzymatic enolization reaction of a carbon acid. Since, within the estimated errors, the individual values for $\Delta G^{\ddagger}_{int}$ are the same, Marcus formalism satisfactorily describes the reaction coordinates for nonenzymatic enolization of carbon acids in solution. The presence of a significant $\Delta G^{\ddagger}_{int}$ in these reactions indicates that, if $\Delta G°$ could be reduced to 0, a substantial $\Delta G^{\ddagger}$ would remain. For example, if $\Delta G° = 0$ and $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, $k \approx 10^4$ s^{-1}.

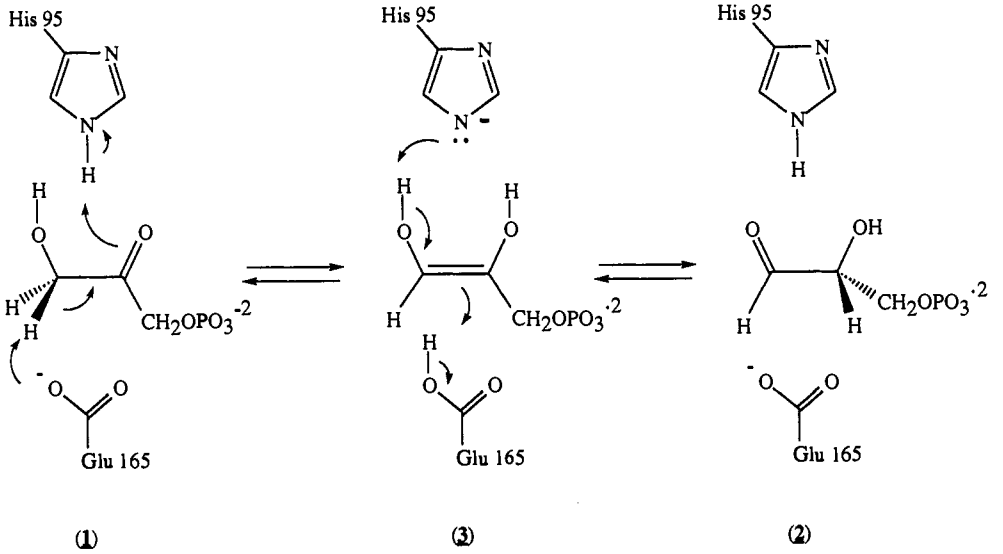
For comparison, the $\Delta G^{\ddagger}_{int}$ for transfer of a proton between heteroatoms is much smaller, ≤ 3 kcal/mol.[11] If $\Delta G° = 0$ and $\Delta G^{\ddagger}_{int} = 1$ kcal/mol, proton transfer could occur at nearly the vibrational frequency, $\sim 10^{12}$ s^{-1}.

The significant difference between the values for $\Delta G^{\ddagger}_{int}$ for proton abstraction from carbon acids (12 kcal/mol) and from heteroatom (normal) acids (≤ 3 kcal/mol) provides an understanding (using Marcus formalism) of the observation that proton abstraction from *almost* all carbon acids is much slower than from normal acids having equivalent pK_as. The origin of the

(36) For unimolecular reactions, Marcus formalism relates the observed $\Delta G^{\ddagger}$ to $\Delta G°$, with $\Delta G^{\ddagger}_{int}$ quantitatively describing the interrelationship. For higher order reactions, e.g., bimolecular and termolecular reactions involving general acidic and/or general basic catalysts, Marcus formalism applies only to the interrelationship between $\Delta G^{\ddagger}$ and $\Delta G°$ within an "encounter" complex of the reacting species. The energy for formation of this complex, w_r, the "work term", is represented in the observed $\Delta G^{\ddagger}$ for the higher order reaction, $\Delta G^{\ddagger}_{obs}$, by $\Delta G^{\ddagger}_{obs} = \Delta G^{\ddagger} + w_r$ where $\Delta G^{\ddagger}$ is the $\Delta G^{\ddagger}$ from the eq 3.[14] For this reason, the analysis described in this article is restricted to understanding the magnitudes of k_{cat} rather than of k_{cat}/K_m.

(37) The work terms for these reactions were either assumed to be small on the basis of statistical considerations[28b] or concluded to be small[29,30] since the dependence of $\Delta G^{\ddagger}$ on $\Delta G°$ did not reveal any systematic deviation from eq 3 that would indicate the presence of a significant work term.

Scheme I



differences in $\Delta G^{\ddagger}_{int}$, and therefore a precise explanation for the kinetic differences between carbon and normal acids, has been attributed to solvation effects as well as changes in structure and bonding.[15,38] We will return to this issue later.

Establishing Requirements for Concerted Catalysis in Enzyme-Catalyzed Reactions: The pKas of the Uncharged Active Site General Acid Catalysts and the OH Groups of the Enol Tautomers of the Substrate Carbon Acids Are Matched

In the context of our proposal that many types of enzyme-catalyzed reactions necessarily involve concerted general acid–general base catalysis to form enolic intermediates, we now consider the identities and pK_as[17] of the general acidic catalysts that are present in enzyme active sites as well as those of the OH groups of the enol tautomers of the substrate carbon acids. We will consider four structurally characterized examples of active sites that catalyze abstraction of the α-protons of carbon acids.

Triose Phosphate Isomerase. We propose that triose phosphate isomerase catalyzes the tautomerization of dihydroxyacetone phosphate (**1**) to glyceraldehyde 3-phosphate (**2**) *via* a cis-enediolic intermediate (**3**) (Scheme I). Crystallographic and enzymological studies suggest that the isomerase utilizes *neutral* histidine (His 95) as the general acidic catalyst and a glutamate anion (Glu 165) as the general basic catalyst.[39]

In analogy with enols formed from aldehydes and ketones, we expect that the first pK_a of the cis-enediol (**3**) is ~10–11.[7] Perhaps counter to chemical intuition, Knowles and his co-workers concluded that the neutral form of His 95 is the general acidic catalyst; the pK_a is thought to be $\leq$11.[40] Thus, we conclude that the pK_as of the general acidic catalyst and of the OH group of the enol tautomer of the substrate carbon acid are closely matched for triose phosphate isomerase.

Δ^5-Ketosteroid Isomerase. We propose that Δ^5-ketosteroid isomerase catalyzes the tautomerization of β,γ- (**4**) and α,β-unsaturated (**5**) ketosteroids *via* a dienolic intermediate (**6**) (Scheme II). Crystallographic and enzymological studies suggest that the ketosteroid isomerase-catalyzed reaction utilizes a tyrosine (Tyr 14) as the general acidic catalyst and an aspartate anion (Asp 38) as the general basic catalyst.[41]

The pK_a of the dienol (**6**) is 10.0.[42] The pK_a of Tyr 14 is >10.9.[3a] Thus, we conclude that the pK_as of the general acidic catalyst and of the OH group of the enol tautomer of the substrate carbon acid are closely matched for ketosteroid isomerase.

Citrate Synthase. We propose that citrate synthase catalyzes the nucleophilic attack of acetyl CoA (**7**) on the carbonyl group of oxalacetate (**8**) *via* an enolic intermediate (**9**) (Scheme III). Crystallographic and enzymological studies suggest that the citrate synthase-catalyzed reaction utilizes a histidine (His 274) as the general acidic catalyst and an aspartate anion (Asp 375) as the general basic catalyst in the enolization reaction.[43]

No data are yet available regarding the pK_a of the OH group of the enol of a thioester (**9**); however, in analogy with ketones, it is likely to be 10–11. In addition, no data are available for either the protonation state or pK_a of His 274.[43] However, in analogy to the reaction catalyzed by triose phosphate isomerase, the proposal has been made that the catalytically relevant state of protonation of His 274 is the neutral species.[43] Since this functional group is located at the N-terminus of an α-helix, its pK_a, like that of His 95 in the active site of triose phosphate isomerase, may be perturbed downward from its normal value of 14. Thus, we conclude that the pK_as of the general acidic catalyst and of the OH group of the enol tautomer of the substrate carbon acid are approximately matched for citrate synthase.

Mandelate Racemase. Recently, we proposed that mandelate racemase catalyzes the interconversion of *R*-mandelate (**10**) and *S*-mandelate (**11**) *via* a geminal enediolic intermediate (**12**) (Scheme IV).[2] Crystallographic and enzymological studies[44] support our proposal that a glutamic acid (Glu 317) functions as the general acidic catalyst and that lysine (Lys 166) and histidine (His 297) function as the general basic catalysts [one for proton transfer to and one for proton transfer from each face of the enediol intermediate (**6**)]. The active site also contains

(38) (a) Bernasconi, C. F.; Terrier, F. *J. Am. Chem. Soc.* **1987**, *109*, 7115. (b) Bernasconi, C. F. *Acc. Chem. Res.* **1987**, *20*, 301.

(39) (a) Knowles, J. R. *Nature* **1991**, *350*, 121. (b) Davenport, R. C.; Bash, P. A.; Seaton, B. A.; Karplus, M. A.; Petsko, G. A., Ringe, D. *Biochemistry* **1991**, *30*, 5821.

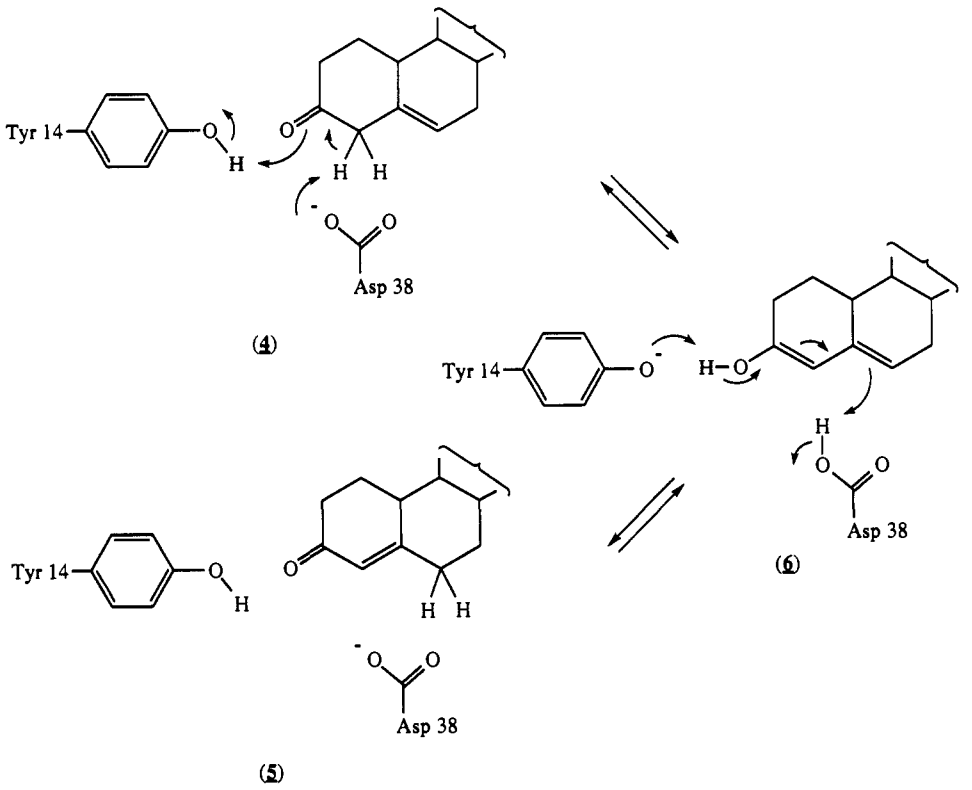
(40) Lodi, P. J.; Knowles, J. R. *Biochemistry* **1991**, *30*, 6948.

(41) (a) Tyr 14, the electrophilic catalyst, has a $pK_a > 10.9$: Kuliopulos, A., Westbrook, E. M.; Talalay, P.; Mildvan, A. S. *Biochemistry* **1987**, *26*, 3927. (b) The carboxylic acid group of Asp 38, the conjugate acid of the general basic catalyst, has a $pK_a < 5$: Kuliopulos, A.; Mildvan, A. S.; Shortle, D.; Talalay, P. *Biochemistry* **1989**, *28*, 149.

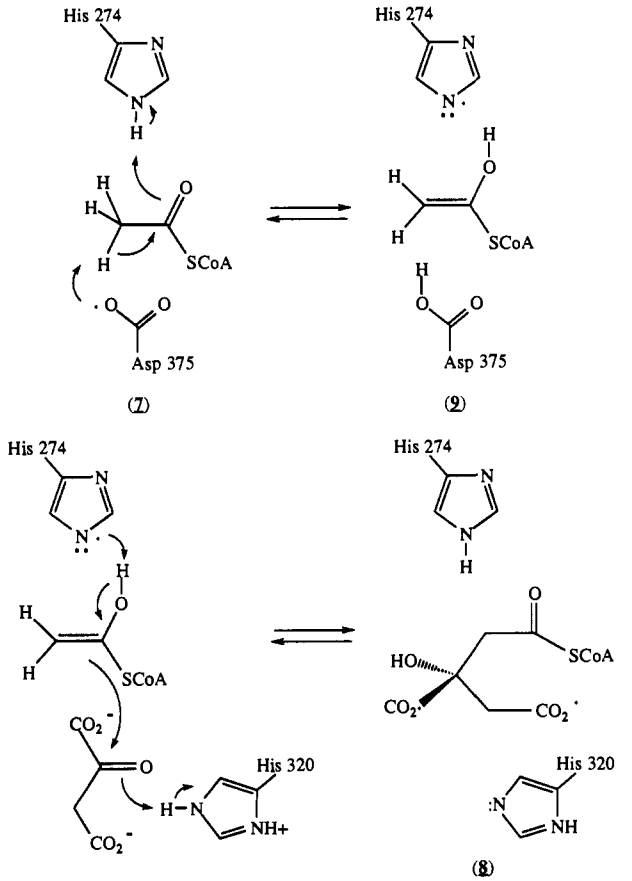
(42) Zeng, B.; Pollack, R. M. *J. Am. Chem. Soc.* **1991**, *113*, 3838.

