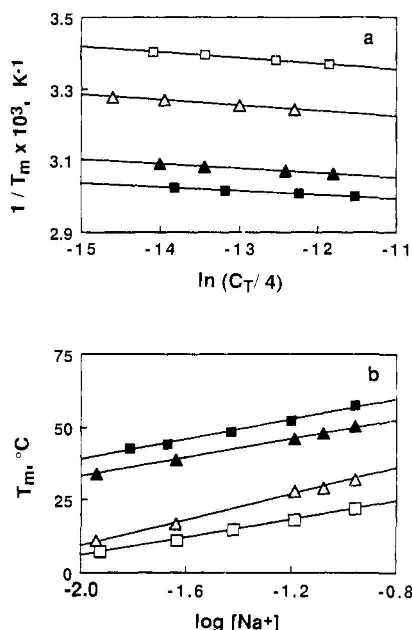


**Figure 3.** Absorbance vs temperature profiles (melting curves) for each oligomer duplex. (a) Dependence of the transition temperature on strand concentration in 10 mM sodium cacodylate buffer containing 0.1 mM $Na_2EDTA$ and 0.1 M NaCl at pH 7.0. The symbols for each duplex are ps-D1·D2 (△), aps-D1·D3 (▲), ps-D5·D6 (□), and aps-D5·D7 (■). (b) Salt dependence of the transition temperature for the dissociation of each duplex; symbols as in panel a.

concentration, the measured enthalpy would have been reduced by 5%.

To confirm the two-state melting behavior of these duplexes, we monitored the temperature dependence of the UV absorbance as a second experimental observable for the measurement of model-dependent transition enthalpies. Such determinations have been reported previously for the same molecules.[4-8] From shape analysis of these curves, we can extract both the $T_m$ and the transition enthalpy, $\Delta H_{shape}$.[13] The slopes of $(1/T_m)$ vs $(\ln C_T/4)$ plots (Figure 3a) provide a more accurate spectroscopic determination of $\Delta H_{vH}$.[13] The resulting model-dependent enthalpies are also given in Table I and are in good agreement with the calorimetric enthalpies, confirming the two-state melting behavior of all duplexes.

From the point of view of statistical mechanical theory of thermal transitions, the two-state melting behavior of the duplexes studied here is surprising.[17] That is, a finite contribution of partly unpaired intermediates would be expected. Unfortunately, direct comparison with literature data cannot be made because calorimetric studies of oligonucleotides as a function of chain length or with lengths comparable to the duplexes of this work have not been performed; the longest reported linear DNA duplexes with a two-state melting behavior are $d(GCGAATTCGC)_2$, $d(GAAGATCTTC)_2$, and $d(ATATATATAT)_2$.[14]

**Standard Thermodynamic Profiles.** The overall destabilization of 17 °C and 34 °C of the ps duplexes relative to the aps duplexes with identical sequences corresponds to $\Delta\Delta G°$ values of 11 and 18 kcal·mol$^{-1}$ for the two pairs of duplexes (see Table I). The nature of this destabilization is purely enthalpic and probably corresponds to the loss of favorable hydrogen-bonding and base-stacking interactions as well as to changes in the degree of hydration. Furthermore, the substitution of four dA·dT base pairs of ps-D1·D2 for four dG·dC base pairs in the ps-D5·D6 duplex results in a destabilization of 11.9 °C, which is entropically driven. The same type of substitution in the aps duplexes results in a stabilization of 4.9 °C, which is enthalpically driven.

**Counterion Binding to Parallel-Stranded DNA.** Figure 3b shows the effect of counterions on the helix–coil transition in these duplexes. These curves indicate the shift of the helix–coil equilibria toward the helical state with increasing salt concentration. The slopes of these lines range from 16.9 to 21.8 °C and correspond, according to eq 1, to $\Delta n_{Na^+}$ values ranging from 0.11–0.14 mol of $Na^+$/mol of phosphate. Since the single strands are similar in sequence, we conclude as in previous studies[4-8] that the ps duplexes and aps duplexes have similar charge densities.

**Acknowledgment.** This work was supported by Grant GM-42223 (L.A.M.) from the National Institutes of Health.

(17) D. Porschke, In *Molecular Biology, Biochemistry and Biophysics*; Pecht, I., Rigler, R., Eds.; Springer-Verlag: Berlin, 1977; Vol. 24, pp 191–218.

# Understanding Enzyme-Catalyzed Proton Abstraction from Carbon Acids: Details of Stepwise Mechanisms for β-Elimination Reactions

**John A. Gerlt* and Paul G. Gassman***

*Contribution from the Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, and Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455.* *Received November 27, 1991*

**Abstract:** The observed rates of enzyme-catalyzed abstraction of a proton from a carbon adjacent to a carbonyl/carboxylic acid group (α-proton of a carbon acid) require that the $pK_a$ of the α-proton be decreased such that it is equal to (±2–3 $pK_a$ units) or less than that of the protonated active site basic catalyst. This can be accomplished by concerted general acid–general base catalysis: Gerlt, J. A.; Kozarich, J. W.; Kenyon, G. L.; Gassman, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9667. Analysis of various enzyme-catalyzed β-elimination reactions using this principle leads to the prediction that these reactions are likely to proceed via stepwise mechanisms which involve initial concerted general acid–general base catalyzed formation of an enol intermediate followed by elimination of the β-substituent. The second step is a vinylogous E2 reaction (1,4-elimination). Thus, these enzymes are not expected to catalyze "simple" E1cb reactions as has often been proposed. The involvement of concerted general acid–general base catalysis in reducing the $pK_a$s of carbon acids may provide insight into the observed stereochemical courses of certain enzyme-catalyzed β-elimination reactions.

## Introduction

The rapid rates of enzyme-catalyzed abstraction of a proton from a carbon adjacent to a carbonyl/carboxylic acid group (α-proton of a carbon acid) have long been puzzling, given the large difference between the $pK_a$s of the substrate carbon acid in solution and the active site base:[1] in solution, the $pK_a$s values of the

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α-protons of most aldehydes, ketones, and thioesters are ~16–20, and the $pK_a$s of those of most carboxylate anions are ≥32; in contrast, the $pK_a$s of active site bases are usually ≤7, as judged by the dependencies of $k_{cat}$s on pH.[1] However, if the substrate carbon acid bound in an active site has the properties of a typical carbon acid in solution, i.e., isoergonic proton transfer occurs with an activation energy barrier of ~14 kcal/mol[2] as a result of a temporal requirement for changes in hybridization and/or solvent structure,[3] the $\Delta pK_a$ between the substrate carbon acid and the conjugate acid of the active site base must be significantly reduced or even entirely eliminated to account for a typical $k_{cat}$ of $10^3$ $s^{-1}$. If the proton transfer were diffusion-controlled or "normal", i.e., isoergonic proton transfer occurs with an activation energy barrier of ~3 kcal/mol, the upper limit on the $\Delta pK_a$ could be as large as ~8.[4]

Thus, although the α-proton of a carbon acid is considerably more acidic than an unactivated aliphatic proton, the acidifying effect of the carbonyl/carboxylic acid group is insufficient to account for the observed rates. While the structure of an active site might perturb the $pK_a$ of the base and/or substrate via proximity of charged groups and exclusion of water, an explicit, but general, explanation for the observed rapid rates of proton transfer in many enzymatic reactions has been lacking.

