4-Oxalocrotonate Tautomerase: pH Dependence of Catalysis and pK_a Values of Active Site Residues†

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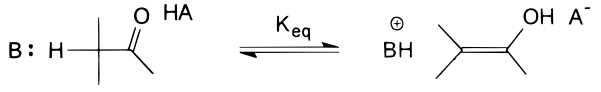
ABSTRACT: The pH-rate profiles for the kinetic parameters of 4-oxalocrotonate tautomerase (4-OT) have been measured using 2-hydroxy-2,4-hexadiendioate (**2a**) and 2-hydroxy-2,4-pentadienoate (**2b**) as substrates. The pH dependences of log (k_{cat}/K_m) and of log k_{cat} for the slow, nonsticky substrate **2b**, which lacks a 6-carboxyl group, were bell-shaped with limiting slopes of unity on both sides of the pH optimum. For **2b**, pK_a values of 6.2 ± 0.3 and 9.0 ± 0.3 for the free enzyme (pK_E) and 7.7 ± 0.3 and 8.5 ± 0.3 for the ES complex (pK_{ES}) were obtained. The pK_E of 6.2 ± 0.3 for **2b** represents a true pK_a for a basic group on the enzyme and is most likely Pro-1 on the basis of inhibition studies with the substrate-based affinity label 3-bromopyruvate (3-BP) [Stivers *et al.* (1996) *Biochemistry 35*, 803−813]. Accordingly, ^{15}N NMR titration of the uniformly ^{15}N-labeled enzyme showed that the pK_a of the amino group of Pro-1 is 6.4 ± 0.2, in reasonable agreement with those found by the effect of pH on k_{cat}/K_m for **2b** (6.2 ± 0.3) and on k_{inact}/K_I for 3-BP (6.7 ± 0.3), but three units lower than the pK_a of the model compound proline amide (pK_a = 9.4 ± 0.2). The pK_a values for the two histidine residues of 4-OT, which were measured by ^{1}H NMR (His-6, pK_a ≤ 5; His-49, pK_a = 5.2 ± 0.2), are at least one pK unit lower than the pK_E, excluding these residues as candidates for the general base. A plot of log (k_{cat}/K_m) vs pH for the 10^4-fold more reactive, but sticky substrate **2a** [$(k_{cat}/K_m)^{max} = 3.9 \times 10^6$ M^{-1} s^{-1}] shows a limiting slope of two on the ascending limb indicating the ionization of two essential groups on the free enzyme and/or substrate. One of these groups, with a pK_a value of 5.4, is reasonably assigned to the 6-carboxylate moiety of **2a** (pK_a^{COOH} = 5.4). This assignment is supported by the slope of unity for the ascending limb of log (k_{cat}/K_m) versus pH for **2b** which lacks this group. Thus a negative charge at the 6-position is important for substrate binding and catalysis. The other group (pK_{a2} = 5.2) most likely represents a perturbed pK_a for the general base Pro-1 (pK_a^{true} = 6.4). The descending limb of log k_{cat}/K_m vs pH for **2a** has a slope of unity and was fit to a single pK_{a3} = 10.3 ± 0.2. The pH dependence of k_{cat} for **2a** gives pK_a values for the ES complex (pK_{ES}) of 6.5 and 9.6. On the basis of these results, an isomerization mechanism involving general-base catalysis by a low pK_a proline-1 and electrophilic catalysis by an as yet unidentified enzymic general-acid (pK_a = 9.0) is proposed.

An intriguing problem in enzymology is the mechanism used by enzymes to accelerate proton abstraction from carbon. Model studies in aqueous solution (Hand & Jencks, 1975; Hegarty & Jencks, 1975; Albery, 1982) as well as studies of enzymes (Xue *et al.*, 1990) have shown that enolization of a carbonyl-activated carbon acid can occur by a concerted general acid−base catalyzed mechanism (Scheme 1).

It is clear from Scheme 1 that the pK_a values for the general base (B) and general acid (HA) are key unknowns in unraveling the thermodynamic as well as the kinetic barriers involved in catalysis. Hence a detailed study of the pH dependence of catalysis is required.

Scheme 1



A straightforward approach to the study of the pH dependence of the 4-oxalocrotonate tautomerase (4-OT)[1] -catalyzed isomerization of α-keto acids (Scheme 2) is complicated by three observations. First, the preferred substrate **1a** as well as the slower substrate **1b** cannot be isolated. They can only be detected in rapid equilibrium with their respective stable dienols, **2a** and **2b**. Hence, kinetic studies of 4-OT have been carried out using the dienol

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[1] Abbreviations: 4-OT, 4-oxalocrotonate tautomerase; **1a**, 2-oxo-4-hexenedioate; **2a**, 2-hydroxy-2,4-hexadienedioate; **3a**, 2-oxo-3-hexenedioate; **2b**, 2-hydroxy-2,4-pentadienoate; 3-BP, 3-bromopyruvate; **4**, 2-oxo-1,6-hexanedioate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; pH*, apparent pH meter reading in ^2H_2O uncorrected for deuterium isotope effects; TSP, 3-(trimethylsilyl)-propionate-*2,2,3,3-d*$_4$; TOCSY, total correlation spectroscopy, NOE, nuclear Overhauser effect.

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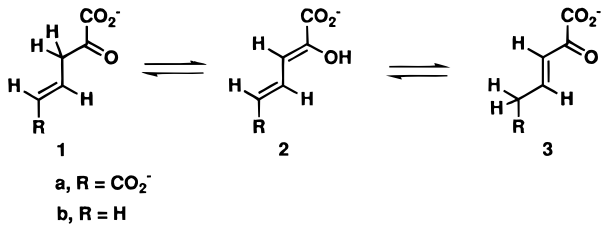
Scheme 2



intermediates (Whitman *et al.*, 1991; Lian & Whitman, 1993), which raise the question of whether the pK_a values of the groups involved in the mechanism can be determined in pH studies using an intermediate in the reaction. In addition, the kinetic parameters for the preferred substrates, **1a** and **2a**, suggest that these substrates are "sticky".[2] For such sticky substrates, the pH dependences of the kinetic parameters are not adequately described by simple ionization curves, and the apparent pK_a values may differ significantly from the true pK_a determined with a nonsticky substrate[2] such as **2b** (Schmidt & Westheimer, 1971; Renard & Fersht, 1973; Cleland, 1977). Finally, **2a** has two carboxylate groups, one of which ionizes in the pH range under study, further complicating the analysis.

In this study, we have resolved the above problems in the following way. Previous kinetic studies have shown that the intermediate **2a** (Scheme 2) equilibrates rapidly with **1a** before it is slowly converted to **3a** in both the enzymatic and nonenzymatic reactions (Whitman *et al.*, 1991). This finding indicates that *the major rate-determining steps in the conversion of* ***1a*** *→* ***3a*** *and* ***2a*** *→* ***3a*** *are the same*. Entirely equivalent results were obtained with the intermediate **2b** (Lian & Whitman, 1993). It may therefore be concluded that, with 4-OT, pH-rate studies using the dienol intermediate will detect pK_a values of groups involved in the overall isomerization of substrate to product. To ensure that true pK_a values for the free enzyme were determined, we have utilized the nonsticky substrate **2b**, which does not ionize in the pH range under study.