(43) The results of both X-ray crystallographic studies (Karpusas, M.; Holland, D.; Remington, S. J. *Biochemistry* **1991**, *30*, 6024) and site-directed mutagenesis studies (Kurz, L. C.; Drysdale, G. R.; Riley, M. C.; Evans, C. T.; Srere, P. A. *Biochemistry* **1992**, *31*, 7908) suggest that His 274 is the electrophilic catalyst and the carboxylate anion of Asp 375 is the general basic catalyst. Asp 375 has a $pK_a \approx 6.5$ (Kosicki, G. W.; Srere, P. A. *J. Biol. Chem.* **1961**, *236*, 2560). The pK_as of His 274 (i.e., for both imidazolium and imidazole as acids) are unknown, although they may be perturbed downward given the location of this functional group at the N-terminus of an α-helix. In analogy with triose phosphate isomerase,[37] Remington recently proposed that the catalytically relevant state of protonation of His 274 is the neutral species (Remington, S. J. *Curr. Opin. Struct. Biol.* **1992**, *2*, 730).

Scheme II



Scheme III



two additional cationic catalysts (Mg^{2+} and Lys 164) that may neutralize the negative charge of the carboxylate group,[44,45] thereby allowing the substrate to react as if it were bound in a form that approached the electronic character of a neutral carboxylic acid rather than that of a carboxylate ion in the active site.

The first pK_a of the geminal enediol (**12**) of mandelic acid is 6.6.[46] While the pK_a of Glu 317 has not yet been measured, it is likely to be approximately 6.[47] Thus, we conclude that the pK_as of the general acidic catalyst and of the OH group of the enol tautomer of the substrate carbon acid are approximately matched for mandelate racemase.

The General Acidic Catalysts Are Uncharged. The general basic catalysts can either be anionic (triose phosphate isomerase, Δ^5-ketosteroid isomerase, and citrate synthase) or neutral (mandelate racemase). In contrast, in each of these active sites, the

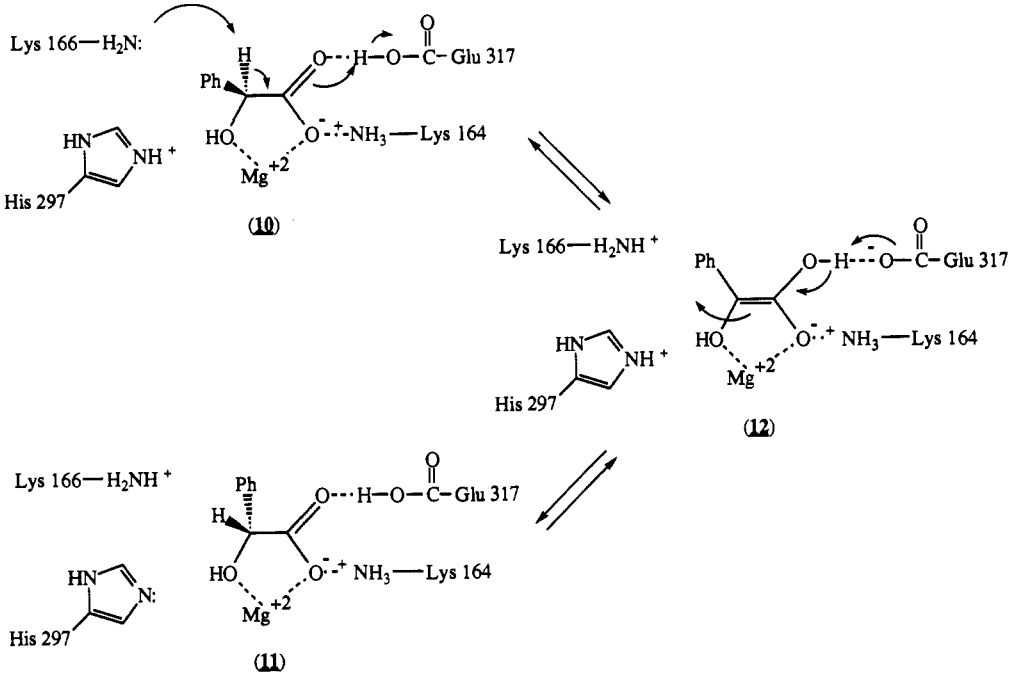
(44) The results of both X-ray crystallographic studies (Neidhart, D. J.; Howell, P. L.; Petsko, G. A.; Powers, V. M.; Li, R.; Kenyon, G. L.; Gerlt, J. A. *Biochemistry* **1991**, *30*, 9264. Neidhart, D. J.; Clifton, J. R.; Petsko, G. A. Unpublished observations) and site-directed mutagenesis studies (Landro, J. A.; Kallarakal, A.; Ransom, S. C.; Gerlt, J. A.; Kozarich, J. W.; Neidhart, D. J.; Kenyon, G. L. *Biochemistry* **1991**, *30*, 9274. Landro, J. A.; Kallarakal, A.; Mitra, B.; Kozarich, J. W.; Gerlt, J. A. Unpublished observations) suggest that Glu 317 is the electrophilic catalyst and both Lys 166 and His 297 are general basic catalysts. The conjugate acids of Lys 166 and His 297 are both estimated to have pK_as $\approx$ 6; the pK_a of Glu 317 is unknown.

(45) The mode of binding of mandelate to the active site shown in Scheme IV, i.e., coordination to the essential Mg^{2+} *via* the hydroxyl group and *one* of the carboxylate oxygens, hydrogen bonding of the metal-coordinated carboxylate oxygen to the ϵ-ammonium group of Lys 164, and hydrogen bonding of the other carboxylate oxygen to the carboxylic acid functional group of Glu 317, is supported by five high-resolution X-ray structures: (1) wild type mandelate racemase complexed with the competitive inhibitor *S*-atrolactate (Neidhart, D. J.; Fugita, S.; Petsko, G. A. Unpublished observations), (2) the E317Q mutant (the general acidic catalyst) complexed with *S*-atrolactate (Clifton, J. R.; Mitra, B.; Gerlt, J. A.; Petsko, G. A. Unpublished observations), (3) the K166R mutant (one of the two general basic catalysts) complexed with the substrate *S*-mandelate (Clifton, J. R.; Kallarakal, A. T.; Gerlt, J. A.; Petsko, G. A. Unpublished observations), (4) the D270N mutant (the carboxylate group hydrogen bonded to His 297, one of the two general basic catalysts) complexed with *S*-atrolactate (Clifton, J. R.; Schafer, S.; Gerlt, J. A.; Petsko, G. A. Unpublished observations), and (5) the covalent adduct between wild-type mandelate racemase and *R*-α-phenylglycidate in which the ϵ-amino group of Lys 166 is alkylated by the epoxide affinity label (Neidhart, D. J.; Landro, J. A.; Gerlt, J. A.; Kozarich, J. W.; Petsko, G. A. Unpublished observations).

(46) Chiang, Y.; Kresge, A. J.; Pruszynski, P.; Schepp, N. P.; Wirz, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 792.

(47) Although the pK_as of glutamic acid residues in proteins can be influenced by environment (Urry, D W.; Gowda, D. C.; Peng, S. Q.; Parker, T. M.; Harris, R. D. *J. Am. Chem. Soc.* **1992**, *114*, 8716), we assume that the pK_a of Glu 317 is likely to be "normal" in the polar environment of the active site of mandelate racemase.

Scheme IV



general acidic catalyst is uncharged.[48] In a later section, we propose a *general mechanism* for reducing ΔG° in enzyme-catalyzed enolization–reketonization reactions that *requires* that the general acidic catalysts be uncharged.

Description of the Transition States for Enzyme-Catalyzed Reactions

Given the matched pK_as of the active site general acidic catalysts and the OH groups of the enol tautomers of the substrate carbon acids, we propose that the transition states for the enzyme-catalyzed reactions resemble the enolic intermediates. This proposal is based upon the expectation that a general acid can act as a catalyst if, and only if, it can transfer protons to the substrate to generate the product,[49,50] in this case the OH group of the enolic intermediate. If the active site general acidic catalysts are to function as proton transfer catalysts, at the transition state on the reaction coordinate that describes the conversion of the keto tautomer of the substrate carbon acid to the enolic intermediate, transfer of the proton from the catalyst to the substrate must be thermodynamically allowed. Since the pK_a of the general acidic catalyst is nearly matched to the pK_a of the OH group of the enol tautomer, the proton associated with the general acidic catalyst can only be transferred to the substrate (carbonyl) oxygen if the transition state resembles the enolic intermediate, i.e., has approximately the same structure and chemical properties. Thus, we propose both that the transition states for the enzyme-catalyzed reactions resemble the enolic intermediates and that these are late on the reaction coordinates for enolization of the substrate carbon acids.

(48) If solvent is excluded from an enzyme active site, the pK_as of cationic acids can be decreased and those of uncharged acids can be increased. In considering the requirements for effective general acidic and/or general basic catalysis in enzyme active sites, Gilbert noted that catalytic advantage could be obtained if general basic catalysts were anionic (carboxylate or thiolate anions) and general acidic catalysts were cationic (lysine or histidine): Gilbert, H. F. *Biochemistry* **1981**, *20*, 5643. While this explanation may partially explain the rapid rates of abstraction of the α-protons by the carboxylate general bases in the active sites of triose phosphate isomerase, Δ^5-ketosteroid isomerase, and citrate synthase, it is not applicable to the proton abstraction reactions catalyzed by the bases in the active site of mandelate racemase. Nor is this explanation applicable to transfer of a proton from the general acidic catalysts to the carbonyl oxygens in any of these active sites. Thus, we do not believe that this effect is generally applicable in explaining the rates of enzyme-catalyzed reactions.

(49) Jencks, W. P. *J. Am. Chem. Soc.* **1972**, *94*, 4731.

(50) Guthrie, J. P. *J. Am. Chem. Soc.* **1980**, *102*, 5286.

However, since the pK_as of the active site general acidic catalysts and the OH groups of the enol tautomers of the substrate carbon acids are nearly (and may be exactly) matched, complete proton transfer from the general acidic catalyst to the carbonyl group of the carbon acid is not possible. As described in later sections of this article, we propose that the actual intermediates in the enzyme-catalyzed reactions are strongly hydrogen bonded species in which the proton of the general acidic catalyst is *shared* by both the conjugate base of the general acidic catalyst and the conjugate base of the OH group of the enol (the enolate anion).

This proposal for the structure of the transition states for the enzyme-catalyzed reactions differs significantly from the proposal formulated by Jencks for the structure of the transition states for nonenzymatic concerted general acid–general base catalyzed reactions of carbonyl compounds, including enolization reactions of carbon acids. For these reactions, Jencks proposed the "libido rule":[49] "concerted general acid–base catalysis of complex reactions in aqueous solution can occur only (a) at sites that undergo a large change in pK_a in the course of the reaction and (b) when this change in pK_a converts an unfavorable to a favorable proton transfer with respect to the catalyst, i.e., *the pK_a of the catalyst is intermediate between the initial and final pK_a values of the substrate site.*" The italicized portion of the rule describes a transition state which is intermediate between the substrate keto tautomer of the carbon acid and the enol tautomer, i.e., the transition state for nonenzymatic reactions is "midway" on the reaction coordinate. For example, in the enolization of ketones, the libido rule predicts that the pK_a of the general acidic catalyst should be $\sim$3, the average of the pK_as of the protonated carbonyl group ($\sim$–4) and the OH group of the enol ($\sim$10). Hegarty and Jencks confirmed this expectation for the enolization of acetone.[51] An explanation for the different transition-state structures for the enzyme-catalyzed and the nonenzymatic reactions is obtained from the analysis that follows in this article.

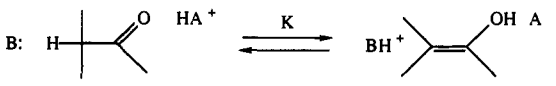
Effect of Concerted General Acid-General Base Catalysis on ΔG° for Enzyme-Catalyzed Reactions

We now examine the origins and magnitudes of both ΔG° and $\Delta G^{\ddagger}_{int}$ for concerted general acid–general base catalyzed enolization reactions of carbon acids in order to understand how an enzyme active site can accomplish reductions in both components of $\Delta G^{\ddagger}$.

(51) Hegarty, A. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1975**, *97*, 7188.