One type of enzyme-catalyzed reaction which is subject to this problem is the β-elimination reaction in which the elimination of a β-leaving group (β-substituent) is accomplished by initial abstraction of the α-proton next to a carbonyl group;[10] the product of this reaction is an α,β-unsaturated carbonyl derivative. In those cases where β-elimination reactions have been concluded to be stepwise, the usual assumption (either implicit or explicit) has been that the mechanism is E1cb.[11] In this article we describe the implications of electrophilic catalysis (*necessary* participation of a general acidic catalyst) on the mechanisms and rates of enzyme-catalyzed β-elimination reactions (and, by microscopic reversibility, Michael addition reactions). We conclude that β-elimination reactions are likely to proceed via a stepwise mechanism but not by the E1cb mechanism that generally has been proposed.

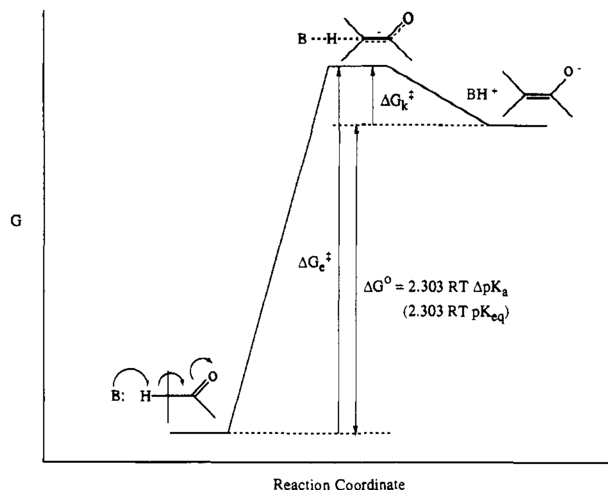



**Figure 1.** Free energy diagram for abstraction of the α-proton from a carbon acid to generate a carbanion. In the direction of carbanion formation (i.e., enolate formation), the total activation energy barrier ($\Delta G_e^\ddagger$) is the sum of the change in free energy [($\Delta G^\circ = 2.303RT\Delta pK_a$) associated with the $pK_a$ difference between the carbon acid and the base ($\Delta pK_a$)] and the activation energy barrier for acid catalyzed-ketonization of the enolate anion ($\Delta G_k^\ddagger$); the latter activation energy barrier is related to the intrinsic barrier for an isoergonic proton transfer as detailed in footnote 4. The negative logarithm of the equilibrium constant for the proton transfer reaction, $pK_{eq}$, is equal to $\Delta pK_a$.

## Carbanions and Carbanionic Intermediates: Stepwise or Concerted Mechanisms

The proposed formation of a discrete "carbanion" or "carbanionic intermediate" by abstraction of a proton α to a carbonyl is implicit in the analysis presented in the Introduction. The instability/lability of such intermediates lead Thibblin and Jencks to conclude that either the enzyme-catalyzed proton abstraction reactions are concerted with a second bond-making event that leads to product formation or the carbanion is somehow stabilized.[12] They concluded that "the carbanion 'intermediates' that have been implicated in a number of enzymic reactions are so unstable as to have, at best, a borderline existence". We believe this to be an understatement!

The observed rates of the Claisen condensation catalyzed by citrate synthase (condensation of acetyl CoA and oxalacetate to yield citrate), the isomerization reaction catalyzed by $\Delta^5$-ketosteroid isomerase (interconversion of α,β- and β,γ-unsaturated ketosteroids), and the racemization of mandelate catalyzed by mandelate racemase were proposed to "suggest that the enzymatic reactions proceed through some mechanism that avoids or stabilizes such unstable species and that the carbanions have an extremely short lifetime if they do exist as intermediates". The

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

(2) (a) Chiang, Y.; Kresge, A. J.; Santaballa, J. A.; Wirz, J. *J. Am. Chem. Soc.* **1988**, *110*, 5506. (b) Albery, W. J. *J. Chem. Soc., Faraday Trans. 1* **1982**, *78*, 1579.

(3) The explanation for the slow rate of proton transfer from most carbon acids remains uncertain, although recent evidence suggests that solvent reorganization and not changes in hybridization *per se* may be the dominant effect: (a) Bernasconi, C. F.; Terrier, F. *J. Am. Chem. Soc.* **1987**, *109*, 7115. (b) Bernasconi, C. F. *Acc. Chem. Res.* **1987**, *20*, 301.

(4) These quantitative conclusions are based upon the fact that for a thermodynamically controlled process the $\Delta G^\circ$ for the proton transfer reaction ($RT\Delta pK_a$) contributes to the activation energy barrier (Figure 1). The value for **k**, the observed rate of transfer of the proton from the substrate acid to the active site base, is related to $\Delta G^*$ (the intrinsic activation energy barrier for an isoergonic proton transfer; ~14 kcal/mol[2] in the case of carbon acids and ~3 kcal/mol[5] for normal acids), $\Delta pK_a$ (the difference in $pK_a$s between the acid and the base), and $\beta_e$ (the Brønsted coefficient for base-catalyzed enolization of the carbon acid) by the equation[6]

$$\mathbf{k} = (kT/h) \exp\{-[(\Delta G^*/RT) + \beta_e 2.303\Delta pK_a]\} \quad (1)$$

where $(kT/h) = 6.2 \times 10^{12}$ $s^{-1}$ ($k$ is Boltzmann's constant and $h$ is Planck's constant). From Marcus theory, $\beta_e$ depends upon the value of $\Delta pK_a$[7]

