Effects of Mutations of the Active Site Arginine Residues in 4-Oxalocrotonate Tautomerase on the pK_a Values of Active Site Residues and on the pH Dependence of Catalysis[†]

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ABSTRACT: The unusually low pK_a value of the general base catalyst Pro-1 (pK_a = 6.4) in 4-oxalocrotonate tautomerase (4-OT) has been ascribed to both a low dielectric constant at the active site and the proximity of the cationic residues Arg-11 and Arg-39 [Stivers, J. T., Abeygunawardana, C., Mildvan, A. S., Hajipour, G., and Whitman, C. P. (1996) *Biochemistry 35*, 814−823]. In addition, the pH−rate profiles in that study showed an unidentified protonated group essential for catalysis with a pK_a of 9.0. To address these issues, the pK_a values of the active site Pro-1 and lower limit pK_a values of arginine residues were determined by direct ^{15}N NMR pH titrations. The pK_a values of Pro-1 and of the essential acid group were determined independently from pH−rate profiles of the kinetic parameters of 4-OT in arginine mutants of 4-OT and compared with those of wild type. The chemical shifts of all of the Arg Nϵ resonances in wild-type 4-OT and in the R11A and R39Q mutants were found to be independent of pH over the range 4.9−9.7, indicating that no arginine is responsible for the kinetically determined pK_a of 9.0 for an acidic group in free 4-OT. With the R11A mutant, where k_{cat}/K_m was reduced by a factor of $10^{2.9}$, the pK_a of Pro-1 was not significantly altered from that of the wild-type enzyme (pK_a = 6.4 ± 0.2) as revealed by both direct ^{15}N NMR titration (pK_a = 6.3 ± 0.1) and the pH dependence of k_{cat}/K_m (pK_a = 6.4 ± 0.2). The pH−rate profiles of both k_{cat}/K_m and k_{cat} for the reaction of the R11A mutant with the dicarboxylate substrate, 2-hydroxymuconate, showed humps, i.e., sharply defined maxima followed by nonzero plateaus. The humps disappeared in the reaction with the monocarboxylate substrate, 2-hydroxy-2,4-pentadienoate, indicating that, unlike the wild-type enzyme which reacts only with the dianionic form of the dicarboxylic substrate, the R11A mutant reacts with both the 6-COOH and 6-COO$^-$ forms, with the 6-COOH form being 12-fold more active. This reversal in the preferred ionization state of the 6-carboxyl group of the substrate that occurs upon mutation of Arg-11 to Ala provides strong evidence that Arg-11 interacts with the 6-carboxylate of the substrate. In the R39Q mutant, where k_{cat}/K_m was reduced by a factor of 10^3, the kinetically determined pK_a value for Pro-1 was 4.6 ± 0.2, while the ionization of Pro-1 showed negative cooperativity with an apparent pK_a of 7.1 ± 0.1 determined by 1D ^{15}N NMR. From the Hill coefficient of 0.54, it can be shown that the apparent pK_a value of 7.1 could result most simply from the averaging of two limiting pK_a values of 4.6 and 8.2. Mutation of Arg-39, by altering the structure of the β-hairpin which covers the active site, could result in an increase in the solvent exposure of Pro-1, raising its upper limit pK_a value to 8.2. In the R39A mutant, the kinetically determined pK_a of Pro-1 was also low, 5.0 ± 0.2, indicating that in both the R39Q and R39A mutants, only the sites with low pK_a values were kinetically operative. With the fully active R61A mutant, the kinetically determined pK_a of Pro-1 (pK_a = 6.5 ± 0.2) agreed with that of wild-type 4-OT. It is concluded that the unusually low pK_a of Pro-1 shows little contribution from electrostatic effects of the nearby cationic Arg-11, Arg-39, and Arg-61 residues but results primarily from a site of low local dielectric constant.

4-Oxalocrotonate tautomerase (4-OT,[1] EC 5.3.2) is a hexameric enzyme, consisting of identical subunits of 62 amino acid residues (*1*, *2*), which catalyzes the isomerization of unconjugated α-keto acids such as 2-oxo-4-hexenedioate (**1**) to its conjugated isomer 2-oxo-3-hexenedioate (**3**) through the dienol intermediate 2-hydroxy-2,4-hexadienedioate (**2**) known commonly as 2-hydroxymuconate (Scheme 1) (*3*). Affinity labeling (*4*), kinetic analysis (*5*), chemical synthesis

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[1] Abbreviations: 4-OT, 4-oxalocrotonate tautomerase; NMR, nuclear magnetic resonance; CCM, *cis*,*cis*-muconate.

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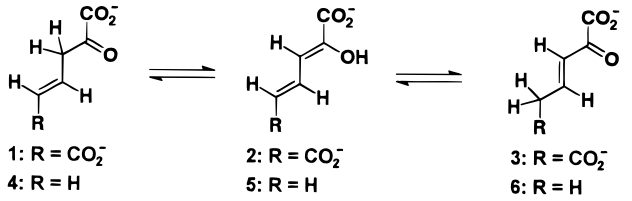
Scheme 1



(*6*), NMR (*7*, *8*), and crystallographic studies (*1*, *2*) identified the amino-terminal proline as the catalytic base in the 4-OT-catalyzed reaction with a pK_a value of 6.4. In addition, the descending limb in the bell-shaped pH−rate profile of wild-type 4-OT yielded a pK_a of 9.0 for an unidentified essential protonated group (*5*), suggesting that 4-OT might utilize a general acid catalyst to polarize the carbonyl group of the substrate.