By its definition, for an endergonic reaction, $\Delta G^{\ddagger}_{int}$ sets a lower limit on $\Delta G^{\ddagger}$ ($\Delta G^{\ddagger} = \Delta G^{\ddagger}_{int}$ when $\Delta G^{\circ} = 0$). An increase in ΔG° from 0 increases $\Delta G^{\ddagger}$ from this lower limit, with the amount of the increase being determined by the precise magnitudes of both ΔG° and $\Delta G^{\ddagger int}$ as described by eq 3. While it might be assumed that $\Delta G^{\ddagger}_{int}$ for abstraction of a proton from a carbon acid will not depend on whether the reaction occurs in solution or in an enzyme active site, we will demonstrate later that this assumption is incorrect. In this and the following section, we consider the components of ΔG°, their expected magnitudes, and a *general mechanism* for reducing ΔG° in enzyme active sites. We then consider the interrelationships between ΔG° and $\Delta G^{\ddagger}_{int}$ in achieving the observed $\Delta G^{\ddagger}$s for enzyme-catalyzed reactions.

Our proposal of concerted general acid–general base catalysis requires that the ΔG° for enzyme-catalyzed reactions be composed of the free energy changes for both proton transfer from the general acidic catalyst to the general basic catalyst and the equilibrium for enol formation. The reaction for enolization of a carbon acid assisted by a general acid and a general base is



The negative logarithm for the equilibrium constant for this composite reaction, pK, is

$$pK = pK_E + \{pK_a(HA^+) - pK_a(BH^+)\} \quad (5)$$

and the expected thermodynamic barrier, ΔG°, is

$$\Delta G^{\circ} = 2.303RT[pK_E + \{pK_a(HA^+) - pK_a(BH^+)\}] \quad (6)$$

where pK_E is the negative logarithm of the equilibrium constant for interconversion of the keto and enol tautomers of the carbon acid, pK_a(HA$^+$) is the pK_a of the general acidic catalyst, and pK_a(BH$^+$) is the pK_a of the conjugate acid of the general basic catalyst.

Experimentally measured values for pK_Es are available for a variety of aldehydes (~6)[7] and ketones (~8)[7] and for mandelic acid (15.4),[46] but not for thioesters. The kinetic similarity of the α-protons of thioesters and ketones led us to previously suggest that the pK_Es for thioesters and ketones are similar.[2]

If pK_a(BH$^+$) > pK_a(HA$^+$), ΔG° as defined by eq 6 can be less than that associated with pK_E, i.e., the apparent equilibrium constant for enol formation can be increased. However, the pK_as of the general acidic catalysts [pK_a(HA$^+$)] significantly *exceed* the pK_as of the conjugate acids of the general basic catalysts [pK_a(BH$^+$)] in the active sites of triose phosphate isomerase (>11 and ~6, respectively),[40,52] Δ^5-ketosteroid isomerase (>10.9 and < 5, respectively),[41] and citrate synthase (>11 and ~6.5, respectively).[43] For all three enzymes the transfer of a proton from HA$^+$ to B is unfavorable by ≥7 kcal/mol, so the catalysts do not provide any reduction in ΔG°. In fact, intercatalyst proton transfer through bifunctional catalysis *increases* the apparent ΔG° for formation of the enolic intermediate in the active sites of these enzymes from that associated with the pK_E (6–8 pK_E units; 8.4–11.2 kcal/mol).

In the active site of mandelate racemase, the pK_a of the electrophilic catalyst (Glu 317)[47] may be similar to the pK_as of the conjugate acids of the general basic catalysts (~6; Lys 166 and His 297),[44] indicating that the intercatalyst proton-transfer reaction may not alter the ΔG° for enol formation from that associated with the pK_E (≥15.4 pK_E units; ≥21.6 kcal/mol).

The calculated values for ΔG° for concerted general acid–general base catalyzed enolization reactions (from eq 6) are 18–22 kcal/mol. Since $\Delta G^{\ddagger} \approx 14$ kcal/mol ($k_{cat} \approx 10^3$ s^{-1}) for these enzyme-catalyzed reactions, ΔG° must be reduced if the rates of the reactions are to be explained.

(52) Plaut, B.; Knowles, J. R. *Biochem. J.* **1972**, *129*, 311.

Reduction of ΔG°: Importance of Short, Strong Hydrogen Bonds

We now propose that the *general* mechanism for reducing ΔG° for concerted general acid–general base catalyzed enolization reactions of carbon acids in enzyme active sites is the differential hydrogen bonding of the conjugate base of the active site general acidic catalyst to the OH group of the enol tautomer relative to the hydrogen bonding of the active site general acidic catalyst to the carbonyl group of the keto tautomer of the substrate.[50]

Unusually strong hydrogen bonds, termed "short, strong hydrogen bonds",[21] can be formed when a proton is shared by two bases whose conjugate acids have equivalent pK_as.[20] In addition, the hydrogen bonded system must possess a net charge (either negative or positive) for the resulting hydrogen bond to be of the short, strong type. We previously noted[10] that, if the pK_a of the general acidic catalyst and the OH group of the enol tautomer of the substrate carbon acid were matched, these could share the proton initially associated with the acidic catalyst in a short, strong hydrogen bond.[20] We have now concluded that the pK_as of the general acidic catalysts in the active sites are, in fact, closely matched to those expected for the OH groups of the enol tautomers of the substrate carbon acids for several enzyme-catalyzed reactions. In each of these active sites, the hydrogen bonded system (O–H–O in the cases of Δ^5-ketosteroid isomerase and mandelate racemase; O–H–N in the cases of triose phosphate isomerase and citrate synthase) would be anionic since the general acidic catalyst is uncharged, thereby satisfying the requirement that the hydrogen bonded system be charged.[20] Thus, the data currently available for enzymes that catalyze abstraction of α-protons of carbon acids indicate that strong, short hydrogen bonds are likely to be formed between the enolic intermediates and the conjugate bases of the general acidic catalysts.

In the well-studied case of symmetrical bicarboxylates sharing a proton [an (O–H–O)$^-$ hydrogen bond; ΔpK_a between the donor and acceptor is necessarily 0], the proton lies in a single-minimum potential well or a double-minimum potential well in which the barrier is less than the zero point energy of the proton, with the O–H distances being equal as judged by X-ray and neutron diffraction studies, i.e., the proton is equally shared by the oxygen atoms.[20] The (O–H–O)$^-$ distance (as short as 2.45 Å) is shorter than that found in a "normal" or weak hydrogen bond (> 2.60 Å) where the proton lies in a double-minimum potential well in which the barrier is greater than the zero point energy. In a weak hydrogen bond, the O–H–O distance is slightly less than the sum of the van der Waals radii of the participating O atoms; in a strong hydrogen bond, the (O–H–O)$^-$ distance is significantly less than the van der Waals radii of the participating O atoms. The short distance reflects the energetics of the hydrogen bonded system, although the local environment, e.g., crystal packing forces, may influence the length of the (O–H–O)$^-$ hydrogen bond.

(53) We also recently noted[10] that stabilization of an enol(ate) intermediate in active sites could involve the formation of a closely approximated anion–cation pair in the active site, e.g., the anionic conjugate base of the electrophilic catalyst resulting from proton transfer to form a neutral enol intermediate could be in close proximity to a preexisting positively charged center in the active site. Alternatively, an enolate anion could be formed without the assistance of an electrophilic catalyst but stabilized by close approximation to a preexisting positively charged center, e.g., a divalent metal ion. Since the electrostatic attraction between opposite charges may be large in an enzyme active site in which the effective dielectric constant may be small,[54] such a mechanism could provide significant stabilization of the enolic intermediate. However, the observed matching of the pK_as of the general acidic catalysts with the OH groups of the enolic intermediates for several enzyme-catalyzed reactions, including the Mg^{2+}-dependent reaction catalyzed by mandelate racemase, suggests that differential hydrogen bonding and not electrostatic stabilization of the enol(ate) intermediate is the *general mechanism* for reducing ΔG°.

(54) If, for example, the two charges were separated by 3 Å in a medium of dielectric constant 6, the differential stabilization of the enol intermediate would be 18.7 kcal/mol.

Measurements in the gas phase as well as calculations indicate that the hydrogen bond strengths of $(O–H–O)^-$ hydrogen bonds can be greater than 20 kcal/mol.[20] However, *in aqueous solution*, short, strong hydrogen bonds between potentially hydrogen bonding solutes are unknown. For example, although the ΔH for formation of the $(F–H–F)^-$ hydrogen bond is 60 kcal/mol in the gas phase,[55] in aqueous solution, the ΔG for formation of this hydrogen bond is ~0 kcal/mol ($\log K_{eq} = 0.598$).[56] Stahl and Jencks developed an equation that relates the strengths of hydrogen bonds *in water* to the pK_as of the donor and acceptor; from their equation, hydrogen bonds in water are expected to be comparatively weak (≤2 kcal/mol), with the strongest hydrogen bonds requiring an interaction involving either a very strong acid or a very strong base.[57] Perrin has attributed the disruption of short, strong hydrogen bonding in the monoanions of dicarboxylic acids in water to the rapidly fluctuating hydrogen bonding interactions between the hydrogen bond donor and acceptor with neighboring water molecules.[20e] Apparently for these reasons, the importance of short, strong hydrogen bonds in enzyme-catalyzed reactions has been largely ignored or discounted by bioorganic chemists and mechanistic enzymologists, with the only exceptions being a recent essay on the importance of the low fractionation factors of short, strong hydrogen bonds (<0.5) in the interpretation of kinetic isotope effects in enzymatic reactions[22] and the discussion in our previous article.[10]

However, crystallographic studies almost always reveal both that active sites are sequestered from bulk solvent and that ordered hydrogen bonding interactions exist between a limited number of water molecules and functional groups in active sites. In these relatively polar environments that lack the disorder that characterizes bulk aqueous solvent and leads to the disruption of the short, strong hydrogen bond,[20e] we propose that short, strong hydrogen bonds can be formed in enzyme active sites and that the strengths of these hydrogen bonds are of sufficient magnitude to reduce the $\Delta G°$ for concerted general acid–general base catalyzed reactions.

In nonaqueous solvents (e.g., methanol, DMSO, DMF, acetonitrile, benzonitrile, and CH_2Cl_2), the existence of short, strong hydrogen bonds is usually diagnosed by IR (characteristic vibrational frequencies at 1600 cm^{-1})[58] and/or NMR (hydrogen isotope effects on the chemical shift of the hydrogen bonded proton)[59] spectroscopies. In these solvents, the $\Delta G°$s for the formation of short, strong hydrogen bonds can be quantitated by measuring stability constants between hydrogen bond donors and acceptors. For example, in benzonitrile, the $\Delta G°$s for formation of various substituted phenol–phenolate homocomplexes (ΔpK_a between the donor and acceptor is 0) can approach –7.0 kcal/mol.[23] However, in these intermolecular reactions, the $\Delta G°$s for formation of the short, strong hydrogen bonds necessarily include unfavorable entropic requirements for association of the donor and acceptor molecules.

The unfavorable entropic contribution to formation of short, strong hydrogen bonds in intermolecular reactions can be reduced by measuring the effects of intramolecular hydrogen bonding on the pK_as of dicarboxylic acids.[60] In the case of the monoanion of phthalic acid, a short, strong intramolecular hydrogen bond between the carboxylate anion and the carboxylic acid is not observed in water. However, in nonaqueous solvents, the $\Delta G°$ for formation of an intramolecular hydrogen bond decreases from –1.8 kcal/mol in methanol to –4.6 kcal/mol in DMSO and –7.5 kcal/mol in acetonitrile.[24] Since, in all of these solvents, some hydrogen bonding competition between the solute monoanion and the solvent can occur, we conclude that the strengths of short, strong hydrogen bonds can exceed 7.5 kcal/mol in the solvent excluded and ordered environments of enzyme active sites, with values of >20 kcal/mol derived from calculations and observed in the gas phase[20] establishing an upper limit on the strengths of these hydrogen bonds. Differential hydrogen bond strengths (between the general acid catalyst and the substrate and intermediate) of approximately 7 kcal/mol are adequate for understanding the rates of typical enzyme-catalyzed reactions of carbon acids (*vide infra*).

Thus, contrary to the expectations derived from the simple thermodynamic arguments presented in the previous section, we suggest that the conjugate bases of the general acidic catalysts present in the active sites of triose phosphate isomerase, Δ^5-ketosteroid isomerase, citrate synthase, and mandelate racemase *are* able to significantly stabilize the enolic intermediates by the formation of short, strong hydrogen bonds. While the strengths of these hydrogen bonds are critically dependent upon the ΔpK_a between the donor and acceptor,[20] the pK_as of the general acid groups are closely matched to those of the OH groups of the enolic intermediates, and the hydrogen bonds that would be formed in the active sites of triose phosphate isomerase $(O–H–N)^-$, Δ^5-ketosteroid isomerase $(O–H–O)^-$, citrate synthase $(O–H–N)^-$, and mandelate racemase $(O–H–O)^-$ would all possess a net negative charge. This should enhance the importance of this overlooked mechanism for reducing $\Delta G°$ (i.e., differential stabilization of the enolic intermediate relative to the substrate). The necessity for closely matched pK_as precludes the formation of short, strong hydrogen bonds between the general acidic catalyst and the carbonyl group of the keto tautomer of the substrate.