$$\beta_e = (1 + \Delta G^\circ/4\Delta G^*)/2 \quad (2)$$

where $\Delta G^\circ$, the free energy difference between the reactants and products, equals $2.303RT\Delta pK_a$. Thus, when $\Delta pK_a = 0$, $\beta_e = 0.5$. When the reaction is endergonic, as shown in Figure 1, $1 > \beta_e > 0.5$; for example, $\beta_e$ is 0.88 for base-catalyzed (carboxylate anion) enolization of acetone[8] and 0.68 for base-catalyzed enolization of acetophenone.[2a] The effect of $\beta_e$ is to reduce the activation energy barrier for the reverse (ketonization) reaction ($\Delta G_k^*$) from the intrinsic barrier ($\Delta G^*$) by $(1 - \beta_e)2.303RT\Delta pK_a$. Despite the complex dependence of **k** on both $\Delta G^*$ and $\Delta pK_a$, a typical $k_{cat}$ of $10^3$ $s^{-1}$ implies that the $\Delta pK_a$ term in the exponential of eq 1 is small or negligible for carbon acids, i.e., $\Delta pK_a \sim 0$ assuming $\Delta G^* \sim 14$ kcal/mol. For normal acids ($\Delta G^* \sim 3$ kcal/mol) eqs 1 and 2 can be solved for $\Delta pK_a$; the result, ~8, indicates that if the active site could "normalize" the behavior of the substrate carbon acid, a larger $\Delta pK_a$ would allow the typical $k_{cat}$. However, the $\Delta pK_a$ is still significantly less than the value observed in solution. Given the uncertainties in the values for the $\Delta G^*$s for carbon acids which are estimated from theories[2,7] such as that proposed by Marcus,[9] rather than actual experimental measurements, we assume that the conclusion that $\Delta pK_a \sim 0$ is also uncertain and may have an uncertainty of perhaps several $\Delta pK_a$ units.

(5) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1.

(6) The equation in footnote 29 of our earlier communication[1] requires clarification; we thank Professor Richard L. Schowen for pointing out this need. Our earlier conclusion that the $\Delta pK_a$ between the substrate acid and the active site base must be small is unchanged.

(7) Keefe, J. R.; Kresge, A. J. In *The Chemistry of Enols*; Rappoport, Z., Ed.; John Wiley & Sons: New York, 1990; Chapter 7.

(8) Venimadhavan, S.; Shelly, K. P.; Stewart, R. *J. Org. Chem.* **1989**, *54*, 2483.

(9) Marcus, R. A. *J. Phys. Chem.* **1968**, *72*, 819.

(10) In the organic chemical literature, the nomenclature for β-elimination reactions is that the proton that is abstracted is bonded to the β-carbon and the leaving group is bonded to the α-carbon. In the present discussion, the designations α and β refer to the position relative to the carbonyl/carboxylic acid group, i.e., the proton that is abstracted is designated the α-proton and the leaving group is designated the β-substituent.

(11) Anderson, V. E. In *Enzyme Mechanisms from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Cleveland, 1991; Chapter 16.

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

reaction mechanism and the nature of the stabilization of the "carbanion intermediates" were not specified. While the kinetic lability of isolated (i.e., unstabilized) carbanionic intermediates would preclude their existence, at least one modern textbook of biochemistry[13] as well as the biochemical literature[11] refer to carbanions or carbanionic intermediates that are obtained by abstraction of $\alpha$-protons of carbon acids. In at least some enzyme-catalyzed reactions, e.g., triose phosphate isomerase[14] and enolase,[15] exchange of the $\alpha$-proton of substrate with solvent is observed, thereby requiring the formation of an intermediate. Thus, while biochemists may have suggested that carbanions cannot exist for long as intermediates in enzyme-catalyzed reactions, a quantitative explanation for both the observed rates of enzyme-catalyzed proton transfer reactions and the occurrence of intermediates has not been put forth. Although "electrophilic catalysis" of $\alpha$-proton abstraction frequently has been proposed to account for the apparent discrepancy between the $pK_a$ of the substrate carbon acid in solution and during the proton transfer event,[16] both its magnitude and importance in solving the problem of facile $\alpha$-proton abstraction from carbon acids were clarified only recently.[1]

### The Magnitude of Electrophilic Catalysis

We provided a useful explanation for the rapid rate of enzyme-catalyzed proton abstraction by pointing out that the $pK_a$s of the $\alpha$-protons of carbon acids would be decreased by $\sim$15 units by protonation of the carbonyl/carboxylic acid group.[1] The incremental effect of protonation of the carbonyl/carboxylic acid group happens to be the same, irrespective of whether the carbon acid is associated with an aldehyde, ketone, thioester, or carbonyl group of a carboxylic acid. This conclusion follows from a thermodynamic analysis which demonstrates that the $pK_a$ of the $\alpha$-proton of a carbonyl-protonated carbon acid is the sum of the $pK_a$ of the carbonyl-bound proton of the carbonyl-protonated carbon acid and the $pK_E$ of the carbon acid (the negative logarithm of the equilibrium concentrations of the keto and enol tautomers of the carbon acid).[1] Thus, electrophilic catalysis is, in fact, extremely effective in enhancing the acidities of carbon acids.

An acidic functional group in an enzyme active site cannot accomplish kinetically significant preequilibrium protonation of the carbonyl/carboxylic acid group, just as a basic functional group cannot accomplish kinetically significant preequilibrium carbanion formation. However, the transition state for concerted abstraction of the $\alpha$-proton and protonation of the carbonyl/carboxylic acid group will resemble the enolic product since the $pK_E$s for carbon acids significantly favor the keto tautomer.[17] In these concerted proton transfer reactions, the $pK_a$ of the $\alpha$-carbon-bound proton decreases as the $pK_a$ of the protonated carbonyl increases. From the Hammond postulate,[18] the $pK_a$ of the $\alpha$-carbon bound proton decreases until it reaches, as a limit, the $pK_a$ of the $\alpha$-proton of the protonated carbon acid, i.e., of the conjugate acid of the enol product. Since the $pK_a$ of the conjugate acid of the enol product is similar to those of active site bases, the thermodynamic barrier to proton transfer determined by the $\Delta pK_a$ between the substrate carbon acid and active site base may well be entirely eliminated.[19]

(13) Voet, D.; Voet, J. G. *Biochemistry*; Wiley and Sons: New York, 1990; Chapters 15, 16, and 19.

(14) (a) Maister, S. G.; Pett, C. P.; Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5607. (b) Fletcher, S. J.; Herlihy, J. M.; Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5612.

(15) Stubbe, J.; Abeles, R. H. *Biochemistry* **1980**, *19*, 5505.

(16) For example, see: (a) Mildvan, A. S.; Kobes, R. D.; Rutter, W. J. *Biochemistry* **1971**, *10*, 1191. (b) Maggio, E. T.; Kenyon, G. L.; Mildvan, A. S.; Hegeman, G. D. *Biochemistry* **1975**, *14*, 1131. (c) Smith, G.; Mildvan, A. S. *Biochemistry* **1981**, *20*, 4340. (d) Webb, M. R.; Knowles, J. R. *Biochem. J.* **1974**, *141*, 589. (e) Belasco, J. G.; Knowles, J. R. *Biochemistry* **1980**, *19*, 472. (f) Komives, E. A.; Chang, L. C.; Lolis, E.; Tilton, R. F.; Petsko, G. A.; Knowles, J. R. *Biochemistry* **1991**, *30*, 3011.

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

(18) Hammond, G. S. *J. Am. Chem. Soc.* **1955**, *77*, 334.