The results obtained in this work show that the true pK_a value of the essential basic residue on the free enzyme, determined from the pH dependence of 4-OT-catalyzed isomerization of **2b,** agrees with both the directly measured pK_a of Pro-1 by ^{15}N NMR titration and the pK_a for inactivation of the free enzyme by bromopyruvate. These findings provide further strong evidence in support of the previous conclusion, based on affinity labeling studies, that Pro-1 is the general-base catalyst (Stivers *et al.*, 1996). The kinetic data also suggest a role for an electrophilic catalyst. A preliminary abstract of this work has been published (Stivers *et al.*, 1995b).

MATERIALS AND METHODS

Materials and General Methods. All chemicals and solvents were purchased from Aldrich Chemical Co. with the following exceptions. The syntheses of **2a** and **2b** are described elsewhere (Whitman *et al.*, 1991; Lian & Whitman, 1993). Biochemicals and buffers were obtained from Sigma Chemical Co. 4-Oxalocrotonate tautomerase was purified to homogeneity as described in the preceding paper (Stivers *et al.*, 1996). Kinetic data were obtained on a Hewlett Packard 8452A Diode Array spectrophotometer at 28 °C. The reported values of pH were determined using a Corning pH meter (model 24) equipped with a Corning semimicro combination electrode and calibrated with Fisher standard buffer solutions. Concentrations of 4-OT were determined by the method of Waddell (1956) and are reported with respect to the monomeric mass (6811).

pH Titration of ***2a***. A quantity of **2a** (113.9 mg), obtained in the fully protonated form (Whitman *et al.*, 1991), was dissolved in methanol (15 mL) and diluted with 25 mL of H_2O. The pH of the solution was 2.77. The titration was carried out by adding 100 μL quantities of a 0.1057 N NaOH solution and recording the pH meter reading. In the reverse direction, 50 μL quantities of a 0.2012 N solution of HCl were added, and the pH meter reading was recorded. The NaOH solution was standardized against potassium hydrogen phthalate, and the HCl solution was standardized using the NaOH solution (Skoog & West, 1975). The pK_a values were determined from the titration curve and were comparable in both directions.

pH Dependence of 4-OT Using ***2a***. The dependence of the rate of ketonization of **2a** to **3a** on pH was determined in 20 mM phosphate buffer, pH 4.8−11.5. Higher buffer concentrations and other buffers significantly accelerate the nonenzymatic ketonization of **2a** to **3a** and preclude accurate measurements of enzymatic rates (Whitman *et al.*, 1991). A portion of enzyme was removed from storage buffer, diluted into the buffer under study, and allowed to incubate for 2 h. In a second experiment, which gave the same results, the enzyme was diluted and incubated in storage buffer for 2 h (Whitman *et al.*, 1991). Subsequently, an aliquot was removed and added to a cuvette containing 1 mL of buffer at the desired pH value. The final concentration of enzyme was 9.5 nM. The reaction was initiated by the addition of a quantity of **2a** (final concentration 0.065−1.16 mM) from a 65 mM stock solution made up in ethanol. The final concentration of ethanol in each reaction mixture did not exceed 2.5% (v/v). No significant inhibition of activity is observed at these concentrations. The cuvettes were mixed by inversion, and the rate of formation of **3a** was monitored at 236 nm (Whitman *et al.*, 1991). Initial rates were determined from plots of absorbance vs time at 236 nm at each pH value as described elsewhere (Whitman *et al.*, 1991). The pH values of reaction mixtures were checked before and after reaction. The pH dependences of the kinetic parameters were fitted to eqs 6 and 7 (see Results) by nonlinear regression analysis with Grafit (Erithacus Software Ltd., Staines, U.K.).

pH Dependence of 4-OT Using ***2b***. The dependence of the rate of ketonization of **2b** to **3b** on pH was determined in 20 mM phosphate buffer, pH 5.5−9.3 as described above with the following modifications. The final concentration of enzyme in each cuvette was 660 nM. The rate of formation of **3b** was monitored at 232 nm ($\epsilon = 5.99 \times 10^3$ M^{-1} cm^{-1}), and initial rates were determined from plots of absorbance vs time at 232 nm at each pH value. The procedure used to generate **2b** results in a mixture of **2b** and **3b** which is dissolved in ethanol (Lian & Whitman,

[2] Using the terminology of Cleland (1977), a sticky or Briggs−Haldane substrate has a rate of dissociation from the ES complex that is comparable to, or less than, the forward rate of its conversion to product. Accordingly, for a nonsticky or Michaelis−Menten substrate, the rate of its dissociation from the ES complex is fast compared to the forward conversion rate. The terms sticky and nonsticky may also be used to describe protons involved in catalysis.

Table 1: Summary of the pH Dependence of Kinetic and Inactivation Parameters for 4-OT[a]

substrate or inactivator	$(k_{cat})^{max}$ (s^{-1})	$(k_{inact})^{max}$ (s^{-1})	$(k_{cat}/K_M)^{max}$ (M^{-1} s^{-1})	$(k_{inact}/K_I)^{max}$ (M^{-1} s^{-1})	pK_{H2E}	pK_{HE}	pK_{H2ES}	pK_{HES}	pK_{H2EI}
2a	$1.10 \pm 0.07 \times 10^3$		$3.9 \pm 0.2 \times 10^6$		5.2 ± 0.1[b]	10.3 ± 0.2[b]	6.5 ± 0.2[b]	9.6 ± 0.3[b]	
2b	0.40 ± 0.02		355 ± 30		6.2 ± 0.3	9.0 ± 0.3	7.7 ± 0.2	8.5 ± 0.3	
3-bromopyruvate[c]		0.06 ± 0.02		14 ± 3	6.7 ± 0.3	ND[d]			5.3 ± 0.4

[a] These rate constants were obtained at 30 °C in 20 mM sodium phosphate buffer, pH 7.3. The rate constants are reported with respect to the concentration of 4-OT monomer as determined by the method of Waddell (1956). The previously reported kinetic parameters for **2a** (Whitman *et al.*, 1991) differ significantly from the values reported here because the previous k_{cat} values were normalized to tetramer concentration using a protein assay standardized against bovine serum albumin. [b] The pK_a values for this substrate are apparent values (Cleland, 1977; see text). [c] These results are from Stivers *et al.* (1996, previous paper). [d] ND, not determined.

1993). The concentration of **2b** is determined as follows. A small quantity of the stock solution is quantitatively converted to **3b** using a large excess of 4-OT. The initial concentration of **3b** is subtracted from the final concentration of **3b**, and the difference corresponds to the concentration of **2b** in the original mixture. By this method, the stock solution was determined to be 25 mM in **2b**. The concentration of **3b** is determined from its absorbance at 232 nm. The pH dependences of the kinetic parameters were fitted to eqs 3 and 4 by nonlinear regression analysis as described for **2a**.

^{15}N-NMR Spectroscopy. The pK_a values for the amino groups of proline-1 and the model compound proline amide were determined by monitoring the pH dependence of the ^{15}N-chemical shift of the secondary amino nitrogen resonance. The ^{15}N chemical shifts were referenced to external liquid ammonia as described (Weber *et al.*, 1993). A Varian Unity-Plus 500 NMR spectrometer operating at 50.659 MHz for ^{15}N was used. Spectra were acquired without proton decoupling using the outer coil of a 5 mm broad-band indirect detection probe. The titrations were performed using samples which were either 3.5 mM in monomers of uniformly ^{15}N-labeled 4-OT or 0.5 M in proline amide (natural abundance ^{15}N) at 30 °C by adding small aliquots of 1 M HCl or NaOH to the samples. The acquisition parameters for the enzyme and proline amide titrations were, respectively, as follows: spectral width, 12 001.2 and 10 000 Hz; acquisition time, 0.683 and 0.202 s; relaxation delay, 0.1 and 1.2 s; total number of transients, 10 800−41 000 and 2048−3600. The titration of 4-OT was found to be reversible, and the enzyme retained 85% of its initial activity at the conclusion of the experiment. The pK_a values were determined from a nonlinear least-squares fit of the data to eq 1, where n is the Hill coefficient and δ_1 and δ_2 are the

$$\delta\ (\mathrm{ppm})^{app} = \frac{\delta_1 + \delta_2(10^{pH-pK_a})^n}{[(10^{pH-pK_a})^n + 1]} \qquad (1)$$

limiting chemical shifts at low and high pH, respectively.