At least four pieces of experimental evidence are available that support our proposal that strong hydrogen bonds stabilize enolic intermediates in enzyme-catalyzed reactions:

(1) Mildvan and his co-workers have characterized the kinetic properties of the Y14F mutant of Δ^5-ketosteroid isomerase.[41b] The k_{cat} using 5-androstene-3,17-dione as the substrate was reduced $10^{4.7}$-fold relative to that of the wild type by this substitution for the general acidic catalyst. If the reduction in k_{cat} reflects a decreased stabilization of the dienolic intermediate (i.e., a smaller reduction in $\Delta G°$), the differential strength of the hydrogen bonds between the general acidic catalyst and the substrate carbonyl oxygen and the OH group of the dienolic intermediate is 6.6 kcal/mol, approximately that we have associated with short, strong hydrogen bonds in solvent-excluded, ordered environments.

(2) Pollack and his co-workers have noted that the pK_a of equilenin (~9 in solution), a phenol analog of the dienol intermediate in the reaction catalyzed by Δ^5-ketosteroid isomerase, is depressed by ≥5.5 pK_a units (≥7.7 kcal/mol) to ≤3.5 when it is bound in the active site.[61] That the structure and pK_a of this inhibitor are similar to those of the dienol intermediate may provide a rare example of an intermediate analog forming a strong hydrogen bond with the active site electrophilic residue (Tyr 14; $pK_a > 10.9$).[41a] With most enzymes, design of an intermediate analog that retains the geometry and pK_a of the OH group of the enol intermediate may be difficult, if not impossible.

(3) In unpublished work from one of our laboratories, substitution of glutamine for the putative electrophilic catalyst Glu 317 in the active site of mandelate racemase (E317Q) decreases k_{cat} by ~10^4-fold (~6 kcal/mol) without detectably altering the X-ray structure of the enzyme with an inhibitor bound in the active site.[62] Since the X-ray structures of both wild-type mandelate racemase and the E317Q mutant reveal a hydrogen bond distance of 2.7 Å between Glu 317/Gln 317 and the carboxylate oxygen of an analog of mandelate, the formation of a short, strong hydrogen bond during enolization of mandelate

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catalyzed by the wild-type racemase is suggested. We favor the interpretation that the reduction in k_{cat} for E317Q reflects a decrease in the differential stabilization of the enolic intermediate *vs* the keto tautomer of the substrate (i.e., a smaller reduction in $\Delta G°$) by increasing the ΔpK_a between the OH group of the enol tautomer of the substrate carbon acid and the general acidic catalyst.

(4) Knowles and his co-workers have characterized the kinetic properties of the H95Q mutant of triose phosphate isomerase.[63] The values for the k_{cat}s characterizing the isomerization of both glyceraldehyde 3-phosphate and dihydroxyacetone phosphate were both reduced by $\geq 10^2$ by this substitution for the general acidic catalyst. While the disruption of a short, strong hydrogen bond should be expected to reduce k_{cat} by more than this amount, mechanistic analysis of the reaction catalyzed by the mutant enzyme suggested that the mechanism had been subtly perturbed, with Glu 165 perhaps catalyzing all of the proton-transfer reactions necessary to accomplish isomerization. The structure of the wild-type isomerase with the tight binding inhibitor phosphoglycolohydroxamate bound in the active site[39b] has been compared with that of the same inhibitor bound in the active site of H95Q.[64] In the wild-type structure,[39b] the hydrogen bonding distance between the imidazole group of His 95 and the anionic oxygen of the inhibitor is 2.7 Å; in the mutant structure,[64] the distance between the carboxamide group of Gln 95 and the anionic oxygen of the inhibitor is 3.2 Å. Assuming that phosphoglycolohydroxamate is an accurate structural analog of the enediol(ate) intermediate, the hydrogen bond observed for the wild-type enzyme is, within error, the distance that would be expected for a short, strong hydrogen bond; in contrast, the hydrogen bond distance observed for H95Q would require structural reorganization of the active site to allow formation of a short, strong hydrogen bond.

Dependence of Transition State Structure on $\Delta G°$ and $\Delta G^{\ddagger}_{int}$

Since the pK_as of the general acidic catalysts and of the OH groups of the enol tautomers of the substrate carbon acids are matched in enzyme active sites, we proposed that the transition states for the enzyme-catalyzed reactions resemble the enolic intermediates. We further proposed that matched pK_as can provide a significant reduction in $\Delta G°$ by the formation of a short, strong hydrogen bond. We now return to Marcus formalism to understand how a late transition state can be achieved and $\Delta G^{\ddagger}$ can be decreased.

The position of the transition state on the reaction coordinate, $x^{\ddagger}$, is predicted to depend on both $\Delta G°$ and $\Delta G^{\ddagger}_{int}$:

$$x^{\ddagger} = 0.5 + \Delta G°/8\Delta G^{\ddagger}_{int} \qquad (2)$$

From eq 2 as $\Delta G°$ increases, the value for $x^{\ddagger}$ increases toward unity. Thus, increases in $x^{\ddagger}$ are accompanied by increases in $\Delta G^{\ddagger}$, i.e., as $\Delta G°$ becomes larger, the rate of the reaction decreases although the transition state occurs later on the reaction coordinate. This dependence of $x^{\ddagger}$ on $\Delta G°$ was first qualitatively predicted by Hammond.[65] Since a late transition state can be achieved only when $\Delta G°$ is very endergonic, the late transition states *and* the rapid rates of enzyme-catalyzed reactions *cannot* simultaneously be satisfactorily explained by increases in $\Delta G°$.

From eq 2, *irrespective of the value for* $\Delta G°$, as $\Delta G^{\ddagger}_{int}$ decreases to its lower limit ($\Delta G°/4$), the value for $x^{\ddagger}$ increases to unity. Thus, increases in $x^{\ddagger}$ are accompanied by decreases in $\Delta G^{\ddagger}$, i.e., as $\Delta G^{\ddagger}_{int}$ becomes smaller, the rate of the reaction increases and the transition state occurs later on the reaction coordinate. Both the late transition states *and* the rapid rates of the enzyme-catalyzed reactions *can* simultaneously be explained if $\Delta G^{\ddagger}_{int}$ can approach its lower limit, $\Delta G°/4$.

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Qualitative Prediction of the Effects of Electrophilic Catalysis on the Magnitude of $\Delta G^{\ddagger}_{int}$ for Formation of Enolic Intermediates

Although we have proposed that reductions in both $\Delta G^{\ddagger}_{int}$ as well as $\Delta G°$ should be able to explain the rates of enzyme-catalyzed abstraction of the α-protons of carbon acids, we have not yet provided insight into the origin of $\Delta G^{\ddagger}_{int}$ and the potential magnitude of its reduction in an active site. Although we are able to provide reasonable estimates of the amount by which $\Delta G°$ can be reduced by the formation of short, strong hydrogen bonds,[20,23,24] we cannot quantitatively predict the amount by which $\Delta G^{\ddagger}_{int}$ can be reduced in an enzyme active site. The reason is that $\Delta G^{\ddagger}_{int}$ measures free energy changes associated with formation of a transition state, a species whose structure cannot be physically characterized but only inferred from $\Delta G^{\ddagger}$ and $\Delta G°$. Solvation effects and changes in hybridization are usually considered to dominate $\Delta G^{\ddagger}_{int}$.[15,38] For example, in base-catalyzed formation of an enolate anion, the usual reference reaction for understanding the rates of enzyme-catalyzed reactions,[8] when the keto tautomer of a carbon acid is converted to the enolate anion, structural reorganization of both the carbon skeleton and of the solvent interacting with the carbonyl oxygen of the substrate and the enolate oxygen of the product must occur.

Can we *qualitatively* predict the effects of electrophilic catalysts on $\Delta G^{\ddagger}_{int}$ in an enzymatic reaction? Since $\Delta G^{\ddagger}_{int}$ is a free energy change, it is composed of enthalpic and entropic components:

$$\Delta G^{\ddagger}_{int} = \Delta H^{\ddagger}_{int} - T\Delta S^{\ddagger}_{int} \qquad (7)$$

With this partitioning, we can associate $\Delta H^{\ddagger}_{int}$ with the ΔH required for changes in bonding (hybridization) and with the ΔH associated with the changing interactions of the substrate and transition state with solvent molecules. In nonenzymatic base-catalyzed formation of an enolate anion, we can associate $\Delta S^{\ddagger}_{int}$ with the entropic requirements for changes in the orientation of solvent molecules that necessarily occur as the electron density increases on the carbonyl oxygen as the α-proton is abstracted. While the sign and magnitude of $\Delta H^{\ddagger}_{int}$ may be difficult to predict, we can predict that $\Delta S^{\ddagger}_{int}$ for the nonenzymatic base-catalyzed reaction will be negative since an increase in electron density on the carbonyl oxygen as it is converted to an (anionic) enolate oxygen is expected to require ordering of solvent molecules to stabilize the developing anionic charge. Since, by definition, $\Delta G^{\ddagger}_{int}$ is positive, the $T\Delta S^{\ddagger}_{int}$ term in eq 7 may dominate $\Delta G^{\ddagger}_{int}$ for the nonenzymatic reaction.

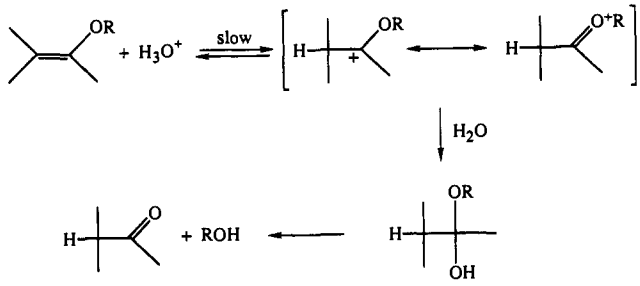
In concerted general acid–general base catalyzed enolization of a substrate carbon acid in an active site, the active site may not be able to influence the enthalpy associated with changes in bonding (hybridization). However, hydrogen bonded networks consisting of substrate functional groups, active site functional groups, and *ordered* water molecules are frequently observed in enzyme active sites by crystallographic studies. Since enzymes exclude bulk solvent from the active site and use *prepositioned* active site functional groups and/or ordered water molecules to "solvate" the reactants, transition states, and reaction intermediates, the contribution of both the component of $\Delta H^{\ddagger}_{int}$ associated with solvent interactions and the entire $\Delta S^{\ddagger}_{int}$ term may differ for the enzymatic *vs* the nonenzymatic reactions. In particular, while the nonenzymatic base-catalyzed reaction will involve the necessary ordering of solvent molecules in response to the developing increase in electron density on the carbonyl oxygen in the transition state for enolate anion formation, the defined positioning of the active site electrophilic catalyst as well as other hydrogen bonding groups (including ordered water molecules) that interact with the substrate, transition state, and enol may require little, if any, repositioning as the enolic intermediate is formed.[15] Thus, $\Delta S^{\ddagger}_{int}$ is expected to be less negative for the concerted enzymatic reaction than for the nonenzymatic base-catalyzed reaction, thereby providing a mechanism for reducing $\Delta G^{\ddagger}_{int}$.

Experimental Assessment of the Effect of a Prepositioned Electrophilic Catalyst on the Value for $\Delta G^{\ddagger}_{int}$

The qualitative reasoning in the previous section argues that the defined structures of active sites should be able to reduce $\Delta G^{\ddagger}_{int}$. Is there an experimental measure of the amount by which $\Delta G^{\ddagger}_{int}$ can be reduced by the defined prepositioning of general acidic or hydrogen bonding groups in an enzyme active site to "solvate" the negative charge that develops on the carbonyl oxygen either before or as the α-proton is abstracted in the concerted iterative process?

If $\Delta S^{\ddagger}_{int}$ dominates $\Delta G^{\ddagger}_{int}$, then $\Delta G^{\ddagger}_{int}$ should be reduced if the structure of the carbon acid provides its own "solvation" of the developing charge on the carbonyl oxygen. One approach by which such solvation can be provided in nonenzymatic reactions is to position a positive charge on the carbonyl oxygen so that no solvent reorganization is required to stabilize the developing negative charge. We propose that both the acid-catalyzed hydrolysis of enol ethers and the base-catalyzed abstraction of the α-protons of iminium ions provide reasonable reactions to quantitate the importance of $\Delta S^{\ddagger}_{int}$ in determining $\Delta G^{\ddagger}_{int}$. In both of these reactions, a full positive charge is localized on the carbonyl or imine group of the substrate carbon acid from which the α-proton is abstracted.

The acid-catalyzed hydrolysis of enol ethers occurs *via* rate-determining protonation of the vinyl carbon atom, subsequent rapid attack of water on the oxocarbonium ion, and finally loss of alcohol to generate the carbonyl product:

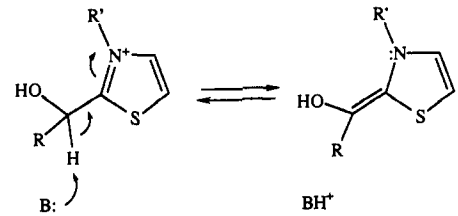


The first step in this mechanism can be used to characterize the importance of $\Delta S^{\ddagger}_{int}$ in determining the value of $\Delta G^{\ddagger}_{int}$ for the abstraction of the α-proton from a carbon acid.