(19) Concerted general acid–general base catalyzed reactions require that the $pK_a$ of the $\alpha$-proton be equal to ($\pm$2–3 $pK_a$ units) or less than that of the protonated active site base to provide a thermodynamic driving force for the reaction: Jencks, W. P. *J. Am. Chem. Soc.* **1972**, *94*, 4731.

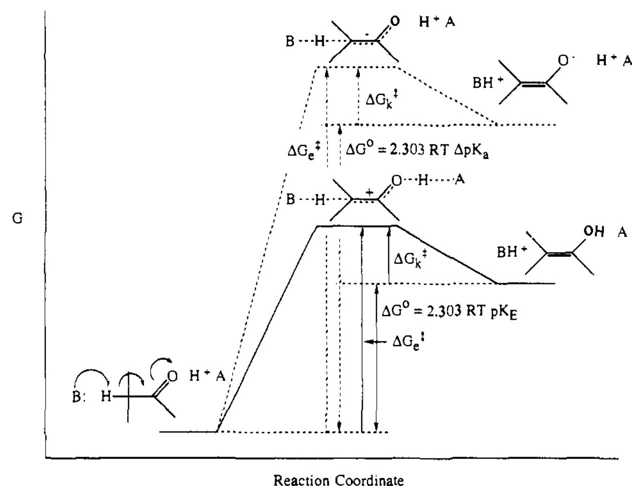


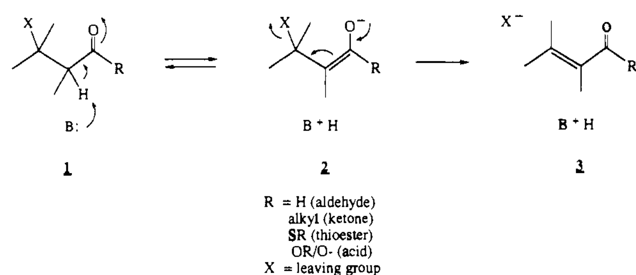

**Figure 2.** Comparison of the free energy diagrams for abstraction of the $\alpha$-proton from a carbon acid to generate a carbanion (enolate) (from Figure 1; dotted lines) and for concerted general acid–general base catalyzed enolization of the carbon acid (solid lines). In the direction of enol formation, the total activation energy barrier ($\Delta G_e^{\ddagger}$) is the sum of the free energy associated with the enolization reaction ($2.303RTpK_E$) and the activation energy barrier for the thermodynamically favorable concerted general acid–general base ketonization of the enol ($\Delta G_k^{\ddagger}$; not necessarily identical to the value of $\Delta G_k^{\ddagger}$ in Figure 1).

The concerted proton transfer reactions will catalyze the conversion of the keto tautomer of the carbon acid to its enol tautomer. Thus, the thermodynamic barrier associated with the $\Delta pK_a$ in the stepwise mechanism involving carbanion formation is necessarily replaced by the thermodynamic barrier associated with the $pK_E$ (Figure 2). Using arguments strictly analogous to those made earlier,[4] a typical $k_{cat}$ of $10^3$ s$^{-1}$ can be achieved as long as the $pK_E$ is $\leq\sim$8 (an upper limit based on the behavior of normal acids). Since the $pK_E$s for most aldehydes, ketones, and thioesters occur in this range and are less than the $\Delta pK_a$ between the bound substrate carbon acid and the active site base in the carbanionic mechanism (>10),[1] the thermodynamic component of the barrier for concerted proton transfer, i.e., tautomerization of the substrate carbon acid, is less than the thermodynamic component of the barrier for carbanion formation. Although the $pK_E$s for carboxylic acids are significantly larger than this range in solution, differential binding of the enolate (enol of the carboxylate or, henceforth, enol/enolate) in the active site can be achieved, thereby reducing the apparent $pK_E$ (vide infra). With this clarification of the mechanism for proton transfer, the reactions catalyzed by citrate synthase, $\Delta^5$-ketosteroid isomerase, and mandelate racemase (and many other enzymes, including those which catalyze $\beta$-elimination reactions) can understandably proceed with their observed $k_{cat}$s; these reactions involve the formation of an enol intermediate of sufficient stability to be kinetically relevant. The thermodynamic requirement that the magnitude of electrophilic catalysis is extremely large provides the necessary foundation for understanding both the rates of enzyme-catalyzed proton transfer reactions and the occurrence of transiently stable intermediates.

Since protonation of the carbonyl group occurs in concert with abstraction of the $\alpha$-proton, the product of the reaction is an enol (in the case of aldehydes, ketones, and thioesters) or an enol/enolate (in the case of carboxylates).[20] Whereas the frequent use of the terms carbanion and carbanionic intermediate may not have necessarily implied that the electron pair resulting from abstraction of the $\alpha$-proton is localized on an $sp^3$ hybridized $\alpha$-carbon, we believe that its usage has contributed to the confusion regarding the basis for the observed rates of proton transfer. The product of $\alpha$-proton abstraction from a carbon acid is (and always has been when the $\sigma$-orbital of the C–H bond to the $\alpha$-proton is or can be parallel to the $\pi$-orbital(s) of the carbonyl/carboxylic

(20) An enolic O–H is generated from one carboxylate oxygen in the concerted proton transfer reactions; the other carboxylate oxygen retains an anionic charge and, thus, can be viewed as an enolate oxygen.

Scheme I




acid group) an enolic species in which the α-carbon is $sp^2$ hybridized, even when an electrophilic catalyst is unavailable. As long as a proton is transferred to the carbonyl during formation of the enol (aldehydes, ketones, and thioesters) or to the carbonyl of the carboxylate anion during formation of the enol/enolate, this intermediate is sufficiently stable to be kinetically significant (with its concentration at steady state specified by the $pK_E$ for the substrate carbon acid *in the active site*). The realization that the $\Delta pK_a$ between the substrate and the active site base can be reduced by a significant amount or even eliminated by concerted transfer of a proton to the carbonyl group allows both the actual identity and the stability of intermediates to be understood.

Concerted general acid–general base catalyzed enolization of simple aldehydes[21] and ketones[21,22] has been observed in nonenzymatic systems but only at high concentrations of acetic acid/acetate buffers. The termolecular rate constants for enolization of aldehydes exceed those for enolization of ketones. This has been attributed both to the fact that the enolization reaction is more favorable (smaller $pK_E$) for aldehydes and to the observation that the carbon acidity is greater for aldehydes. Catalysts containing both acidic and basic functional groups have also been reported.[23] While these examples constitute chemical precedent for the efficacy of concerted general acid–general base catalyzed abstraction of the α-proton from a carbon acid, the observed rate enhancements do not approach those of enzymatic systems. The rates of the enzyme-catalyzed reactions presumably reflect significant optimization of both the geometry for the intramolecular reaction as well as the $pK_a$s of the acidic and basic catalysts.