Titration of His Residues by ^{1}H-NMR. Proton NMR spectra at 600 MHz were obtained on a Bruker AM 600 NMR spectrometer by collecting 4K data points over a spectral width of 7246 or 6849 Hz using acquisition times of 0.276 or 0.292 s, respectively. The sample contained, in ^2H_2O, 1.75 mM unlabeled 4-OT, and 2 mM Tris-d_{11}, at an initial pH* = 7.26 in a total volume of 0.475 mL. The pH titrations were performed at 30 °C by the addition of 1−5 μL portions of 0.1 M KOD or DCl to the sample. The pH* of the sample was measured before and after the spectrum was acquired; the observed ΔpH was always ≤0.05 units. The spectra were referenced to internal TSP. The pK_a values were determined from a nonlinear least-squares fit to eq 1 of the pH dependences of the chemical shifts of the assigned imidazole proton resonances of His-6 and His-49 (Stivers *et al.*, 1994, 1995a). No corrections for the deuterium isotope effect on the imidazolium pK_a values in ^2H_2O were made because imidazolium pK_a's determined by uncorrected glass electrode readings in ^2H_2O agree well with those determined in H_2O due to equal and opposite isotope effects on the pK_a values and the glass electrode reading (Meadows, 1972).

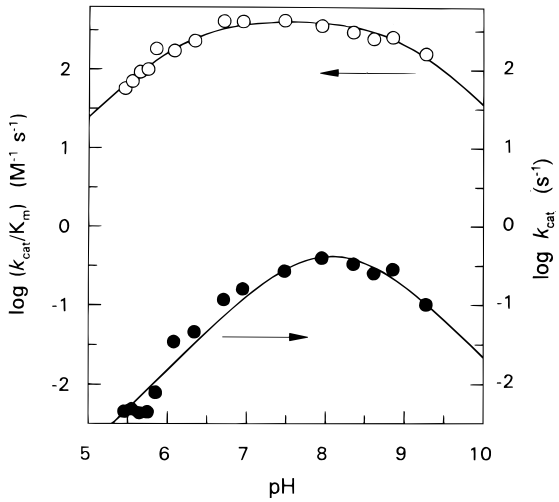
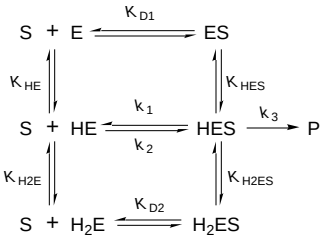


FIGURE 1: pH dependence of the kinetic parameters for the 4-OT-catalyzed isomerization of **2b**. The pH dependence of log(k_{cat}/K_m) (open circles) and log k_{cat} (filled circles) are shown. The curves were computed from a nonlinear least-squares fit of the data to eqs 3 and 4, respectively. The pK_a values are reported in Table 1.

RESULTS

*pH Dependence of the Kinetic Parameters for the Isomerization of **2b***. The values for k_{cat} and k_{cat}/K_m were determined over the pH range 5.4−9.5. The relatively low k_{cat} = 0.40 ± 0.02 s^{-1} and the high K_m = 1.1 mM for **2b** (Table 1), which are, respectively, ∼2700-fold less than k_{cat} and 4-fold greater than K_M of **2a**, indicate that the 4-OT·**2b** complex is in rapid equilibrium with free 4-OT and **2b**. Therefore, the pH dependence of the kinetic parameters for **2b** can be analyzed using equilibrium assumptions. The data for **2b** (Figure 1) show a bell-shaped dependence on pH with limiting slopes of unity on either side of the pH maximum, indicating that a basic and acidic group on the enzyme are important for catalysis and that the HE form of the enzyme (eq 2) is catalytically active. The substrate (pK^{COOH} ∼2.4) does not titrate in the pH range studied. The data were modeled using eq 2 where K_{HE} and K_{H2E} are the ionization constants for the free enzyme, K_{HES} and K_{H2ES} are the ionization constants of the E·**2b** complex, and K_{D1} and K_{D2}



(2)

are the dissociation constants of **2b** from the E and H_2E forms of the enzyme, respectively.

For this model, $K_m = (k_2 + k_3)/k_1$, and $k_3 = k_{cat}$. Under rapid equilibrium conditions, $k_2 \gg k_3$ and K_m will simplify to $k_2/k_1 = K_S$, the dissociation constant of **2b** from the HES complex. The pH dependences of (k_{cat}/K_m) and k_{cat} are given by eqs 3 and 4 (Cleland, 1977):

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)^{max}}{1 + \frac{[H^+]}{K_{H_2E}} + \frac{K_{HE}}{[H^+]}} \quad (3)$$

$$k_{cat} = \frac{(k_{cat})^{max}}{1 + \frac{[H^+]}{K_{H_2ES}} + \frac{K_{HES}}{[H^+]}} \quad (4)$$

where $(k_{cat}/K_m)^{max}$ and $(k_{cat})^{max}$ are the maximal values for these parameters. A nonlinear least-squares fit of the pH dependence of k_{cat}/K_m to the logarithmic form of eq 3 gave pK_a values for the free enzyme of pK_{H2E} = 6.2 ± 0.3 and pK_{HE} = 9.0 ± 0.3. These values can be confidently assigned to the free enzyme because **2b** has no ionizable groups in the pH range 5.5−9.5.

A fit of the pH dependence of k_{cat} to the logarithmic form of eq 4 gave pK_a values for the enzyme·**2b** complex of pK_{H2ES} = 7.7 ± 0.3 and pK_{HES} = 8.5 ± 0.3. Correction of these pK_a values using the equations describing partially overlapping ionizations (Cleland, 1977) gives pK_{H2ES} and pK_{HES} values of 7.8 and 8.4, which are not significantly different from the uncorrected values.

*pH Dependence of the Kinetic Parameters for the Isomerization of **2a***. For the preferred substrate **2a** the k_{cat}/K_m = 3.9×10^6 M^{-1} s^{-1} and $k_{cat} = 1.1 \times 10^3$ s^{-1} are 10^4-fold and 2700-fold greater, respectively, than the values for the nonsticky substrate **2b**. These larger k_{cat} and k_{cat}/K_m values for **2a** and the 4-fold smaller value for K_m, as compared to **2b,** suggest that **2a** is a sticky substrate. The expressions for k_{cat}/K_m and k_{cat} under steady-state conditions are complex, and a rigorous analysis would require knowledge of the relative stickiness of the substrate and of protons to the enzyme, which are unknown. In the absence of this information, it is more reasonable to model the data using equilibrium assumptions and then qualitatively interpret the deviations from nonideality using the steady-state equations and the true pK_a values obtained with **2b**.