Kresge[25] and Toullec[26] studied the effects of varying both the acid catalyst[25a] and the enol ether substrate[25b,26] on the rate of enol ether hydrolysis and used Marcus formalism to obtain $\Delta G^{\ddagger}_{int}$ for the rate-determining step. The values obtained in these studies are 4.0 ± 1.2 kcal/mol (hydrolysis of ethyl isopropenyl ether),[25a] 2.1 ± 0.3 kcal/mol (hydrolysis of ethyl cyclopentenyl ether),[25a] 4.9 ± 1.2 kcal/mol (isotope effects of acid-catalyzed hydrolysis of a series of enol ethers),[25b] and ~3.6 kcal/mol (hydrolyses of α-methoxystyrenes).[27] Within the estimated errors, the individual values for $\Delta G^{\ddagger}_{int}$ are similar, thereby indicating that Marcus formalism adequately describes the reaction coordinates for carbon-protonation of enol ethers.[66] Since $\Delta G^{\ddagger}_{int}$ is necessarily identical for the forward and reverse reactions proceeding through the same transition state, these values approximate those for $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton from a carbonyl-protonated carbon acid. Since $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton is 12 kcal/mol when no mechanism is available to reduce $\Delta S^{\ddagger}_{int}$ (*vide infra*), a full positive charge on the carbonyl oxygen of a carbon acid can reduce $\Delta G^{\ddagger}_{int}$ by ~8.4 kcal/mol.

Washabaugh and his co-workers have studied the kinetics of proton abstraction from 2-(1-hydroxyethyl)thiamin,[27] an analog of an intermediate in thiamin pyrophosphate-mediated reactions of α-ketoacids.

(66) In contrast to the negligible values for the work terms either assumed or deduced for formation of enols[28b] or enolate anions,[29,30] the work terms for these reactions were concluded to be larger (~10 kcal/mol) than $\Delta G^{\ddagger}_{int}$ since the dependence of $\Delta G^{\ddagger}$ on ΔG° revealed a systematic deviation from eq 3 that could be described by a significant work term.



The quaternary iminium group of this reactant is also a model for a reaction in which the developing negative charge that accompanies abstraction of the α-proton is "presolvated" by the full positive charge of the quaternary iminium group. Although a value for $\Delta G^{\ddagger}_{int}$ was not reported for this reaction, general base-catalyzed abstraction of the α-proton is characterized by a Brønsted β value of ≥ 0.9. This behavior is characteristic of normal (heteroatom) acids for which the value of $\Delta G^{\ddagger}_{int}$ is ≤ 3 kcal/mol.[11] Thus, the β value implies that $\Delta G^{\ddagger}_{int}$ can be reduced by ≥ 9 kcal/mol by the quaternary iminium group.

Thus, we propose that $\Delta G^{\ddagger}_{int}$ for abstraction of the α-proton can be reduced by interactions of the carbonyl group with prepositioned hydrogen bonding groups and/or general acidic catalysts. In the concerted processes we have proposed for enzyme-catalyzed reactions, $\Delta G^{\ddagger}_{int}$ will have a value of less than 12 kcal/mol and more than 3.6 kcal/mol, with the exact value being dependent on the exact position of the general acidic catalyst and/or hydrogen bonding group relative to the carbonyl group of the substrate carbon acid in the active site. Accordingly, in the enzyme-catalyzed reactions, we expect that $\Delta G^{\ddagger}$ can be decreased from the value that describes the nonenzymatic reactions by a reduction in $\Delta G^{\ddagger}_{int}$.

We emphasize that the reduction in $\Delta G^{\ddagger}_{int}$ allows the rate of transfer of the α-proton from the substrate to an active site general basic catalyst to be determined primarily by ΔG°, i.e., the substrate carbon acid can be transformed to a normal acid if $\Delta G^{\ddagger}_{int}$ can be reduced toward $\Delta G^{\circ}/4$.

Rates of the Enzyme-Catalyzed Reactions Can Be Quantitatively Explained

In the absence of differential stabilization of the enolic intermediate, we predict that the magnitude of ΔG° for concerted general acid–general base catalyzed enolization of a carbon acid is 18–22 kcal/mol (*vide infra*). From eq 3, if $\Delta G^{\circ} = 20$ kcal/mol and $\Delta G^{\ddagger}_{int} = 12$ kcal/mol, $\Delta G^{\ddagger}$ is expected to be 24.1 kcal/mol. Thus, $\Delta G^{\ddagger}$ must be reduced by 10.1 kcal/mol to explain the rates of a typical enzyme-catalyzed reaction ($\Delta G^{\ddagger} = 14$ kcal/mol). If we assume that the general acidic catalyst in the active site can both decrease ΔG° and reduce $\Delta G^{\ddagger}_{int}$ toward the lower limit allowed by Marcus formalism, $\Delta G^{\circ}/4$, we now demonstrate that the reduction in $\Delta G^{\ddagger}$ from 24.1 kcal/mol to 14 kcal/mol can be *quantitatively* explained by the analysis we have described.

We assume that ΔG° can be reduced from 20 to 13 kcal/mol in the active site, i.e., the differential stabilization of the enolic intermediate relative to the substrate carbon acid by hydrogen bonding is 7 kcal/mol. This necessary difference between the strength of the weak hydrogen bond between the general acidic catalyst and the carbonyl group of the substrate carbon acid and the short, strong hydrogen bond between the enolic intermediate and the conjugate base of the general acidic catalyst is similar to the strengths of the hydrogen bonds measured for substituted phenol–phenolate homoconjugates[23] and the monoanions of dicarboxylic acids[24] in nonaqueous solvents.

If $\Delta G^{\circ} = 13$ kcal/mol and $\Delta G^{\ddagger} = 14$ kcal/mol, $\Delta G^{\ddagger}_{int} = 5.62$ kcal/mol. Thus, $\Delta G^{\ddagger}_{int}$ need not be reduced to its lower limit ($\Delta G^{\circ}/4 = 3.25$ kcal/mol) to explain the observed rates of the enzyme-catalyzed reactions. However, this reduction in $\Delta G^{\ddagger}_{int}$ does allow $\Delta G^{\ddagger}$ to be determined almost entirely by ΔG°.

$\Delta G^{\ddagger}_{int}$s for Enzyme-Catalyzed Reactions

Experimental verification of a reduced value for $\Delta G^{\ddagger}_{int}$ in an enzyme active site is desirable but will be difficult to achieve.

One approach would be to determine the slope of a plot of $\Delta G^{\ddagger}$ *vs* ΔG° since the slope of this plot (the Brønsted β) is related to $\Delta G^{\ddagger}_{int}$ by eq 4. However, only in special instances, e.g., by using ring-substituted derivatives of mandelate in the reaction catalyzed by mandelate racemase or by changing the pK_as of the active site general acidic and general basic catalysts by unnatural mutagenesis,[67] will it be possible to vary ΔG° in enzyme active sites.

With less reliability, $\Delta G^{\ddagger}_{int}$ could be obtained from values for $\Delta G^{\ddagger}$ and ΔG° for a single reaction in place of a series of reactions in which ΔG° is varied. Unfortunately, values for the ΔG°s for formation of enolic intermediates are not yet available for any enzyme-catalyzed reactions, including the isomerization reactions catalyzed by both triose phosphate isomerase[5] and Δ^5-ketosteroid isomerase,[68] despite attempts to quantitatively define the reaction coordinates for these reactions.

Limitations on the Value of $x^{\ddagger}$ in Enzyme-Catalyzed Reactions

The description of the reaction coordinate by eq 1 allows the value for $x^{\ddagger}$ to equal 1 if $\Delta G^{\ddagger}_{int}$ is reduced to the lower limit, $\Delta G^{\circ}/4$. We now consider just how close $x^{\ddagger}$ can approach this limit in enzyme-catalyzed reactions.

In principle, the conversion of the keto tautomer of the substrate carbon acid to the keto tautomer of the product carbon acid could be concerted, i.e., if $\Delta G^{\ddagger}_{int} = \Delta G^{\circ}/4, x^{\ddagger} = 1$. In this case, Marcus formalism suggests that the *transition state* for the concerted general acid–general base catalyzed proton abstraction reaction would have the character of an enol, but an enol *per se* would necessarily cease to be a transiently stable intermediate.[69] However, in the reactions catalyzed by both triose phosphate isomerase and mandelate racemase, the hydrogen bonded enol cannot be a transition state since it must have sufficient lifetime to undergo exchange reactions. For triose phosphate isomerase, the acidic proton of Glu 165 must be able to exchange with solvent.[70] For mandelate racemase, the conjugate acid of Lys 166 must be minimally able to undergo rotation about the C–N bond.[44] However, the hydrogen bonded enol must be close in structure and energy to the transition state since the pK_as of the OH group of the enol tautomer and of the electrophilic catalyst are closely matched. The $\Delta G^{\ddagger}$ for ketonization of the enolic intermediate to form product (tautomerized or racemized substrate) need not be very large to allow solvent exchange or bond rotation to compete with reketonization, i.e., these processes need not have $\Delta G^{\ddagger}$s larger than 1 kcal/mol. Thus, these proton abstraction reactions cannot be concerted, and $x^{\ddagger}$ cannot equal 1.

However, we propose that while $x^{\ddagger}$ can approach 1 it is unlikely to equal 1 for *any* enzyme-catalyzed reaction that involves interconversion of the keto tautomers of substrate and product carbon acids (e.g., racemization, tautomerization, elimination, carboxylation, or Claisen condensation reactions). If $x^{\ddagger}$ were equal to 1, the enzyme-catalyzed reaction would be concerted. In a concerted mechanism, the bond breaking and the bond making processes required to convert the keto tautomer of the substrate carbon acid to the keto tautomer of the product carbon acid would both necessarily occur in the single transition state for the reaction. Since the transition state for the reaction is symmetrical for the bond making and breaking processes that interconvert the keto tautomers of the substrate and product carbon acids (e.g., consider the reaction catalyzed by mandelate racemase), the charge on the carbonyl oxygen cannot increase as the transition state is achieved. Without development of negative charge on the carbonyl oxygen, the pK_a of the conjugate acid of this oxygen cannot increase to match those of the active site general acidic catalysts that have been observed in enzyme active sites, thereby preventing the decrease in ΔG° by the formation of the short, strong hydrogen bond that is necessary to achieve the observed $\Delta G^{\ddagger}$ for the enzyme-catalyzed reactions. We, therefore, propose that the reduction in ΔG° that is required to achieve the observed $\Delta G^{\ddagger}$ can be attained only if a stabilized enolic intermediate is on the reaction coordinate, i.e., the enolization and reketonization processes are distinct, i.e., stepwise, reactions.[71]

Our proposal that enzymatic reactions are expected to be stepwise agrees with the experimental observations that the mechanisms of the enzyme-catalyzed reactions are almost always[71] stepwise, not concerted, e.g., the mechanisms of enzyme-catalyzed β-elimination reactions.[10] Thus, while $x^{\ddagger}$ must approach 1 to take advantage of electrophilic catalysis, it cannot equal 1.

Effect of Short, Strong Hydrogen Bonding of the Enol Intermediate on the pK_a of the α-Proton of the Carbon Acid

In our earlier analysis of the effect of protonation of the carbonyl group on the pK_a of the α-proton, we concluded that the effects could be large (15 pK_a units).[2] However, in this article, we propose that the intermediates in the enzyme-catalyzed reactions are hydrogen bonded species (enolic intermediates)[16] in which the proton of the general acidic catalyst is *shared* by both the conjugate base of the general acidic catalyst and the conjugate base of the OH group of the enol tautomer (the enolate anion). Thus, the extent of proton transfer to the carbonyl group from the general acidic catalyst will be approximately 0.5, so the reduction in the pK_a of the α-proton in the transition state for the concerted general acid–general base catalyzed enolization reaction is expected to be less than that which accompanies complete protonation of the carbonyl group.

To determine whether the k_{cat}s of the enzyme-catalyzed reactions are consistent with partial protonation of the carbonyl group, we will first assume that the effect of protonation of the carbonyl group on the pK_a of the α-proton is proportional to the extent of protonation of the carbonyl group. We then examine whether this assumption is in accord with the pK_as of the general basic catalysts present in the active site and the observed k_{cat}s of the enzyme-catalyzed reactions.

In the transition state that we propose for enzyme-catalyzed enolization, the extent of protonation of the carbonyl group will be approximately one-half. With the assumption described in the previous paragraph, we predict that the pK_a of the α-proton of the carbon acid substrate is reduced by $\sim$7 pK_a units to $\sim$13 for aldehydes, ketones, and thioesters and to $\sim$15 for mandelic acid in the transition states for the enzyme-catalyzed reactions. Since a kinetic consequence of reducing $\Delta G^{\ddagger}_{int}$ is to make $\Delta G^{\ddagger}$ primarily dependent on ΔG°, the rate of the concerted general acid–general base catalyzed reaction would be determined by the difference in pK_as between the α-proton of the substrate carbon acid in the transition state and the conjugate acid of the general basic catalyst (ΔpK_a). If $\Delta G^{\ddagger} \approx 14$ kcal/mol ($k_{cat} \approx 10^3\ s^{-1}$), from transition-state theory $\Delta pK_a \approx 10$.

(67) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182.

(68) Hawkinson, D. C.; Eames, T. C. M.; Pollack, R. M. *Biochemistry* **1991**, *30*, 10849.

(69) Jencks, W. P. *Chem. Soc. Rev.* **1981**, *10*, 345.

(70) Fisher, L. M.; Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5621.