## Nonenzymatic vs Enzyme-Catalyzed β-Elimination Reactions

Certain nonenzymatic β-elimination reactions proceed via an E1cb mechanism in which a strong base abstracts the α-proton to generate an enolate (carbanionic intermediate),[24] i.e., the conjugate base of the carbon acid (**2** in Scheme I[25]). The evidence for the E1cb mechanism, i.e., the formation of a kinetically significant intermediate derived by abstraction of the proton, rests primarily upon the observation that solvent hydrogen isotope exchange successfully competes with departure of the β-substituent. This enolate then undergoes elimination of the β-substituent, in the rate determining step, to yield the α,β-unsaturated product (**3**). In nonenzymatic systems, the $pK_a$s of the α-protons are those associated with neutral aldehydes, ketones, thioesters, and carboxylate anions, ≈16, ≈18, ≈18, and ≥32, respectively,[1] and these require the use of very strong bases for rapid reaction in order to minimize the $\Delta pK_a$ between the substrate and the protonated base and, therefore, maximize the rate of the reaction. Despite the fact that the enolate intermediates generated in these reactions are labile, the observed exchange of the α-proton with solvent demands their existence for more than a vibration prior to the departure of the β-substituent; otherwise, the reactions would necessarily occur by a concerted E2 mechanism. Since the mechanisms of nonenzymatic reactions (E1cb vs E2) depend upon the relative rates of recapture of the enolate by solvent hydrogen and departure of the β-substituent, the mechanism can be changed by modulating the $pK_a$ of the α-proton and/or the $pK_a$ of the protonated β-substituent. A number of investigators, including Jencks, Kirby, Saunders, Spencer, and Stirling,[26] have observed that the mechanism of a β-elimination reaction is finely balanced between E1cb and E2, since it is a function of the identity and properties of the β-substituent.

In enzyme-catalyzed β-elimination reactions, the absence of isotope exchange does not constitute unequivocal evidence for the absence of an E1cb mechanism because the conjugate acid of the active site base need not be polyprotic and/or the active site may not contain solvent molecules that can exchange with the conjugate acid of the active site base. Despite this experimental uncertainty, a large number of enzyme-catalyzed β-elimination reactions have been proposed to proceed via E1cb mechanisms.[11] However, the fundamental problem associated with an E1cb mechanism in enzyme-catalyzed reactions is that the $\Delta pK_a$ between the substrate carbon acid and the protonated active site base is too large to account for the observed rates (vide infra).

## Proposed Mechanism for Enzyme-Catalyzed β-Elimination Reactions of Aldehydes, Ketones, and Thioesters

To circumvent the problem associated with E1cb mechanisms and thereby to illuminate the mechanisms of the enzyme-catalyzed reactions, we propose that certain enzyme-catalyzed β-elimination reactions do proceed by stepwise mechanisms but not by a stepwise E1cb mechanism. The premise on which our proposal is based is the thermodynamic requirement that protonation of a neutral aldehyde, ketone, or thioester would reduce the $pK_a$s of the α-protons to ≈2, ≈5, and ≈4, respectively.[1] With the full understanding that protonation of the carbonyl group and deprotonation of the α-carbon will be gradual processes in the concerted reaction as a result of the changes in their $pK_a$s as the reaction coordinate is traversed, we propose that if the substrate is an aldehyde, ketone, or thioester (**4**; Scheme II), rapid ($k_{cat} \approx 10^1$–$10^4$ $s^{-1}$) enzyme-catalyzed β-elimination reactions will proceed by a stepwise mechanism involving (1) initial concerted general acid–general base catalyzed enolization of the carbon acid to generate an enol intermediate (**5**) followed by (2) a vinylogous E2 reaction in which the β-substituent (e.g., X = $-OH$, $-NH_2$, $-OPO_3^{2-}$, or $-SR$) is eliminated from the enol.

Since the rates of ketonization of enolates exceed those of enols,[17] we expect that the elimination of the β-substituent from the enol intermediate will be catalyzed by the conjugate base of the acidic group catalyzing enol formation ($^-$A–E). We are unaware of any quantitative experimental data which exist regarding the relative rates of elimination of β-substituents from enols and enolates. The general acidic group participating in the enolization of the substrate carbon acid (HA–E) is likely to have a $pK_a$ matched to that of the enol; under these conditions the overall rate of the successive enolization vinylogous E2 reactions can be maximized.[27] The $pK_a$s of enols are usually ~10,[17] so

(21) Hegarty, A. F.; Dowling, J. *J. Chem. Soc., Chem. Commun.* **1991**, 996.

(22) (a) Hegarty, A. F.; Jencks, W. P. *J. Am. Chem. Soc.* **1975**, *97*, 7189. (b) Albery, W. J. *J. Chem. Soc., Faraday Trans. I* **1982**, 1579.

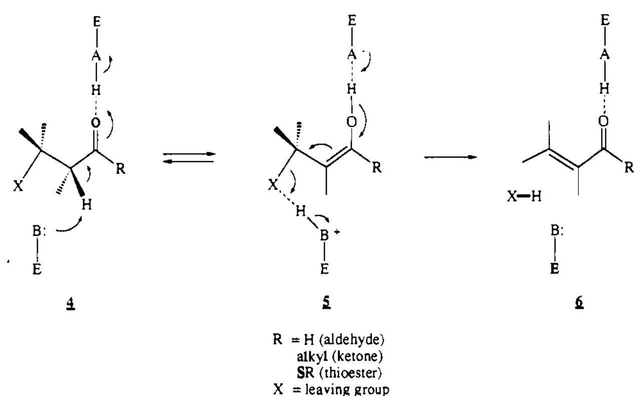
(23) Rebek and co-workers described an interesting but only modestly effective catalyst for enolization of quinuclidinone: Wolfe, J.; Muehldorf, A.; Rebek, J. *J. Am. Chem. Soc.* **1991**, *113*, 1453. The catalyst contains both a carboxylic acid group and an acridine basic group held in a geometry appropriate for enolization of the ketone. This catalyst increases the rate of enolization approximately $10^3$-fold relative to the uncatalyzed rate; the effect of adding an acidic functional group to a basic functional groups in the same catalyst provides an 8- to 40-fold enhancement in rate.

(24) For example, see: Fedor, L. R.; Glave, W. R. *J. Am. Chem. Soc.* **1971**, *93*, 985.

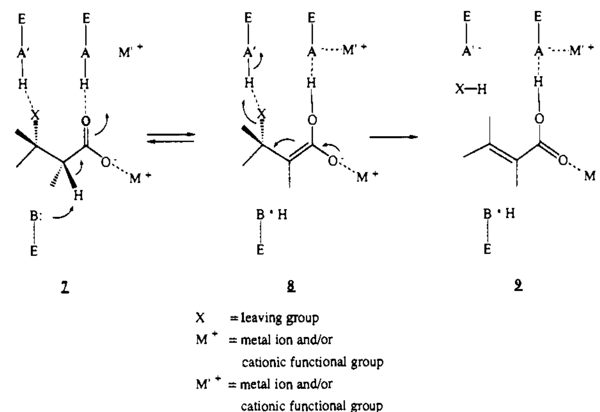
(25) The structures in the schemes are not intended to convey stereochemical information, e.g., *E/Z* isomerism of the olefins and enol(ate)s and syn/anti directions of the carboxylate groups.