A plot of log(k_{cat}/K_m) for **2a** (Figure 2) shows a bell-shaped dependence on pH with limiting slopes of two and one on the ascending and descending limbs, respectively. This pH response indicates the importance of two ionizable groups in the free enzyme or free substrate at pH values <6 and a single ionizable group at pH values >9. The plot of log k_{cat} vs pH is also bell-shaped, but the limiting slopes are unity

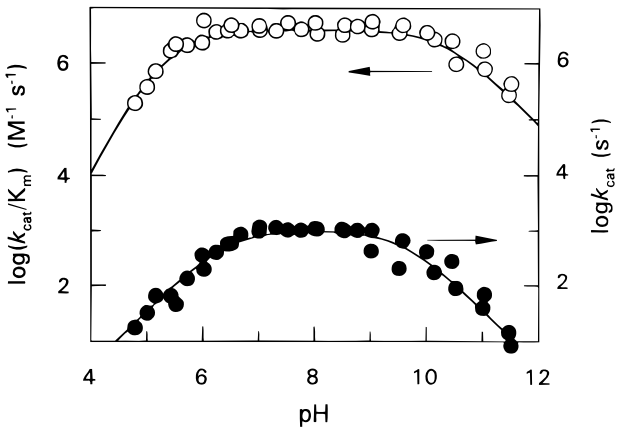
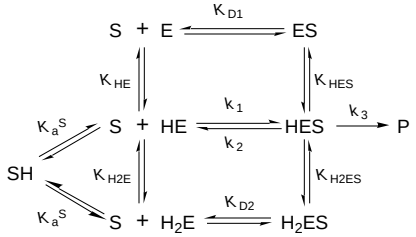


FIGURE 2: pH dependence of the kinetic parameters for the 4-OT-catalyzed isomerization of **2a**. The pH dependence of log(k_{cat}/K_m) (open circles) and log k_{cat} (filled circles) are shown. The curves were computed from a nonlinear least-squares fit of the data to eqs 6 and 7, respectively. The pK_a values are reported in Table 1.

on both sides of the pH maximum indicating the importance of a basic and acidic group in the enzyme−substrate complex.

The data for **2a** were modeled according to eq 5, where the ionization constants (K_{HE}, K_{H2E}, K_{HES}, K_{H2ES}), dissociation constants (K_{D2}, K_{D1}), and maximal kinetic constants have the same definitions as in eq 2. An additional constant (K_a^S) describing the ionization of the 6-carboxylic acid group of **2a** (SH, pK_a^S = 5.4) is also included in eq 5.[3] The ionizations for the 1-carboxylic acid group (pK_a = 2.9) and the 2-hydroxyl group (pK_a = 12.2) of **2a** would not be detected in the pH range studied.



(5)

The pH dependences of k_{cat}/K_m and k_{cat} using the model described by eq 5 are given by eqs 6 and 7 (Segel, 1975).

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)^{max}}{\left(1 + \frac{[H^+]}{K_a^S}\right)\left(1 + \frac{[H^+]}{K_{HE}} + \frac{K_{H2E}}{[H^+]}\right)} \quad (6)$$

$$k_{cat} = \frac{(k_{cat})^{max}}{1 + \frac{[H^+]}{K_{HES}} + \frac{K_{H2ES}}{[H^+]}} \quad (7)$$

In this analysis it is reasonably assumed that only the dianionic form of **2a** (S, eq 5) is a competent substrate, because the monoanionic substrate **2b** is 2700-fold less active under k_{cat} conditions (Table 1). Furthermore, it is reasonable to exclude all complexes involving the protonated substrate

[3] Intramolecular general-base catalysis of C-3 proton transfer by the 6-carboxyl group of **1a** in aqueous solution or on 4-OT is unlikely because this group is positioned *trans* to the C-3 methylene group (Whitman *et al.*, 1991).

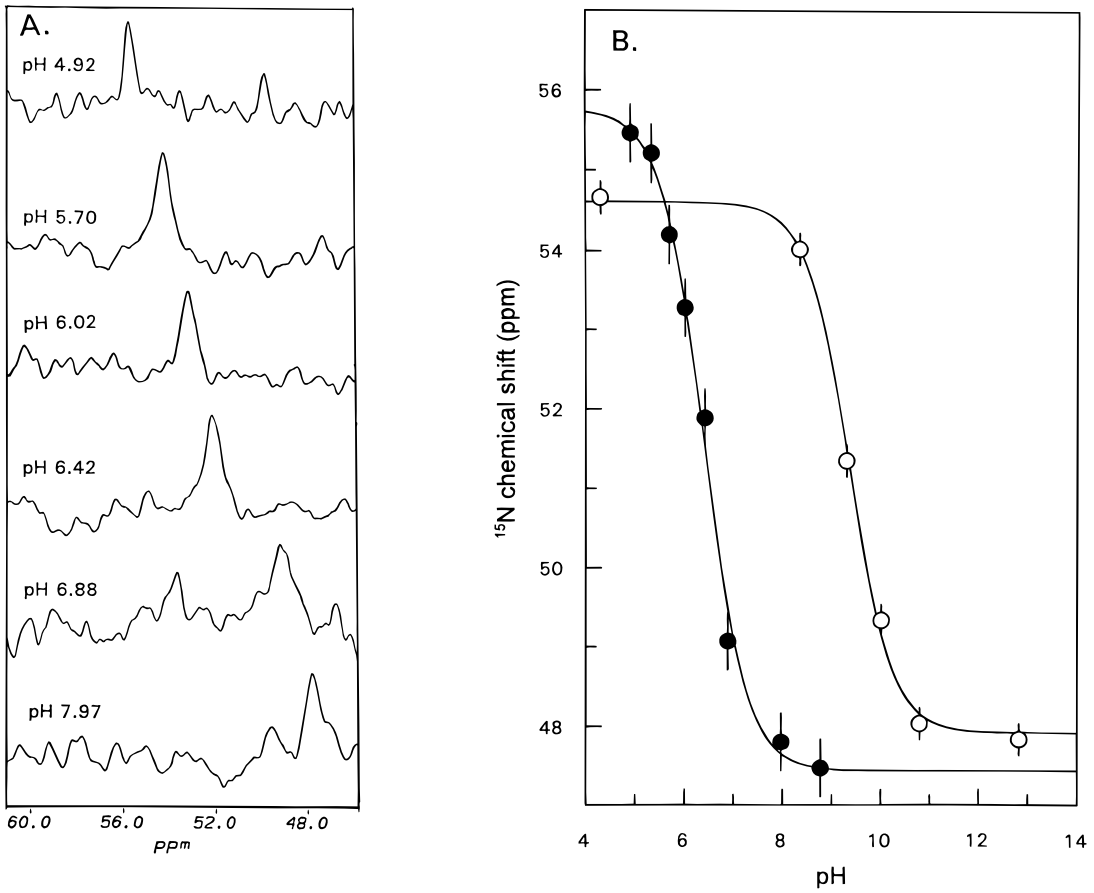


FIGURE 3: Determination of the pK_a of the amino proton of proline-1 and free proline amide by ^{15}N NMR spectroscopy (T = 30 °C). (A) ^{15}N NMR spectra showing the pH dependence of the chemical shift of the amino nitrogen atom of proline-1. The spectra were processed using 10 Hz line broadening. (B) pH titration curves for the amino nitrogen atom of proline-1 (closed circles) and the model compound proline amide (open circles). The curves computed are from a nonlinear least-squares fit to eq 1.