(71) The partial increase in the electron density on the carbonyl oxygen that would occur in a reaction in which the bond breaking and making reactions have partial asynchronous character could allow differential stabilization of the transition state by hydrogen bonding to an active site general acidic catalyst (according to Marcus formalism, by decreasing $\Delta G^{\ddagger}_{int}$), thereby accomplishing catalysis of the reaction. Such catalysis has been observed in the addition of nucleophiles to carbonyl groups: Fishbein, J. C.; Baum, H.; Cox, M. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1987**, *109*, 5790. Presumably, the degree of asynchronicity in bond breaking and making reactions is determined, at least in part, by the pK_a of the general acidic catalyst positioned adjacent to the carbonyl oxygen of the substrate: if the pK_a is matched to that of the conjugate acid of the enolic intermediate, the reaction will be stepwise (completely asynchronous) with formation of an enolic intermediate; if the pK_as are not matched, the bond breaking and making reactions will be partially asynchronous or, in the unlikely extreme, truly concerted. This distinction may explain the conclusion that the β-elimination reaction catalyzed by crotonase apparently does not occur *via* a stepwise mechanism: Bahnson, B. J.; Anderson, V. L. *Biochemistry* **1989**, *28*, 4173; **1991**, *30*, 5894.

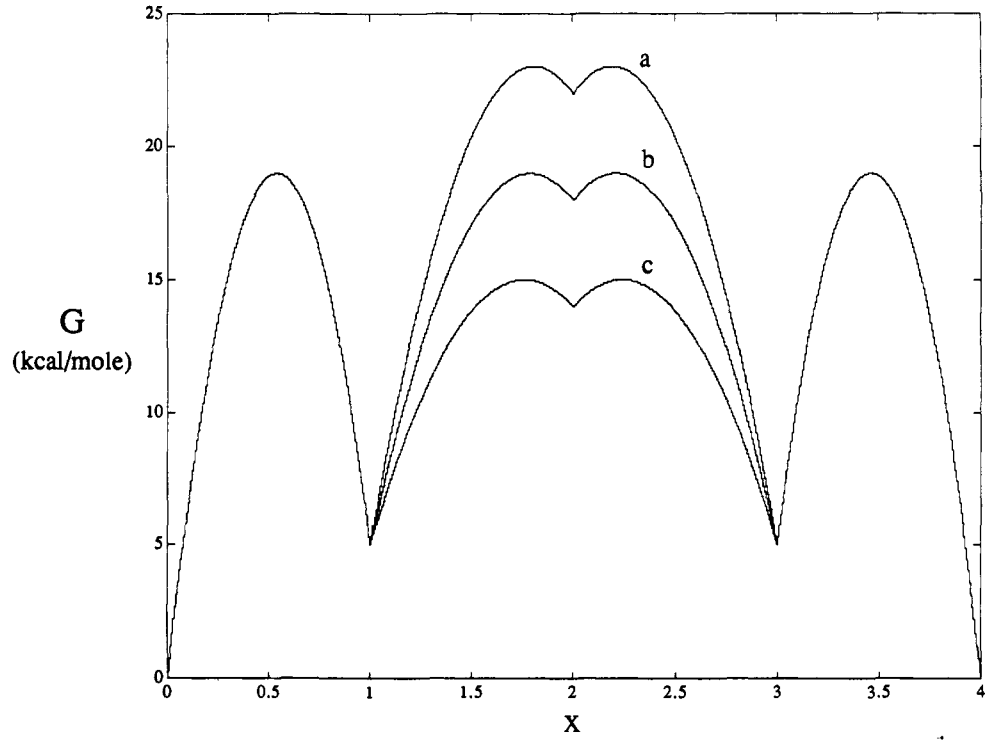


Figure 2. Dependence of G on the position of the reaction coordinate, x, for binding of the substrate carbon acid, enolization, reketonization of the enolic intermediate, and dissociation of the product carbon acid. The free substrate carbon acid is at $x = 0$; the bound substrate carbon acid is at $x = 1$; the bound enolic intermediate is at $x = 2$; the bound product carbon acid is at $x = 3$; and the free product carbon acid is at $x = 4$. For all four reaction profiles, the free energies of the bound substrate and bound product are equal, and the free energies of the transition state for substrate dissociation (first transition state) and product dissociation (fourth transition state) are 14 kcal/mol. The parameters used for enolization of the substrate carbon acid (second transition state) and enolization of the product carbon acid (third transition state) are as follows: profile a, $\Delta G^\circ = 17$ kcal/mol or $\Delta pK_a = 12.5$, $\Delta G^\ddagger_{int} = 6.88$ kcal/mol, $\Delta G^\ddagger = 18$ kcal/mol; profile b, $\Delta G^\circ = 13$ kcal/mol or $\Delta pK_a = 9.6$, $\Delta G^\ddagger_{int} = 5.62$ kcal/mol, $\Delta G^\ddagger = 14$ kcal/mol; profile c, $\Delta G^\circ = 9$ kcal/mol or $\Delta pK_a = 6.6$, $\Delta G^\ddagger_{int} = 4.34$ kcal/mol, $\Delta G^\ddagger = 10$ kcal/mol.

The pK_as of the general basic catalysts in the active sites of triose phosphate isomerase (Glu 165; $pK_a \sim 6$),[52] ketosteroid isomerase (Asp 38; $pK_a < 5$),[41b] citrate synthase (Asp 375; $pK_a \sim 6.5$),[43] and mandelate racemase (Lys 166/His 297; $pK_a \sim 6$)[44] are all ~ 7–9 pK_a units less than those predicted for the α-protons of the substrate carbon acids assuming one-half protonation of the carbonyl group in the transition state. Thus, the observed k_{cat}s of the enzyme-catalyzed reactions are consistent with our assumption that the pK_a of the α-proton in the transition state is proportional to the expected extent of protonation of the carbonyl group in the transition state (approaching one-half).

Importance of the pK_a of the Conjugate Acid of the General Basic Catalyst

The analysis in the previous section suggests that the rates of the enzyme-catalyzed reactions can be increased by increasing the pK_a of the conjugate acid of the general basic catalyst since this would decrease both ΔG° (ΔpK_a) and, therefore, $\Delta G^\ddagger$. Why are the pK_as of the conjugate acids of the general basic catalysts not greater than those observed in the enzyme-catalyzed reactions?

We propose that the answer to this question is at least 3-fold:

(1) The transition states for the successive enolization and reketonization reactions are not expected to be lower in energy than the transition states for the diffusion-controlled binding of substrate to and/or dissociation of product from the active site. According to the principles for development of enzymatic function described by Albery and Knowles,[72] evolutionary pressure to increase the rates of the chemical steps in an enzyme-catalyzed reaction is possible only if the chemical steps are at least partially rate determining. When the rates of the chemical steps exceed those of the substrate binding and/or product dissociation steps, no further pressure to increase the k_{cat} of the enzyme-catalyzed reaction is possible since the rates of the latter steps are limited by diffusive processes that cannot be altered by the enzyme molecule. Thus, in the concerted general acid–general base catalyzed enolization reactions we have proposed, the pK_a of the conjugate acid of the general basic catalyst is expected to be that which will decrease ΔG° for formation of the hydrogen bonded enolic intermediate so that the free energies of the transition states for the successive enolization and reketonization reactions (as determined primarily by ΔG°) are approximately equal to free energies of the transition states for substrate binding and/or product dissociation.

We illustrate this point with the reaction profiles shown in Figure 2, where the $\Delta G^\ddagger$s for substrate and product dissociation (first and fourth transition states) are each 14 kcal/mol.[73] The reaction profiles were calculated assuming that the $\Delta G^\ddagger$ for ketonization of the enolic intermediate is 1 kcal/mol (also the contribution of $\Delta G^\ddagger_{int}$ to the $\Delta G^\ddagger$ for enolization of the substrate and product carbon acids). In reaction profile b, $\Delta pK_a = 9.6$ and the energies of the transition states for enolization (second transition state) and reketonization (third transition state) are equal to those of the transition states for substrate and product dissociation. If the pK_a of the active site general basic catalyst is decreased (profile a, $\Delta pK_a = 12.5$), both ΔpK_a and ΔG° are increased, and the rate of the overall reaction is determined by the successive enolization and reketonization steps. If the pK_a of the active site general basic catalyst is increased (profile c, $\Delta pK_a = 6.6$), both ΔpK_a and ΔG° are decreased, and the rate of the overall reaction is determined by the substrate binding and product dissociation steps.

(72) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631.

(73) For example, for triose phosphate isomerase, the rates of both substrate and product dissociation from the enzyme are $\sim 10^3$ s^{-1}.[5] From transition state theory, $\Delta G^\ddagger \approx 14$ kcal/mol for these steps. Since the free energies of bound substrate and bound product are approximately equal in the active site of triose phosphate isomerase, the $\Delta G^\ddagger$s for the chemical steps interconverting bound substrate and bound product, i.e., enolization, are not expected to and do not differ significantly from 14 kcal/mol.

(2) If the pK_a of the conjugate acid of the general basic catalyst were comparable to that of the general acidic catalyst, a significant amount of intercatalyst proton transfer could occur, placing the active site acidic and basic catalysts in the incorrect state of protonation for catalysis. This situation is avoided since the pK_as of the general acidic catalyst greatly exceed those of the general basic catalysts in most active sites. Of those enzymatic examples described in this article, only mandelate racemase deviates from this principle. However, in the active site of mandelate racemase binding of the substrate should be possible only if the catalysts are in the correct state of protonation, since the general acidic catalyst (Glu 317) forms a (weak) hydrogen bond to the substrate when it is bound, thereby "enforcing" the general acidic catalyst to be protonated.

(3) If the pK_a of the conjugate acid of the general basic catalyst were greater than 7, the functional group would be protonated and unable to function as a general basic catalyst at neutral pH.

Substrate Isotope Effects[74] in the Enzyme-Catalyzed Reactions

In our mechanism for concerted general acid–general base catalyzed enolization of substrate carbon acids, the pK_a of the α-proton in the transition state (13–15) is significantly greater than the pK_a of the conjugate acid of the general basic catalyst (<7). As a result, the position of this proton in the transition state is expected to be highly unsymmetrical with respect to the donor and acceptor, i.e., the proton is significantly closer to the general basic catalyst than to the α-carbon in the late transition state for proton transfer. This asymmetry in the transition state requires that the intrinsic substrate isotope effect be less than the maximum value that would be observed if the transition state occurred midway on the reaction coordinate and the proton transfer reaction were symmetrical in the transition state.[75]

Isotope effects approaching the maximum possible value are rarely observed in the types of enzyme-catalyzed reactions described in this article. However, since the rates of the (isotopically insensitive) substrate binding and/or product dissociation steps are expected to be comparable to those of enolization of the substrate carbon acid and reketonization to form the product carbon acid, the observed substrate isotope effects are expected to be less than those of the intrinsic isotope effects in the chemical steps since the chemical steps are not cleanly rate determining. Thus, in the absence of information regarding the relative rates of the enolization–reketonization and substrate binding/product dissociation steps, the magnitudes of substrate isotope effects are difficult to interpret. However, that large substrate isotope effects are rarely observed in enzyme-catalyzed reactions does support our prediction that the transition states for transfer of the α-proton to the general basic catalyst are unsymmetrical, i.e., late on the reaction coordinate for the concerted general acid–general base catalyzed reaction.

Solvent Isotope Effects[74] in the Enzyme-Catalyzed Enolization Reactions

Our proposal for the mechanisms of the enzyme-catalyzed enolization reactions requires that a proton be *partially* transferred from the general acidic catalyst to the carbonyl group of the substrate to form a strongly hydrogen bonded enolic intermediate. Can the necessary proton transfer associated with formation of this intermediate be detected by a solvent isotope effect on the k_{cat} for the enzyme-catalyzed reaction?

In many enzyme-catalyzed enolization–reketonization reactions, a solvent-derived proton is incorporated into a C–H bond of the carbon acid product. For example, in the reaction catalyzed by triose phosphate isomerase, facile exchange of the conjugate acid of the general basic catalyst with solvent occurs so that, if the reaction is conducted in D_2O, deuterium is incorporated into the C–H bond of the product (Scheme I).[70] Thus, any solvent isotope effect that could be measured is a composite of the isotope effect on transfer of a deuteron to/from the carbonyl group by the general acidic catalyst *and* the isotope effect on the transfer of a deuteron from the conjugate acid of the general basic catalyst to the stabilized enolic intermediate to form the product. In the reaction catalyzed by mandelate racemase, a solvent-derived proton is incorporated into the product mandelate since the stabilized enolic intermediate is protonated by a general acidic catalyst that differs from the conjugate acid of the general basic catalyst that generated the enolic intermediate (Scheme IV).[44] The solvent isotope effect that is measured ($k_{H_2O}/k_{D_2O} \approx 4$)[44] is expected to be a composite of the isotope effect on transfer of a deuteron to/from the carbonyl group from the general acidic catalyst *and* the isotope effect on the transfer of a deuteron from the conjugate acid of one of the general basic catalysts to the stabilized enolic intermediate to form the product. Thus, multiple isotopically sensitive steps are frequently represented in the observed solvent isotope effects, and these must be deconvoluted to obtain that for transfer of a proton to/from the carbonyl group that might be diagnostic of the formation of the stabilized enolic intermediate.