(26) (a) Gandler, J. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1982**, *104*, 1937. Fishbein, J. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1988**, *110*, 5075. Fishbein, J. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1988**, *110*, 5087. Thibblin, A. *J. Am. Chem. Soc.* **1988**, *110*, 4582. Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1990**, *112*, 6950. (b) More O'Ferrall; Warren, P. J. *J. Chem. Soc., Chem. Commun.* **1975**, 483. (c) Marshall, D. R.; Thomas, P. J.; Stirling, C. J. M. *J. Chem. Soc., Perkin Trans. II* **1977**, 1914. Barlow, K. N.; Marshall, D. R.; Stirling, C. J. M. *J. Chem. Soc., Perkin Trans. II* **1977**, 1920. (d) Saunders, W. H.; Cockerill, A. F. *Mechanism of Elimination Reactions*; Wiley-Interscience: New York, 1973. Saunders, W. H. *Acc. Chem. Res.* **1976**, *9*, 19. (e) Mayer, B. J.; Spencer, T. A.; Onan, K. D. *J. Am. Chem. Soc.* **1984**, *106*, 6343. Mayer, B. J.; Spencer, T. A. *J. Am. Chem. Soc.* **1984**, *106*, 6349.

**Scheme II**




**Scheme III**




the phenolic hydroxyl groups of tyrosines, the $\epsilon$-ammonium groups of lysines, the guanidinium groups of arginines, and either the neutral imidazole or the cationic imidazolium groups of histidines are reasonable candidates for the required general acidic catalyst. In the case of histidine, the $pK_a$ of a neutral imidazole functional group is $\sim$14; the $pK_a$ of an imidazolium functional group is $\sim$7.[28] Depending upon the electrostatic environment in the active site, either the neutral or the cationic form of the histidine functional group could be an effective catalyst for the successive enolization-vinylogous E2 components of our proposed $\beta$-elimination mechanism.

Depending upon the identity (basicity) of the $\beta$-substituent, the elimination of the $\beta$-substituent may be general acid catalyzed through protonation of the $\beta$-substituent. Since the $\alpha$-protons of aldehydes, ketones, and thioesters are expected to be quite acidic during concerted general acid–general base catalyzed enolization (vide infra), the conjugate acid of the basic catalyst catalyzing enol formation ($HB^+$–E) is expected to be sufficiently acidic to catalyze the departure of most $\beta$-substituents. The ability of this functional group to participate in both the enolization (as :B–E) and E2 elimination (as $HB^+$–E) half-reactions is clearly dependent on the geometry of the enzyme–substrate complex: as shown in Scheme II, the $\alpha$-proton and the $\beta$-substituent would need to be located adjacent (gauche) to one another and in close proximity to the general basic catalyst that facilitates enol formation (:B–E). In fact, *all* enzyme-catalyzed $\beta$-elimination reactions of aldehydes, ketones, and thioesters proceed via the syn stereochemical course[29] that would be expected if this scenario were an accurate description of the enzyme–substrate complex. While the syn stereochemical courses of these reactions are in accord with the minimal number of functional groups (i.e., one) that might be required for efficient catalysis, assuming that evolution of catalytic efficiency was achieved by natural selection,[30] the relationship between the observed stereochemistries and $pK_a$s of the $\alpha$-protons provides a possible, but not necessary, chemical explanation for the uniformity of stereochemistry.

### Proposed Mechanism for Enzyme-Catalyzed $\beta$-Elimination Reactions of Carboxylic Acids (Carboxylates)

A variation of the mechanism in Scheme II will apply when the substrate is a carboxylate anion (thermodynamics requires that the $pK_a$ of the $\alpha$-proton of a protonated carboxylic acid is $\geq$10–12[1]). In this case, an E1cb mechanism would require the highly unfavorable formation of a dianion by removal of an $\alpha$-proton which has a $pK_a \geq 32$.[31] In addition, as we noted earlier, the $pK_E$s of carboxylic acids significantly exceed those of aldehydes, ketones, and thioesters: we estimate that the $pK_E$s of aliphatic carboxylic acids will be $\geq$18, and the $pK_E$s of aliphatic carboxylate anions will be even larger ($\geq$20[32]). Thus, the enzyme active site must be able to stabilize the enol/enolate intermediate so that the effective $pK_E$ can be reduced to $\leq \sim$8 (vide infra). As depicted in Scheme III, we propose the anionic carboxylate group of the substrate will interact directly with a cation (**7**; $M^+$ is a metal ion and/or cationic amino acid functional group) upon binding in the active site. Concerted general acid–general base catalysis will allow formation of the enol/enolate intermediate **8** from **7** in the same manner as the formation of the enol intermediate **5** from **4** in Scheme II.

In the case of the enol of mandelic acid, the first $pK_a$ of a geminal enediol is 6.6;[33] by analogy to the difference in the $pK_a$s of carbonic acid, the second $pK_a$ of a geminal enediol is expected to be $\geq$10.[34] Therefore, the transfer of a proton from the general acidic catalyst to the coordinated anionic carboxylate group accompanied by the removal of the $\alpha$-proton by the general basic catalyst to generate an enol/enolate (**8**) is thermodynamically possible for the strongly and weakly acidic functional groups found in proteins. If the acidic catalyst is neutral (carboxylic acid, neutral imidazole, or thiol), the product of this proton transfer reaction will be an anion ($^-$A–E; carboxylate anion, imidazolate anion, or thiolate). Close proximity of this newly formed negatively charged

(27) (a) If the $pK_a$s are matched, the proton may be equally shared/oscillating between the enolate and the conjugate base of the active site acid and involved in a "strong" hydrogen bond: Emsley, J. *Chem. Soc. Rev.* **1980**, *9*, 91. Kreevoy, M. M.; Liang, T. M. *J. Am. Chem. Soc.* **1980**, *102*, 3315. Emsley, J. *Struct. Bonding (Berlin)* **1984**, *57*, 147. While such an environment for the proton should not change the reactivity of the intermediate with respect to the vinylogous E2 elimination reaction, it could stabilize the intermediate, thereby facilitating both its formation and decomposition. The amount of stabilization afforded by the hydrogen bonding environment depends upon the strength of the hydrogen bond between the carbonyl group of the substrate and the acidic catalyst, the strength of the X–H bond in the acidic catalyst, and the strengths of the hydrogen bonds between the proton and both the intermediate and the conjugate base of the acidic catalyst. Potential evidence for stabilization of an intermediate by strong hydrogen bonding may be provided by the observation that the active site of $\Delta^5$-ketosteroid isomerase appears to decrease the $pK_a$ of the hydroxyl group of an analog of the presumed enediol intermediate by $\geq$5.5 $pK_a$ units ($\geq$7.7 kcal/mol): Zhang, B.; Bounds, P. L.; Steiner, R. F.; Pollack, R. M. *Biochemistry* **1992**, *31*, 1521. (b) The apparent occurrence and potential importance of strong hydrogen bonds in enzyme-catalyzed reactions was recently noted: Cleland, W. W. *Biochemistry* **1992**, *31*, 317.