SH from the mechanism because the slope of unity on the ascending limb of log k_{cat} vs pH (Figure 2) indicates that a single ionizable group is important in the enzyme−substrate complex. For **2a**, a nonlinear least-squares fit of the pH dependence of k_{cat}/K_m to the logarithmic form of eq 6 yields pK_a values of 5.2 ± 0.1 and 5.4 ± 0.1 on the acidic limb and pK_{HE} = 10.3 ± 0.2 on the alkaline limb. The two pK_a values of 5.2 and 5.4 on the acidic limb may reasonably be assigned to the free enzyme (pK_{H2E}) and free substrate (pK_a^S), respectively, because the higher pK_a is identical to that determined by direct titration of the substrate. Accordingly, if a pK_a^S = 5.4 is explicitly assigned during the curve fitting process, a pK_{H2E} = 5.24 ± 0.08 is also obtained. Alternatively, as indicated by these overlapping pK_a values, the data are equally well fit by assuming pK_{H2E} = pK_a^S = 5.32 ± 0.04. However, the possibility cannot be excluded that *both* pK_a^S and pK_{H2E} are perturbed from their true values of 5.4 and 6.2, respectively. Hence an alternative fit of the data assuming equal perturbations yields values for pK_a^S = 4.9 ± 0.2 and pK_{H2E} = 5.6 ± 0.2, i.e., both are perturbed by −0.6 units. A nonlinear least-squares fit of the data for the pH dependence of k_{cat} to the logarithmic form of eq 7 gives pK_{H2ES} = 6.5 ± 0.2 and pK_{HES} = 9.6 ± 0.3.

pK_a *Value of Pro-1*. The similar pK_a values of k_{cat}/K_m for substrate **2b** (6.2 ± 0.3) and of k_{inact}/K_I for modification of Pro-1 by 3-BP [6.7 ± 0.3 (Stivers *et al.*, 1996)] indicates that the pK_a of the ammonium group of Pro-1 in the free enzyme is in the range 6.2−6.7. These kinetically determined pK_a values are confirmed by direct pH titration of the uniformly ^{15}N-labeled enzyme using ^{15}N NMR (Figure 3A). The ^{15}N-chemical shift of Pro-1 is well resolved from the other ^{15}N resonances of 4-OT over the pH range 4.9−8.8, and pH titration monitoring this resonance yields a pK_a = 6.4 ± 0.2 and a Hill coefficient (n) of 1.0 ± 0.05 (closed circles, Figure 3B). This experiment directly demonstrates that Pro-1 has the correct pK_a and is in the required protonation state to serve as the general base in the reaction mechanism. In contrast, titration of the ammonium group of proline amide using natural abundance ^{15}N NMR spectroscopy yielded a pK_a = 9.4 ± 0.2 (open circles, Figure 3B). Therefore, the enzymatic environment lowers the pK_a of the ammonium group of Pro-1 by approximately 3 units as compared to proline amide, a suitable model compound.

pH Titrations of Other Active Site Residues by NMR. The pK_a values for the imidazolium groups of the two histidine residues and the ϵ-ammonium groups of the three lysine residues of 4-OT are of interest for two reasons. First, the pH dependence of k_{cat}/K_m for **2b** indicates the importance of a basic group on the free enzyme with a pK_a = 6.2 ± 0.3, which suggests an unperturbed histidine residue. Secondly, His-49 and Lys-47 are nearby in the linear sequence of 4-OT to Phe-50, which has been identified as an active site residue by NMR studies (Stivers *et al.*, 1994).

The pH titrations of His-6 and His-49 by proton NMR are shown in Figure 4A. The sequence-specific assignments for the two aromatic ring protons of both histidines were made on the basis of intraresidue NOEs to their own β-protons, which were then linked to the intraresidue

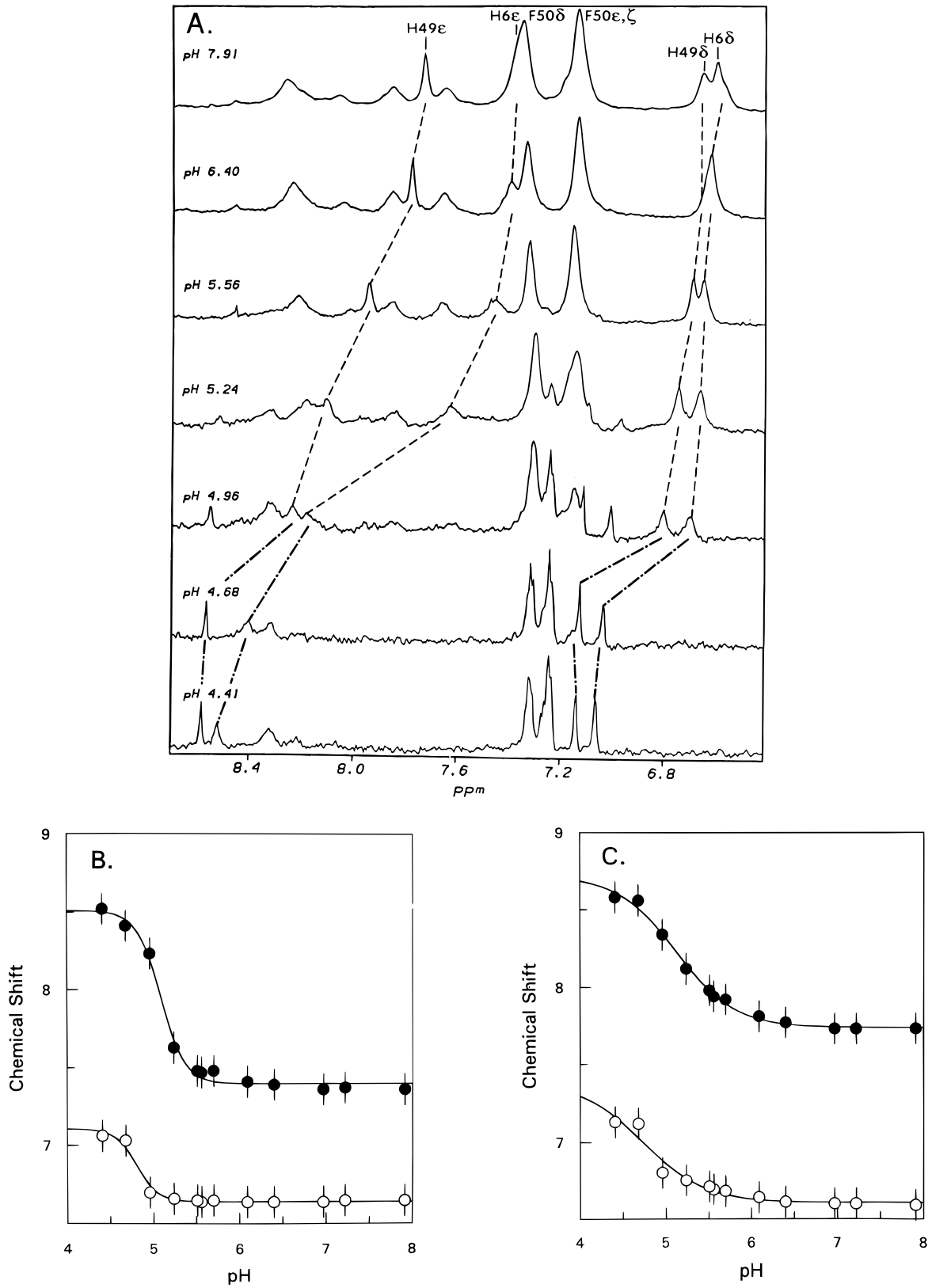


FIGURE 4: Titration of the histidine residues of 4-OT by ^{1}H NMR (T = 30 °C). (A) Downfield region of the 1D proton NMR spectra of 4-OT in D_2O in the range pH* = 4.41−7.91. Below ~pH 4.96 the enzyme is in slow exchange between two forms resulting in two sets of resonances for the ϵ and δ protons of H49 and H6, as well as the ring protons of F50. (B and C), Plots of the pH dependence of the chemical shifts for the ϵ (closed circles) and δ (open circles) ring protons of H6 (B) and H49 (C). The curves are computed from a nonlinear least-squares fit to eq 1.