However, we predict that the isotope effect for proton transfer to/from the carbonyl group will be small even if formation of the strongly hydrogen bonded enolic intermediate is uniquely rate determining. In our mechanism, the proton of the general acidic catalyst remains partially bound (strongly hydrogen bonded) to the general acidic catalyst in the transition state. Thus, the fractionation factor of the proton in the transition state will lie between the value for the fractionation factor for the proton of the general acidic catalyst weakly hydrogen bonded to the carbonyl group of the substrate carbon acid (≤ 1.0)[76] and the fractionation factor for the same proton in the strongly hydrogen bonded enolic intermediate (0.3–0.5).[20b,22] The former fractionation factor is subject to uncertainty since no data are presently available for weak hydrogen bonds that are excluded from bulk solvent. The latter fractionation factor is also subject to uncertainty since the precise value will depend upon the potential energy function that describes the hydrogen bonded enol.[20b] In any event, the solvent isotope effect is not expected to exceed 2–3 for formation of the strongly hydrogen bonded enolic intermediate and may be significantly less than this value.[77]

For reketonization of the strongly hydrogen bonded enolic intermediate, the fractionation factor for the strongly hydrogen bonded proton of the enolic intermediate is not expected to change significantly in the transition state since the transition state is early on the reaction coordinate for reketonization and resembles the strongly hydrogen bonded enolic intermediate. Thus, in reketonization of the stabilized enolic intermediate, the solvent isotope effect is not expected to differ significantly from unity. Thus, we conclude that large solvent isotope effects are not expected if formation of the strongly hydrogen bonded enolic intermediate is rate determining.

(74) In this article, the term "substrate isotope effect" refers to changes in k_{cat} associated with incorporation of deuterium (or tritium) into a nonexchangeable C–H bond of the substrate. The term "solvent isotope effect" refers to changes in k_{cat} associated with the use of D_2O (or HTO) as solvent. The latter isotope effect includes both medium effects and the chemical effects of incorporation of deuterium (or tritium) into exchangeable positions, e.g., the general acidic catalyst or the conjugate acid of the general basic catalyst.

(75) When $\Delta pK_a = 0$ between carbon acids and heteroatom bases (a symmetrical transition state), the maximum primary deuterium isotope effect ranges from approximately 8 to 10. *Large* increases or decreases in ΔpK_a are necessary to produce large decreases in the primary deuterium isotope effect: (a) Westheimer, F. H. *Chem. Rev.* **1961**, *61*, 265. (b) Klinman, J. P. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; Chapter 4.

(76) Schowen, K. B.; Schowen, R. L. *Methods Enzymol.* **1982**, *64*, 551.

(77) In solution, when $\Delta pK_a = 0$ between normal acids and heteroatom bases (a symmetrical transition state), the maximum primary deuterium isotope effect is approximately 4. *Small* increases or decreases in ΔpK_a are necessary to produce large decreases in the primary deuterium isotope effect. The maximum deuterium isotope effect is less than that observed for carbon acids[75] because proton transfer is never completely rate determining: (a) Bergman, N.-Å.; Chiang, Y.; Kresge, A. J. *J. Am. Chem. Soc.* **1978**, *100*, 5954. (b) Cox, M. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1978**, *100*, 5956.

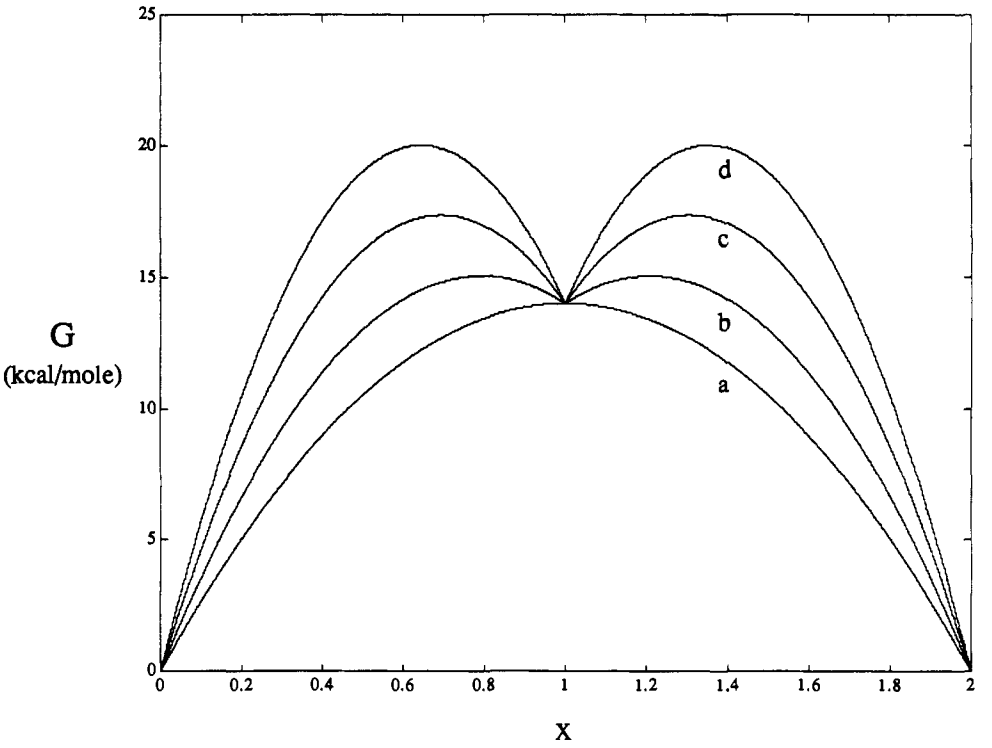


Figure 3. Dependence of G on the position of the reaction coordinate, x, for the successive enolization and ketonization of a carbon acid assuming $\Delta G^\circ = 0$ for interconversion of the substrate carbon acid and the product carbon acid. The keto tautomer of the substrate carbon acid is at $x = 0$; the enolic intermediate is at $x = 1$; and the keto tautomer of the product carbon acid is at $x = 2$. Reaction coordinates are generated for $\Delta G^\circ = 14$ kcal/mol at several values of $\Delta G^\ddagger_{int}$ (a, 3.5; b, 6; c, 9 ; d, 12 kcal/mol). The pairwise values for $x^\ddagger$ and $\Delta G^\ddagger$ (kcal/mol) described by the curves are (1.000, 14.00), (0.792, 15.04), (0.694, 17.36), and (0.646, 20.02), respectively. Note that the reaction described by reaction coordinate a is concerted whereas the reactions described by the remaining reaction coordinates are stepwise.

In the reaction catalyzed by Δ^5-ketosteroid isomerase (Scheme II), little exchange of the conjugate acid of the general basic catalyst, Asp 38, occurs during the course of the reaction. Since the k_{cat} of the reaction catalyzed by the Y14F mutant of the isomerase in which the putative general acidic catalyst has been deleted is reduced by a factor of $10^{4.7}$,[32] general acidic catalysis is important in this reaction. Under conditions in which the substrate isotope effect for the reaction catalyzed by the wild-type isomerase is large ($k_H/k_D = 6.1$), the solvent isotope effect is small ($k_{H_2O}/k_{D_2O} = 1.6$). Thus, the substrate and solvent isotope effects measured for Δ^5-ketosteroid isomerase are in accord with the predictions based upon concerted general acid–general base catalysis to form a strongly hydrogen bonded enolic intermediate.

A Rule for Concerted General Acid–General Base Catalysis in Enzyme-Catalyzed Reactions

The energetics of formation of strongly hydrogen bonded enolic intermediates in enzyme active sites do not satisfy Jenck's "libido rule" for concerted general acid–general base catalysis.[49] On the basis of the analysis presented in this article, the libido rule describes catalysis in reactions in which the transition state for the reaction is midway along the reaction coordinate, not late as observed in enzyme-catalyzed reactions. Since $\Delta G^\ddagger_{int}$ is large for the nonenzymatic enolization of carbon acids (12 kcal/mol), $x^\ddagger$ is expected to be close to midway on the reaction coordinate for most likely values of ΔG°. Since proton transfers from general acidic catalysts and to general basic catalysts must be thermodynamically favorable in aqueous solution[49,50] and, therefore, occur at the reaction coordinate of the transition state, $x^\ddagger$, the pK_a of the general acidic catalyst is expected to be approximately the average of the pK_as of the protonated carbonyl group of the substrate and the OH group of the enol in nonenzymatic reactions. For enzyme-catalyzed reactions, any rule regarding the pK_as required for general acid–general base catalysis must be formulated to reflect that $\Delta G^\ddagger_{int}$ is smaller and, therefore, that the transition states are later on the reaction coordinate.

The mechanisms of the enzyme-catalyzed reactions involve successive enolization and reketonization of the substrate. If $\Delta G^\ddagger_{int}$ can be reduced toward its lower limit, $\Delta G^\circ/4$, the rate of the successive reactions will be determined primarily by ΔG° for formation of the enolic intermediate since $\Delta G^\ddagger$ for reketonization of the enolic intermediate will necessarily be small (Figure 3).[78] Under these conditions, the rate of the reaction can be further increased only if mechanisms are available to reduce ΔG°. Such reductions in ΔG° are expected only if the rates of the proton-transfer steps for enol formation are rate determining,[72] i.e., less than those of substrate binding or product dissociation processes.

We propose the following rule for concerted general acid–general base catalysis for abstraction of the α-protons of carbon acids (enolization) in enzyme active sites:

The rates for concerted general acid–general base catalysis in the defined geometries of enzyme active sites are maximized when

(1) *the pK_a of the general acidic catalyst is matched to that of the OH group of the enol tautomer of the substrate carbon acid and*

(2) *the pK_a of the conjugate acid of the general basic catalyst allows the rate of enolization of the substrate carbon acid to be competitive with the rate of substrate binding to and/or product dissociation from the active site.*

This rule, which we call "the late transition state rule" for concerted general acid–general base catalysis in enzyme active sites, both ensures reductions in ΔG° for the enolization reaction, the primary determinant of $\Delta G^\ddagger$ when $\Delta G^\ddagger_{int}$ is reduced by the active site geometry, and allows the rate of the reaction to be determined by the rates of diffusive processes.

(78) As shown in Figure 3, the transition state for reketonization of the bound enolic intermediate will be early on the reaction coordinate, since by microscopic reversibility the mechanism of the ketonization reaction will be identical to that of the reverse of the enolization reaction.

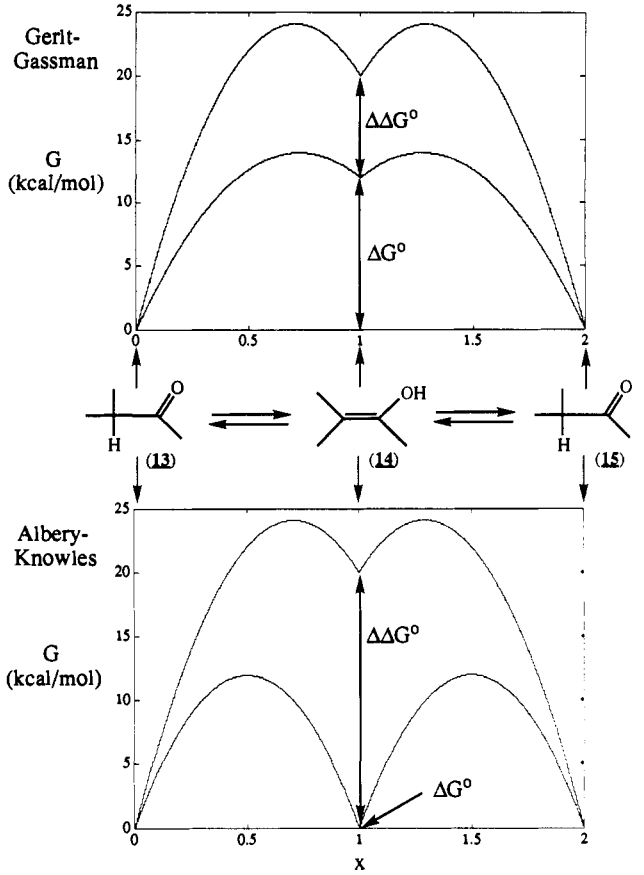


Figure 4. Dependence of G on the position of the reaction coordinate, x, for successive enolization and ketonization reactions of a carbon acid in an enzyme active site as predicted by the Albery–Knowles proposal for optimization of catalytic efficiency (bottom panel) and our "late transition state rule" (top panel). The keto tautomer of the bound substrate carbon acid (**13**) is at $x = 0$; the bound enolic intermediate (**14**) is at $x = 1$; and the bound keto tautomer of the product carbon acid (**15**) is at $x = 2$. In both panels, the interconversion of the substrate carbon acid and the product carbon acid is isoenergetic. The free energy difference between the enolic intermediate and the substrate carbon acid is labeled as $\Delta G°$. In both panels, the upper reaction coordinates are identical and were calculated from eq 1 assuming that $\Delta G^{\ddagger}_{int} = 12$ kcal/mol and $\Delta G° = 20$ kcal/mol; this value for $\Delta G°$ is that calculated from eq 6 for a nonenzymatic reaction involving concerted general acid–general base catalyzed enolization. In the bottom panel, the lower reaction coordinate was calculated from eq 1 assuming that $\Delta G^{\ddagger}_{int} = 12$ kcal/mol and $\Delta G° = 0$ kcal/mol. In the top panel, the lower reaction coordinate was calculated from eq 3 assuming that $\Delta G^{\ddagger}_{int} = 6.65$ kcal/mol and $\Delta G° = 12$ kcal/mol. The free energy difference, $\Delta\Delta G°$, between the enolic intermediates in the upper and lower reaction coordinates in each panel is the stabilization of the enol intermediate required to produce the observed rates of the enzyme-catalyzed reactions.