(28) (a) Bruice, T. C.; Schmir, G. *J. Am. Chem. Soc.* **1958**, *80*, 148. (b) Walba, H.; Isensee, R. W. *J. Org. Chem.* **1961**, *26*, 2789.

(29) (a) Schwab, J. M.; Klassen, J. B.; Habib, A. *J. Chem. Soc., Chem. Commun.* **1986**, 357. (b) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299. (c) Mazumder, A.; Gerlt, J. A.; Absalon, M. J.; Stubbe, J.; Cunningham, R. P.; Withka, J.; Bolton, P. H. *Biochemistry* **1991**, *30*, 1119.

(30) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1.

(31) The $pK_a$ of the $\alpha$-proton of mandelate anion is $\geq$29. This value is the result of two thermodynamics cycles: (1) the $pK_E$ for a carboxylate anion (18.6) is the difference between the $pK_a$ of the $\alpha$-proton (22) and the $pK_a$ of the OH proton of the carboxylic acid (3.4), and (2) the $pK_a$ of the $\alpha$-proton of a carboxylate anion ($\geq$29) is the sum of the $pK_E$ for the carboxylate anion (18.6) and the second $pK_a$ of the enol tautomer (geminal enediol) of the carboxylic acid ($\geq$10) (vide infra). The $pK_a$s of the $\alpha$-protons of aliphatic acids/carboxylate anions are expected to be greater than the value for mandelic acid/mandelate anion value by $\geq$3–5 $pK_a$ units.[1]

(32) The $pK_E$ of a carboxylate anion is the difference between the $pK_a$s of the $\alpha$-proton of the carboxylic acid ($\sim$25–27) and of the hydroxyl proton of the carboxylic acid ($\sim$5).

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

(34) Assuming that the difference of 3.5 $pK_a$ units between the first and second $pK_a$s of carbonic acid is the *lower* limit for the difference between the first and second $pK_a$s of geminal enediols.

amino acid functional group to positive charge, either the cation to which the substrate carboxylate group is coordinated ($M^+$) and/or a second cationic group ($M'^+$),[35] will result in differential binding of **8**, using the terminology of Albery and Knowles.[36] The electrostatic attraction between the anionic functional group ($^-$A–E; whose presence is strictly dependent upon formation of the enol/enolate intermediate) and the positively charged center ($M^+$ and/or $M'^+$)[35] will stabilize **8** and thereby decrease the $pK_E$ of the substrate in the active site.[37,38] Once **8** is formed, the second step of the mechanism (the vinylogous E2 elimination) can diverge from that shown in Scheme II since general base catalysis of the formation of **9** may not be needed since intermediate **8** is already an enolate anion.

The departure of the β-substituent from carboxylic acid substrates may also be general acid catalyzed through protonation of the β-substituent. In contrast to aldehydes, ketones, and thioesters, the α-protons of carboxylic acids and carboxylates are not very acidic.[1] Thus, the conjugate acid of the basic catalyst catalyzing enol/enolate formation ($HB^+$–E) may not be sufficiently acidic to protonate the β-substituent as was proposed for aldehydes, ketones, and thioesters. A second, more acidic catalyst adjacent to the β-substituent (HA′–E) may be required for its protonation and departure. In order to avoid an unproductive transfer of a proton from this catalyst (HA′–E) to the more basic group catalyzing enol/enolate formation (:B–E), these catalysts would need to be isolated from one another, i.e., possibly located on opposite sides of the bound substrate and enol/enolate intermediate. This implies that the α-proton and the β-substituent would need to be antiperiplanar in the enzyme–substrate complex as shown in Scheme III. Assuming these constraints, it follows that the stereochemical course of a β-elimination reaction from a carboxylic acid may be anti, unless other mechanistic or evolutionary constraints apply. In fact, there is only one known exception to the anti stereochemistry, the reaction catalyzed by muconate lactonizing enzyme.[39,40] In this case the β-substituent is a carboxylate group, so its rapid departure may not require acid catalysis.[41]

## Rationalization of the Proposed Mechanisms with Structural and Mechanistic Data

With the exception of muconate lactonizing enzyme and enolase, structural information is presently unavailable for enzymes that catalyze β-elimination reactions. In the case of the muconate lactonizing enzyme that catalyzes a syn β-elimination reaction (although both the physiological reaction and the equilibrium position are in the direction of addition to the α,β-unsaturated species, i.e., formation of muconolactone from *cis,cis*-muconate), the active site contains, as we predicted, both a general basic (Lys 169) and a general acidic (Glu 327) catalyst that can assist in the enolization of the substrate, muconolactone.[43] A cationic residue (the ε-ammonium group of Lys 167) is also present in the active site adjacent to the carboxylic acid group of Glu 327; thus, the enol/enolate intermediate in the β-elimination reaction can be electrostatically stabilized, thereby lowering the effective $pK_E$ by the mechanism we have proposed.[44] The only acidic group that could catalyze the departure of the carboxylate leaving group is the conjugate acid of Lys 169. Although an X-ray structure has been reported for enolase,[45] the "catalytic" $Mg^{2+}$ is not visible, so definitive identification of active site acidic and basic catalysts is not yet possible.