backbone C_α-H and N-H resonances using a TOCSY experiment. Since all of the backbone resonances of 4-OT have been assigned using heteronuclear NMR methods (Stivers *et al.*, 1995a), the δ and ϵ ring protons of His-6 and His-49 were assigned unambiguously. The pH titration curves for these residues are shown in Figure 4, panels B and C, respectively. A nonlinear least-squares fit of the data to eq 1 gave a pK_a = 5.1 ± 0.2 (n = 1.2 ± 0.2) for His-49

and pK_a ≤5 (n = 3.0 ± 0.5) for His-6. The NMR spectra in Figure 4A show that the resonances of His-6, His-49, and Phe-50 are in slow exchange between two chemical environments at low pH values, between 4.5 and 5.0. Since the resonances are significantly narrower in one of the environments (∼10 Hz) than in the other (∼27 Hz), it is likely that this slow exchange represents the reversible dissociation of the hexamer into subunits. Therefore, in the pH range 4.5−5.0, the broader resonances of the native hexamer were used to define the titration curve. Cooperativity in subunit dissociation at low pH values would account for the large Hill coefficient in the titration of His-6.

Lower limit pK_a values for the side chain ammonium groups of Lys-47 and the other two lysine residues (Lys-16, Lys-59) of ≥9.8 can be made on the basis of the observation that the ^{15}N chemical shifts of the side chain nitrogens of these residues did not change in the pH range 4.9−8.8 when uniformly ^{15}N-labeled 4-OT was titrated (data not shown). These lower limit values were calculated using the Henderson−Hasselbalch equation assuming a detection limit of $\Delta\delta^{15}$N = 1 ppm and a maximal $\Delta\delta^{15}$N = 10 ppm for deprotonation of a lysine residue, as found for lysine methyl ester (Martin *et al.*, 1981).

DISCUSSION

A goal in the study of the pH dependence of an enzyme-catalyzed reaction is to determine the pK_a values of essential acidic and basic groups on the enzyme in order to provide a chemical basis for catalysis. The kinetically determined pK_a values for k_{cat}/K_m, which must be obtained using a nonsticky substrate, should reflect catalytically important ionizations in the free enzyme. These kinetically determined pK_a values may then be compared with the pK_a values of specific groups on the free enzyme which can be measured directly by NMR spectroscopy or indirectly by the pH dependence of k_{inact}/K_I in affinity labeling studies.

*pH Dependence of the Kinetic Parameters for **2b***. On the basis of the ascending slope of unity for the pH dependence of log (k_{cat}/K_m) for the nonsticky substrate **2b** (Figure 1), it is concluded that one basic group on the free enzyme, with a pK_a of 6.2 ± 0.3, is important for activity. This assignment is reasonable because there are no ionizable groups on the free substrate in this pH range. Similarly, the pK_a on the descending limb indicates that an acidic group on the free enzyme with a pK_a of 9.0 ± 0.3 is required for activity. The pK_a of 6.2 ± 0.3 for the essential basic group on the free enzyme is the same, within error, as that determined from the pH dependence of k_{inact}/K_I for affinity labeling of Pro-1 by 3-BP (6.7 ± 0.3, Table 1) and the pK_a for Pro-1 measured directly by ^{15}N NMR titration (pK_a = 6.4 ± 0.2). These agreements provide cogent evidence that **2b** is a nonsticky substrate. These results, and the proximity of Pro-1 to the active site as determined by X-ray crystallography (Subramanya *et al.*, 1996; Whitman *et al.*, 1995), establish that this residue is the essential basic group on the enzyme. The identity of the acidic group with a pK_a = 9.0 ± 0.3 on the free enzyme has not yet been established. This pK_a most likely represents a true pK_a of a group on the enzyme, and not the denaturation of the enzyme at high pH, because the k_{cat}/K_m using **2a** is unaffected until pH ∼10 (Figure 2).

While the hydroxyl groups of an amino-terminal serine and threonine have been shown to serve as the nucleophiles in penicillin acylase (Duggleby *et al.*, 1995) and in a large bacterial proteinase (Seemüller *et al.*, 1995), respectively, and the α-amino group of an N-terminal threonine has been shown to function as a nucleophile in the T4 endonuclease V reaction (Schrock & Lloyd, 1993), to our knowledge, 4-OT provides the first reported example of an amino-terminal proline residue serving as a general base. Because such a role is unprecedented, both for an amino-terminal residue and for proline, its role was established by several independent methods: mechanism-based modification of Pro-1 in the crystalline state (Whitman *et al.*, 1995); affinity labeling in solution which targets only Pro-1 and blocks the amino terminus of 4-OT; observation of comparable pK_a values for k_{inact}/K_I in affinity labeling studies (Stivers *et al.*, 1996, previous paper), for k_{cat}/K_m in catalysis with a nonsticky substrate, and for Pro-1 itself, as directly measured by ^{15}N NMR titration. The observation of comparable pK_a values for the free enzyme determined with an α-keto substrate analog, 3-BP, and with the dienolic intermediate **2b** further validates the use of intermediates in pH rate studies of 4-OT.

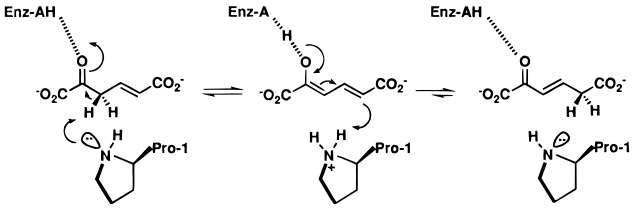


FIGURE 5: Minimal catalytic mechanism of 4-OT. Proline-1 (pK_a = 6.4) is proposed to remove the 3-proton of substrate **1a** concerted with electrophilic catalysis at the C-2 oxygen atom by a putative enzymic general acid (pK_a = 9.0). The dienolic intermediate **2a** is then reketonized and protonated stereospecifically at the 5-position to yield the *pro-[S]* enantiomer of **3a** (Whitman *et al.*, 1992).

The pH dependence of k_{cat} for **2b**, which yields the important pK_a values in the ES complex, also shows a bell-shaped profile, confirming the requirement of a basic and an acidic group in the isomerization mechanism. The increase in pK_a of the basic group and decrease in pK_a of the acidic group on substrate binding most simply reflect changes in the environment of the catalytic residues in the enzyme−substrate complex, although pK_{ES} values may also be perturbed when the ratio of nonproductive to productive ES complexation is pH dependent (Fersht, 1985).