Implications of Our "Late Transition State Rule" on the Attainment of Catalytic Efficiency in Enzyme Active Sites

In their analysis of the evolution of enzyme function and development of catalytic efficiency,[72] Albery and Knowles proposed three *successive* strategies by which the rates of *reversible* enzyme-catalyzed reactions have been optimized in the course of evolution: (1) uniform binding of substrate, reaction intermediates, and products, (2) differential binding of reaction intermediates; and (3) catalysis of elementary steps. The analysis presented in this article has important implications for the Albery–Knowles proposal regarding optimization of enzymatic efficiency in reversible reactions involving enolization of carbon acids. In the discussion that follows, we emphasize that *we are considering only bound substrate carbon acid, bound enolic intermediate, and subsequently formed bound product carbon acid.* These species are shown in the reaction coordinates of Figure 4 as structures **13**, **14**, and **15**, respectively. In both panels of Figure 4, the substrate carbon acid (**13**) and the product carbon acid (**15**) are approximated to have the same free energies (e.g., *R*- and *S*-mandelates bound in the active site of mandelate racemase[79]); the energy difference designated $\Delta G°$ is the instability of the enolic intermediate (**14**) relative to either the substrate carbon acid or the product carbon acid.

The Albery–Knowles proposal that differential binding of a reaction intermediate will accelerate the rate of an enzyme-catalyzed reaction was based on the Hammond postulate, i.e., decreasing the endergonicity of a reaction will necessarily lower the energy of the transition state and decrease $\Delta G^{\ddagger}$. On the basis of this analysis, Albery and Knowles suggested that optimization of catalysis would occur if the $\Delta G°$s for the reactions interconverting substrate, reaction intermediates, and product bound in the active sites of enzymes all approach 0 (Figure 4, lower panel).

While this expectation has been verified for bound substrate carbon acid and bound product carbon acid in enzyme-catalyzed enolization reactions of carbon acids,[5,68,79] *the concentrations of the reactive enolic intermediates bound in the active sites of these enzymes have not been measured.* In order to meet the requirement that the $\Delta G°$s for the enolization and reketonization reactions also approach 0, the enzyme active site must accomplish substantial differential stabilization of the enolic intermediate (~20 kcal/mol), designated as $\Delta\Delta G°$ in Figure 4 (lower panel), in order to counterbalance the instabilities of the enol tautomers of carbon acids[7] and the unfavorable thermodynamics for transfer of a proton from the general acidic catalyst to the general basic catalyst in enzyme active sites (eq 6). Because the concentrations of the enolic intermediates in enzyme active sites have not yet been measured, critical examination of the Albery–Knowles proposal, which requires that the final stage in the reduction in $\Delta G^{\ddagger}$ will occur *via* stabilization of a transition state that interconverts *a substrate carbon acid (or a product carbon acid) and an enolic intermediate of equal energy* (catalysis of an elementary step), has not been possible.[80] We emphasize, however, that the Albery–Knowles proposal that $\Delta G°$ for interconversion of a bound substrate carbon acid (**13**) and a bound enolic intermediate (**14**) approach 0 in an enzyme active site *requires* that the transition state for the enolization reaction be midway, not late, on the reaction coordinate. This requirement *is not supported* by the matched pK_as of the active site catalysts and the enolic intermediates that have now been observed in several enzyme active sites (*vide infra*).[78]

We do not disagree with the Albery–Knowles proposal that $\Delta G°$ for interconversion of *bound substrate carbon acid (7) and bound product carbon acid (15)* should approach 0 for optimized catalysis. When this $\Delta G°$ approaches 0, the Hammond postulate predicts that the transition states for enolization of the bound substrate carbon acid and for reketonization of the bound enolic intermediate should have similar structures (microscopic reversibility). In this situation, both enolization and reketonization

(79) In the reaction catalyzed by mandelate racemase, both the k_{cat}s and K_ms are approximately equal using either *R*- or *S*-mandelate as the substrate.[3d] Since the proton-transfer reactions are largely rate determining,[6] the equivalence of the K_ms suggests that the $\Delta G°$ relating bound *R*- and *S*-mandelates is 0.

(80) A consequence of a late transition state for formation of the enolic intermediate in an enzyme-catalyzed reaction is that the value for $\Delta G°$ of the bound enolic intermediate *vs* the substrate carbon acid will approach, but cannot equal, the value for $\Delta G^{\ddagger}$ for the enzyme-catalyzed reaction, if the concerted proton transfer reactions involved in enolization of the substrate carbon acid are rate determining. Since the $\Delta G^{\ddagger}$s are ~14 kcal/mol for the enzyme-catalyzed reactions,[3] when $\Delta G°$ approaches this value, the concentrations of the bound enolic intermediates will be small (e.g., $K_{eq} \sim 10^{-8}$ if $\Delta G° = 12$ kcal/mol). A low concentration of the enolic intermediate is catalytically desirable because a greater proportion of the enzyme active sites will be available to bind substrate carbon acid for conversion to product carbon acid *via* the enolic intermediate.

(81) Albery and Knowles formulated their principles[72] at a time (1976) when the only structure available for an active site that catalyzes abstraction of the α-proton of a carbon acid was that of triose phosphate isomerase. Since that time, as discussed in this article, structures for additional active sites have become available as have data regarding the pK_as of enol intermediates and active site functional groups.

are equally rate determining, and the rate of the reaction will be optimized for specific values of ΔG° and $\Delta G^\ddagger_{int}$ for formation of the enolic intermediate.

However, a consequence of our conclusion that the transition states for the enzyme-catalyzed reactions of carbon acids are late on the reaction coordinate is that the ΔG°s for reactions involving both the formation of the bound enolic intermediate (**14**) from the substrate carbon acid (**13**) or the product carbon acid (**15**) *cannot* approach 0 (Figure 4, top panel). It is, however, necessary for these to be reduced from the values calculated from eq 6 for nonenzymatic reactions since these calculated values are too large to explain the observed $\Delta G^\ddagger$s. As noted earlier in our discussion of eq 6, $\Delta\Delta G^\circ$, the necessary reduction in ΔG°, can be as small as 7 kcal/mol if $\Delta G^\ddagger_{int}$ can be reduced from 12 kcal/mol, the value for the nonenzymatic, base-catalyzed reaction, toward 3.6 kcal/mol, the value expected for the enzyme-catalyzed reactions in which the developing negative charge on the carbonyl oxygen is "solvated" by a general acidic or hydrogen bonding group in the defined geometry of the active site.

We propose that reductions in $\Delta G^\ddagger$ are achieved by *coupled* reductions in both ΔG° and $\Delta G^\ddagger_{int}$. In particular, a reduction in $\Delta G^\ddagger_{int}$ accomplishes catalysis of an elementary step, i.e., reducing $\Delta G^\ddagger$ for interconversion of a bound substrate carbon acid and a bound enolic intermediate. If $\Delta G^\circ > 0$ for formation of the enolic intermediate, a reduction in $\Delta G^\ddagger_{int}$ will necessarily increase $x^\ddagger$. As this ΔG° becomes smaller, a reduction in $\Delta G^\ddagger_{int}$ increases $x^\ddagger$ to a lesser extent. We emphasize that *if ΔG° for formation of the bound enolic intermediate from substrate carbon acid or product carbon acid could be reduced to 0, as proposed by Albery and Knowles,[72] a reduction in $\Delta G^\ddagger_{int}$ would not change $x^\ddagger$* (Figure 4, bottom panel). Since we believe that a short, strong hydrogen bond between the OH group of the bound enolic intermediate and the electrophilic catalyst is the most likely mechanism for reducing ΔG° for formation of the enolic intermediate, given the observed nearly matched pK_as of the electrophilic catalysts and enol tautomers of the substrate carbon acids, the increase in $x^\ddagger$, which is possible only if $\Delta G^\circ > 0$, will allow the reduction in ΔG° that is necessary to produce the observed rates of the enzyme-catalyzed reactions. Thus, we propose that differential binding of the enolic intermediate and catalysis of an elementary step are not necessarily independent stages in achieving perfection in enzyme-catalyzed reactions.

A comparison of the energetic requirements for catalysis summarized in the top panel (the "late transition state rule" described in this article) and in the bottom panel (the Albery–Knowles proposal) of Figure 4 illustrates that the amount of stabilization of the enolic intermediate, $\Delta\Delta G^\circ$, required to achieve the observed $\Delta G^\ddagger$s for the enzyme-catalyzed reactions is much less when the transition state for the enolization reaction is late (top panel; our proposal) rather than midway (bottom panel; Albery–Knowles proposal) on the reaction coordinate. Since the available evidence indicates that $\Delta G^\ddagger_{int}$ is also mutable by the environment of an enzyme active site, we believe that our proposal that large changes in $\Delta G^\ddagger$ can be achieved by modest values for $\Delta\Delta G^\circ$ (7 kcal/mol) is energetically more plausible than the Albery–Knowles proposal that implicitly requires large values for $\Delta\Delta G^\circ$ (~20 kcal/mol).[82,83]

Thus, while we believe that the concepts of differential binding of reaction intermediates and catalysis of elementary steps are useful for understanding optimization of catalysis, we propose that the manner in which these are accomplished in enzyme active sites differs in significant detail from that described by Albery and Knowles. At least for the active sites of enzymes catalyzing enolization of carbon acids, we do not believe that application of the Hammond postulate is central to understanding the rates of enzyme-catalyzed reactions. Instead, we believe that differential binding of intermediates and catalysis of elementary steps are interdependent processes, with both the late transition states made possible by decreases in $\Delta G^\ddagger_{int}$, and the decreases in ΔG°, which are then possible by the formation of short, strong hydrogen bonds, explaining the observed rates of the enzyme-catalyzed reactions.[84]

Conclusions

The partitioning of $\Delta G^\ddagger$ by Marcus formalism into contributions from ΔG°, the thermodynamic barrier, and $\Delta G^\ddagger_{int}$, the intrinsic kinetic barrier, is instrumental in understanding the rapid rates of concerted general acid–general base catalyzed abstraction of the α-protons of carbon acids in enzyme active sites. Our analysis reveals the importance of late transition states and the following associated requirements in these enzyme-catalyzed reactions:

(1) A reduction in ΔG° is accomplished by stabilization of the enolic intermediate by a short, strong hydrogen bond between the OH group of the enolic intermediate and the conjugate base of the active site electrophilic catalyst.

(2) A reduction in $\Delta G^\ddagger_{int}$ is accomplished by positioning the general acidic catalyst adjacent to the carbonyl, carboxylic acid, or carboxylate group of the substrate carbon acid so that the negative charge that develops as the α-proton is abstracted can be stabilized in a concerted (iterative) process. Partial protonation of the carbonyl, carboxylic acid, or carboxylate anion group in the transition state for enolization causes a significant decrease in the pK_a of the α-proton so that it can be transferred to the weakly basic active site general basic catalyst. The late transition states that are observed in the enzyme-catalyzed reactions are a direct consequence of a reduction in the value for $\Delta G^\ddagger_{int}$.

We believe that this article, together with our earlier descriptions of the effects of electrophilic catalysis on the pK_as of the α-protons of carbon acids[2] and of the nature of the stepwise mechanisms of β-elimination reactions,[10] provides the intellectual basis for understanding the rates and mechanisms of certain enzyme-catalyzed reactions of carbon acids.

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(82) If the Albery and Knowles proposal[72] that $\Delta G^\circ = 0$ for formation of the bound enolic intermediate from the bound substrate carbon acid were correct, the observed $\Delta G^\ddagger$s (~14 kcal/mol) for the reactions catalyzed by triose phosphate isomerase, mandelate racemase, and Δ^5-ketosteroid isomerase would indicate that $\Delta G^\ddagger_{int}$ for the enzyme-catalyzed abstraction of the α-proton of the bound substrate carbon acid is the same as that for the nonenzymatic base-catalyzed reaction. This large value for $\Delta G^\ddagger_{int}$ requires a transition state that is midway on the reaction coordinate, which is incompatible with the conclusion that the pK_as of the active site electrophilic catalyst and the conjugate acid of the general basic catalyst are closely matched with the pK_as of the OH group of the enolic intermediate and the α-carbon proton of the carbonyl-protonated substrate, respectively.

(83) While the strengths of short, strong hydrogen bonds in the *gas phase* do approach or exceed 20 kcal/mol[20] so that a reduction in ΔG° of this magnitude in an enzyme active site is, in principle, possible, our proposal for concerted general acid–general base catalysis does not require as significant a reduction in ΔG° (~7 kcal/mol).

(84) Albery and Knowles[72] suggested that catalysis of an elementary step may involve development of "a new catalytic pathway, for instance, by going from monofunctional to bifunctional (e.g., push–pull) catalysis." They further noted that "the presence of enzyme groups that allow specific solvation (i.e., stabilization) of intermediates and/or transition states" may be involved. While this strategy does, in fact, qualitatively describe the analysis we have described in this article, we emphasize that the basic assumption and the quantitative aspects of our analysis differ substantially from the treatment described by Albery and Knowles.