Despite this paucity of structural information, the mechanisms of several enzymes that catalyze β-elimination reactions have been scrutinized by kinetic isotope effect studies.[11] In all but one case, the reaction catalyzed by crotonase (enoyl CoA dehydrase; $k_{cat} \approx 570\ s^{-1}$ [46]), the reactions have been deduced to proceed by *stepwise E1cb mechanisms* in which the α-proton is abstracted to form a carbanion before the β-substituent departs; for crotonase, α-proton abstraction and β-substituent departure have been suggested to be concerted.[47] In principle, a mechanism involving concerted general base catalyzed abstraction of the α-proton and general acid catalyzed loss of the β-substituent (E2 elimination) could account for the observed $k_{cat}$ for the reaction catalyzed by crotonase. General acid catalysis of the departure of the hydroxylic β-substituent would also decrease the effective $pK_a$ of the α-proton of the substrate and direct the mechanism to be E2; this effect is reminiscent of the change in mechanism from E1cb to E2 observed in nonenzymatic reactions as the $pK_a$ of the protonated β-substituent is decreased.[26] However, we suspect that the pronounced acidifying effect of the carbonyl/carboxylic acid group on the $pK_a$ of the α-protons of carbon acids in the presence of a general acid catalyst has dominated the evolution of enzyme function and that crotonase also operates by a two-step process.[48]

## Conclusions

We propose that both the mechanisms and rates of enzyme-catalyzed β-elimination reactions can be best rationalized by a stepwise mechanism involving (1) initial concerted general acid–general base catalyzed enolization of the carbon acid to generate an *enol* intermediate followed by (2) a vinylogous E2 reaction in which the β-substituent is eliminated from the enol. The first step allows the full acidifying effect of the protonated carbonyl/carboxylic acid group on the $pK_a$ of the α-proton to be realized, thereby decreasing both the thermodynamic and kinetic barriers to proton abstraction. This mechanism differs substantively from the E1cb mechanism that has been often proposed since it both

(35) Since the carboxylate group of the substrate is coordinated to $M^+$, the effective positive charge available for differential binding could be less than if a second cation (i.e., $M'^+$) were present in the vicinity of $^-$A–E. In addition, if $M^+$ were a (divalent) metal ion, its positive charge might also be partially neutralized by carboxylate ligands.

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(37) If, for example, the two charges were separated by 3 Å in a medium of dielectric constant 6, the differential stabilization of the enol/enolate intermediate would be 18.7 kcal/mol, thereby causing a reduction of 13.3 units in the effective $pK_E$ in the active site. This reduction in $pK_E$ is sufficient to allow abstraction of the α-proton at a rate compatible with observed $k_{cat}$s.

(38) We note an analogous mechanism for differential stabilization of an enol intermediate might also occur in the active sites of enzymes catalyzing β-elimination reactions of aldehydes, ketones, and thioesters, although the $pK_E$s of these substrate carbon acids are sufficiently low that such stabilization may not be necessary.

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(41) The stereochemical course of the seemingly closely related enzyme, carboxymuconate lactonizing enzyme, is the expected *anti*;[40] this lactonizing enzyme, but not muconate lactonizing enzyme, is evolutionarily related to a family of enzymes which catalyze β-elimination reactions, including fumarase, adenylosuccinate lyase, and arginosuccinate lyase.[42] In the reactions catalyzed by these enzymes, the departure of the strongly basic β-substituent is expected to require acid catalysis.

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(43) (a) Goldman, A.; Ollis, D. L.; Steitz, T. A. *J. Mol. Biol.* **1987**, *194*, 143. (b) Schliming, I.; Goldman, A.; Ringe, D., personal communications.

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(46) Waterson, R. M.; Hill, R. L. *J. Biol. Chem.* **1972**, *247*, 5258.

(47) (a) Bahnson, B. J.; Anderson, V. L. *Biochemistry* **1989**, *28*, 4173. (b) Bahnson, B. J.; Anderson, V. L. *Biochemistry* **1991**, *30*, 5894.

(48) This suspicion is strengthened by the recent finding that the primary sequences of crotonase, 4-chlorobenzoyl CoA dehalogenase (electrophilic substitution of an aromatic C–Cl bond), and enoyl CoA isomerase (isomerization of α,β- and β,γ-unsaturated CoA esters) are homologous (Babbitt, P. A.; Kenyon, G. L.; Dunaway-Mariano, D., personal communication). Since the reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase can best be rationalized by a stepwise mechanism in which an *enol* thioester is formed as an intermediate, the mechanism of the reaction catalyzed by crotonase can be expected to be stepwise. This sequence analysis revealed that these enzymes are not homologous to other CoA ester-dependent enzymes, e.g., citrate synthase, thereby suggesting that the relationships between crotonase, the dehalogenase, and the isomerase do not simply reflect the ability of the enzymes to bind the CoA portion of the substrate.

avoids a *discrete* carbanion intermediate and allows the rates of the reactions to be explained. Our proposal provides a possible explanation for the observed stereochemical courses of enzyme-catalyzed $\beta$-elimination reactions that is based on the $pK_a$s of the $\alpha$-protons of the carbon acid substrates.

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# Sequence-Specific Double-Strand Alkylation and Cleavage of DNA Mediated by Triple-Helix Formation

**Thomas J. Povsic, Scott A. Strobel, and Peter B. Dervan***

*Contribution from the Arnold and Mabel Beckman Laboratories of Chemical Synthesis, California Institute of Technology, Pasadena, California 91125.* *Received February 12, 1992*

**Abstract:** Attachment of the nondiffusible electrophile *N*-bromoacetyl to the 5-position of a thymine at the 5′-end of a pyrimidine oligodeoxyribonucleotide affords sequence specific alkylation of a guanine two base pairs to the 5′-side of a local triple-helix complex in >96% yield. *N*-Bromoacetyloligodeoxyribonucleotides bind adjacent inverted purine tracts on double-helical DNA by triple-helix formation and alkylate single guanine positions on opposite strands at 37 °C (pH 7.4). After depurination, double-strand cleavage at a single site within plasmid DNA (4 kp in size) occurs in greater than 85% yield. The resulting DNA fragments from site-specific alkylation and cleavage can be ligated with DNA fragments generated by restriction endonuclease digestion. This nonenzymatic approach which couples sequence-specific recognition with sequence-dependent cleavage affords double-strand site-specific cleavage in megabase size DNA. A yeast chromosome, 340 000 base pairs in size, was cleaved at a single site in 85–90% yield.

Reliable models for the sequence-specific recognition of double-helical DNA by low molecular weight peptides,[1,2] small protein–DNA binding domains,[3,4] and oligonucleotide triple-helix motifs[5–8] now exist. This is due, in part, to a combination of footprinting and affinity cleaving methods[1] for determining sequence specificities, groove locations, and binding orientations of ligands on DNA and the direct structural characterization of some of these complexes by nuclear magnetic resonance spectroscopy[8] and X-ray diffraction analyses.[9] The design of sequence-specific DNA *cleaving* molecules requires the integration of two separate functions, recognition and cleavage in a single molecule. One approach is to combine DNA binding molecules with reactive functionalities capable of oxidation of the deoxyribose,[10,11] electrophilic modification of the bases,[12,13] or hydrolysis of the phosphodiester backbone.[14]

Two criteria for successful bifunctional design are (i) the incorporation of a specific reaction at a designated atom within the bound ligand–DNA complex and (ii) cleavage yields that are quantitative under physiologically relevant conditions. In order to approach quantitative reactions on DNA, the cleaving functionality must be sufficiently reactive to allow modification on DNA at reasonable rates at 37 °C, be inert to aqueous media and buffer components, and not suffer unimolecular decomposition in competition with the desired reaction on DNA. In order to position reactive moieties within angstroms of a single atom

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