A reasonable mechanistic interpretation for the requirement of an enzymic base and acid in the isomerization reaction is that the enzyme utilizes a general base to remove the carbon bound 3-*pro-R* proton of **1a** (Lian & Whitman, 1993) and a general acid to donate a proton or hydrogen bond in order to stabilize the developing negative charge on the C-2 oxygen atom of the putative dienolic intermediate (Figure 5). A similar requirement for general acid−base catalysis has been established for the enzyme Δ^5-3-ketosteroid isomerase, which catalyzes the isomerization of β,γ-unsaturated ketosteroids, where Asp-38 and Tyr-14 have been identified as the general base and acid, respectively (Kuliopulos *et al.*, 1989, 1990).

*pH Dependence of the Kinetic Parameters for **2a***. The preferred dienolic substrate, **2a**, also shows a bell-shaped pH dependence for k_{cat}/K_m, but significantly different pK_a values from those of **2b** (Table 1). A plausible explanation is that the altered pK_a values for **2a** reflect the differential partitioning of the EH·**2a** complex as a function of pH between the forward catalytic step (k_3, eq 5) and dissociation (k_2, eq 5) to form free enzyme and **2a**, i.e., **2a** is a sticky

substrate. The apparent values for pK_{HE} and pK_{H2E} (eq 5) obtained under these conditions would be expected to be lower and higher, respectively, than the true values. The directions and magnitudes of the observed shifts are consistent with this mechanism (Table 1).[4]

As observed with the pH dependence of k_{cat}/K_m for **2a** and **2b**, the observed pK_a values for k_{cat} (Table 1) differ significantly with these two substrates. In general, it is not unexpected to find a different pH dependence of k_{cat} for substrates of different reactivity. These pK_a differences may arise from the stickiness of **2a** or a proton in the HES complex (Cleland, 1977), from a pH dependence of nonproductive binding for either substrate (Fersht, 1985), or from differences in the structure of the ES complex for each substrate, causing different perturbations in the pK_a values for the active site residues in the two complexes.

*Catalytic Contribution of the 6-Carboxylate Group of **2a***. The 10^4-fold greater k_{cat}/K_m value for **2a** as compared to **2b**, which corresponds to a 5.6 kcal/mol lowering of the kinetic barrier, cannot be attributed to a significant difference in the intrinsic chemical reactivity of these two compounds, because the kinetics of the nonenzymatic reactions are similar (Whitman *et al.*, 1991; Lian & Whitman, 1993), nor can it be attributed solely to an increase in affinity because K_D for **2a** is 21 ± 7 μM [5] while the K_S of **2b** is 1.7 mM (Table 1).

Since the affinity of 4-OT for **2a** and **2b** differs by at most 120-fold, while the reactivity on the enzyme differs by 10^4-fold, it can be shown that ~60% of the total binding energy provided by the additional carboxylate group of **2a** is not expressed as tighter ground-state binding but is used to decrease the overall kinetic barrier for formation of **3a** by 5.6 kcal/mol.[3] Further support for a relatively small effect of the 6-carboxylate group on ground state affinity is provided by the weak K_s = 2.7 mM for the dicarboxylic partial substrate 2-oxo-1,6-hexanedioate (Stivers *et al.*, 1994). Thus, **2a** provides an example of the utilization of substrate binding energy to facilitate catalysis (Moore & Jencks, 1982; Jencks, 1987). A strong electrostatic interaction between the 6-carboxylate group of the substrate and a cationic residue of the enzyme which either distorts the ground state toward the transition state or stabilizes the transition state would explain the lower kinetic barrier for **2a** versus **2b**. The preliminary X-ray structure shows that two arginine residues at the active site are positioned to interact with the C-1 and C-6 carboxylate groups of the substrate (Subramanya *et al.*, 1996).

Kinetic Competence of the Dienolic Intermediate. The kinetic and NMR results presented above and those reported previously (Whitman *et al.*, 1991) show that the dienolic intermediate **2a** reacts with 4-OT under k_{cat}/K_m conditions nearly as fast as substrate **1a**, with a pH dependence indicating the assistance of two enzymic groups with pK_a values of 6.4 and 9.0. From these pK_a values it can be calculated that only 0.22% of the total enzyme present at pH 7.3 is in the correct protonation state to catalyze the ketonization of **2a** to form **1a** (Figure 5). Correcting the observed value of k_{cat}/K_m for **2a** (3.9×10^6 M^{-1} s^{-1}) by the fraction of the enzyme in the correct protonation state (0.0022) yields a second-order rate constant of 1.8×10^9 M^{-1} s^{-1} for **2a** which is at least 5-fold greater than encounter rates for diffusion-controlled enzyme reactions (Fersht, 1985; Xue *et al.*, 1990). This larger value suggests that an alternative mechanism, involving the predominant protonation state of 4-OT, facilitates the conversion of **2a** to **1a**. While this mechanism is unknown, one possibility is that Pro-1 in its neutral state receives the enolic hydroxyl proton of **2a** and transfers it to C-3 of the carbanion to yield **1a**. A similar mechanism has been proposed by Nickbarg *et al.* (1988) to explain the activity of the H95Q mutant of triosephosphate isomerase as well as by Hawkinson *et al.* (1991) to explain the rapid reaction of the externally generated dienol intermediate with ketosteroid isomerase. Alternatively, in the sequestered active site, strain (Cardinale & Abeles, 1968; Washabaugh & Jencks, 1989a,b and references cited therein) and substrate desolvation (Stivers & Washabaugh, 1991; Gerlt & Gassman, 1993) may facilitate the conversion of **2a** to **1a** without general acid−base catalysis.

A Low pK_a for Pro-1. As shown by Figure 3B, the pK_a of 6.4 for Pro-1 is ~3 units lower than that of the model compound proline amide, corresponding to a $\Delta\Delta G^o$ of 4.0 kcal/mol. Plausible mechanisms by which the pK_a of the amino group of Pro-1 might be lowered on the enzyme include (1) the location of Pro-1 near the positive (amino) end of a helix dipole, thereby shifting the protonation equilibrium toward the neutral form (Hol, 1985; Lodi & Knowles, 1993), (2) an active site environment of low dielectric constant, and (3) juxtaposition of Pro-1 near other cationic residues causing unfavorable electrostatic interactions which favor the neutral form of Pro-1.

The first mechanism is eliminated by the crystal structure of 4-OT (Subramanya *et al.*, 1996) and by the solution secondary structure as determined by NMR (Stivers *et al.*, 1995a) which show that Pro-1 is the first residue of a parallel β-strand and is more than 15Å from the nearest positive end of an α-helix. The second mechanism is suggested by the 1.3 nm red-shifted absorption maximum of Phe-50, the sole UV chromophore of 4-OT, which is located at the active site 6Å from Pro-1 (Subramanya *et al.*, 1996). This red-shift, when correlated with the absorption maxima of *N*-acetylphenylalanine ethyl ester found in organic solvents of varying dielectric constants (ϵ), yields a local ϵ_{prot} = 22 ± 4 at the active site of 4-OT,[6] similar to the value of 18 ± 2 found on ketosteroid isomerase (Li *et al.*, 1993). Using the Born approximation (eq 8; Rashin & Honig, 1985; Friedman & Krishan, 1973)

$$\Delta\Delta G^o = \frac{164.9(1/\epsilon_{prot} - 1/\epsilon_{water})}{r} \qquad (8)$$

with ϵ_{prot} = 22 ± 4, r = 2.17Å for secondary amines and ϵ_{water} = 77.9 at 25 °C, we calculate a $\Delta\Delta G^o$ of 2.4 kcal/mol, which is significantly lower than the 4.0 kcal/mol observed. Hence the third mechanism is also required. The additional

[4] Using the equation $pK_{H2E}^{app} = pK_{H2E}^{true} + \log(1 + k_3/k_2)$ (Cleland, 1977), it is possible to estimate the partitioning ratio k_3/k_2 (eq 5) using the measured pK_{H2E}^{true} = 6.2 for **2b** and the pK_{H2E}^{app} = 5.2 for **2a**. Such an analysis yields a k_3/k_2 ratio of ~9. A similar calculation using pK_{HE}^{app} and pK_{HE}^{true} yields a k_3/k_2 ratio of ~19.

[5] This K_D value for **2a** was calculated from the measured $k_{cat} = k_3$ and $K_m = (k_2 + k_3)/k_1$, the ratio $9 \le k_3/k_2 \le 19$ estimated from the pH dependence of k_{cat}/K_m for **2a** and **2b** (see footnote 4), and the relationship $K_D = K_m - k_3/k_1$. From this calculation $k_1 = 4.2 \times 10^6$ M^{-1} s^{-1} and $k_2 = 90$ s^{-1} for **2a**.

[6] J. T. Stivers, unpublished observations, 1995.

Scheme 3

K - G - **H** - **F** - G - I - G - G
47 49 50 54

effects of cationic arginine residues positioned at ~7.1Å (1.04 kcal/mol) and at ~8.6Å (0.86 kcal/mol) from the nitrogen of Pro-1 (Subramanya *et al.*, 1996), as calculated with a form of eq 8 lacking the term $1/\epsilon_{water}$, yield a total $\Delta\Delta G^o$ value of 4.3 kcal/mol, comparable to that observed. Therefore, the unusually low pK_a of Pro-1 may result from a site of low dielectric constant and the electrostatic effects of nearby cationic residues.

The low pK_a values (Figure 4) for both histidines of 4-OT exclude these residues as the general base. Nevertheless, His-49 is near the active site in a glycine-rich loop (Scheme 3) adjacent to Phe-50, a known active site residue which receives transferred NOEs from the bound partial substrate 2-oxo-1,6-hexanedioate (Stivers *et al.*, 1994). Hence the low pK_a of 5.1 ± 0.2 for His-49 may result from the same factors that lower the pK_a of Pro-1. In contrast, the pK_a ≥9.8 for the ϵ-ammonium group of Lys-47 (Scheme 3) is not significantly lowered, suggesting this residue to be in a different environment.

Implications for Enzyme-Catalyzed Enolization. Two general mechanistic strategies have been proposed to explain the large rate enhancements observed for enzyme-catalyzed enolizations (Scheme 1). In the first mechanism (Albery & Knowles, 1976) the enzyme accelerates the reaction by lowering the thermodynamic barrier for formation of the enolic intermediate, such that $K_{eq} \approx 1$ on the enzyme (Scheme 1). An alternative mechanism (Gerlt & Gassman, 1993) proposes a substantial reduction in the intrinsic kinetic barrier[7] to formation of the enolic intermediate on the enzyme as compared to the intrinsic kinetic barrier in solution. The fundamental distinction between these mechanisms is that the first predicts a *stable enolic intermediate and the second requires an unstable intermediate*.

Assuming a mechanism of general acid-base catalysis (Scheme 1), an upper limit value for ΔG^o of 3.6 kcal/mol for the keto−enol tautomerization of **1a** to **2a** on 4-OT can be estimated from the equilibria shown in eq 9 (and

$$K_{eq} = \frac{K_a^{CH}}{K_a^{OH}}\frac{K_a^{HA}}{K_a^{BH}} = (K_{enol})\frac{K_a^{HA}}{K_a^{BH}} \quad (9)$$

$$\Delta G^o = 2.303RT[pK_{enol} + pK_a^{HA} - pK_a^{BH}] \quad (10)$$

equivalently expressed in terms of a free energy difference in eq 10), using the known keto−enol equilibrium constant $K_{enol} \approx 1$ (p$K_{enol} = 0$) in aqueous solution (Lian & Whitman, 1993), the pK_a value for Pro-1 (p$K_a^{BH} = 6.4$), and the pK_a value for the putative electrophilic catalyst (p$K_a^{HA} = 9.0$) (Figure 4 and Table 1). In eq 9 K_a^{CH} and K_a^{OH} are the acid dissociation constants of the 3-proton of **1a** and the enol hydroxyl, and the ratio K_a^{CH}/K_a^{OH} is K_{enol}, the keto−enol equilibrium constant. Thus, by lowering the thermodynamic barrier to enolization ($\Delta G_{enol} = -RT \ln K_{enol}$) such that K_{eq} is near unity, 4-OT would achieve a maximal catalytic advantage of only 3.6 kcal/mol. Further stabilization of the intermediate **2a** such that $K_{eq} \gg 1$ is unlikely because no catalytic advantage is gained if an enzyme binds an intermediate significantly tighter than substrate or product (Albery & Knowles, 1976). By itself, this purely thermodynamic mechanism is therefore not sufficient to explain the catalytic power of the enzyme because 4-OT lowers the kinetic barrier to enolization of **1a** to **2a** by at least 8.0 kcal/mol.[8] These calculations suggest that the *kinetic barrier* for formation of **2a** from **1a** must be lowered by at least an additional 4.4 kcal/mol. This conclusion differs significantly from that obtained with ketosteroid isomerase (Hawkinson *et al.*, 1994), where it was concluded that the primary function of the enzyme was to decrease the *thermodynamic barrier* for formation of the dienolic intermediate derived from 5-androstene-3,17-dione by lowering ΔG^o by ~10 kcal/mol, compared to the nonenzymatic reaction. The difference in the catalytic mechanism for these two enzymes is due in large part to the ~10^3-fold greater stability of the dienol **2a** as compared to the dienol derived from 5-androstene-3,17-dione in aqueous solution (Lian & Whitman, 1993; Hawkinson *et al.*, 1994). Hence, the catalytic mechanism utilized by these enzymes depends upon the relative stability of the intermediate and substrate when free in solution (Jencks, 1980).

Both the thermodynamic and kinetic barriers to enolization on an enzyme may be lowered by either of two mechanisms. In the first mechanism, concerted general acid−base catalysis of enolization occurs resulting in an enolic intermediate which is stabilized by a strong, low barrier hydrogen bond from the general acid (Cleland, 1992; Li *et al.*, 1993; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994; Zhao *et al.*, 1995). In the second mechanism, general base catalysis results in an enolate ion which is stabilized electrostatically by a cationic residue in an environment of low dielectric constant (Guthrie & Kluger, 1993). While for ketosteroid isomerase the first mechanism is likely (Li *et al.*, 1993; Zhao *et al.*, 1995), and the second mechanism can be excluded because the active site has no cationic residues (Kuliopulos *et al.*, 1989), for 4-OT, neither of these mechanisms can be excluded.

ADDED IN PROOF

Tchou and Grollman (1995) have recently suggested that the amino-terminal proline of the enzyme formamidopyrimidine−DNA glycosylase may function as a nucleophile.

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[7] The intrinsic kinetic barrier is the activation energy barrier in the absence of a thermodynamic driving force (i.e., when $\Delta G^o = 0$).

[8] This lower limit value of 8.0 kcal/mol was obtained by comparing k_{cat} (1100 s^{-1}) with the nonenzymatic rate constant for enolization (1.6 × 10^{-3} s^{-1}) (Whitman *et al.*, 1991).

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