The X-ray structure of the complex of 4-OT with the affinity label 2-oxo-3-pentynoate showed Arg-11, Arg-39, and Arg-61 to be at the active site, making them potential candidates for the putative acid catalyst as well as ligands for the two carboxylate groups of the substrate (*2*). In the previous paper (*9*), the $^{15}N\epsilon$ and $^{15}N\epsilon H$ resonances of all six arginine residues in NMR spectra of wild-type 4-OT were assigned, the contributions to catalysis of the three active site arginines (Arg-11, -39, and -61) were determined by site-directed mutagenesis, and a mechanism was proposed which provides a quantitative explanation of the 10^7-fold catalytic power of 4-OT. In this mechanism, Arg-11 interacts with the 6-carboxylate of substrate **1** to facilitate both substrate binding and catalysis, and Arg-39 interacts with the 1-carboxylate and the 2-keto group of substrate **1** to promote carbonyl polarization and catalysis, while Pro-1 mediates proton transfer from C-3 to C-5. However, the pK_a of 9.0 for the essential protonated group is much lower than that of a typical arginine (12.5), raising the possibility that Arg-39, like Pro-1, might have an unusually low pK_a (*1*, *5*).

The pK_a of 6.4 for Pro-1 is 3 units lower than that of the model compound proline amide, corresponding to a $\Delta\Delta G°$ of 4.0 kcal/mol. The decreased pK_a value of Pro-1 ensures that it is largely deprotonated at physiological pH so that it can function as the catalytic base. The mechanisms by which the pK_a of the amino group of Pro-1 might be lowered on the enzyme include (i) an active site environment of low dielectric constant and (ii) juxtaposition of Pro-1 near other cationic residues causing unfavorable electrostatic interactions which favor the neutral form of Pro-1 (*1*, *5*). Assuming that the 3 unit lower pK_a value of Pro-1 results only from a lower local dielectric constant, a dielectric constant near Pro-1 of $\epsilon_{prot} = 15.7$ is calculated using the Born approximation:

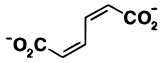
$$\Delta\Delta G° = \frac{164.9(1/\epsilon_{prot} - 1/\epsilon_{water})}{r} \qquad (1)$$

with $\Delta\Delta G° = 4.0$ kcal/mol, $\epsilon_{water} = 77.9$ at 25 °C, and $r = 2.17$ Å for secondary amines (*5*, *10*, *11*). An independent estimate of the dielectric constant near Pro-1 has been measured from the 1.3 nm red-shifted absorption maximum of Phe-50 (*5*), the sole UV chromophore of 4-OT, which is located at the active site 6 Å from Pro-1 (*2*). This red shift, when correlated with the absorption maxima of *N*-acetylphenylalanine ethyl ester found in organic solvents of varying dielectric constants, yields a local $\epsilon_{prot} = 22 \pm 4$, corresponding to a $\Delta\Delta G°$ value of only 2.4 ± 1.0 kcal/mol. Hence the unusually low pK_a of Pro-1 was proposed to result from both a site of low dielectric constant and the electrostatic effects of nearby cationic residues (*5*).

In this paper, we report the effects of mutations of these active site arginine residues on pH−rate profiles and on the pK_a values of Pro-1 determined by direct ^{15}N NMR titrations. It is concluded that no arginine residue is responsible for either the essential acid group with pK_a of 9.0 in the pH−rate profile or the unusually low pK_a of Pro-1.

EXPERIMENTAL PROCEDURES

Materials. Wild-type 4-OT and the four single mutants of 4-OT (R11A, R39A, R39Q, and R61A) were prepared as described in the preceding paper (*9*). The uniformly ^{15}N-labeled wild-type 4-OT and the R11A and R39Q mutants were prepared as described in the preceding paper (*9*). The syntheses of *cis*,*cis*-muconate (CCM) (**7**), an analogue of the



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reaction intermediate (*8*), and of substrates **2** (*3*, *12*) and **5** (*13*) have been described.

General Methods. Kinetic data were obtained on a Hewlett-Packard 8452A Diode Array spectrophotometer at 23 °C. Enzyme activity was monitored by following the formation of **3** at 236 nm (*3*). The cuvettes were mixed by a stir/add cuvette mixer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. Protein was analyzed by tricine sodium dodecyl sulfate−polyacrylamide gel electrophoresis under denaturing conditions on 16% gels on a vertical gel electrophoresis apparatus obtained from Gibco (*14*). Trichloroacetic acid was used instead of acetic acid in the staining and destaining solutions. The concentration of wild-type 4-OT and the mutant forms of 4-OT (in micrograms per milliliter) was determined from the difference in absorption measurements at 215 and 225 nm multiplied by the factor 144 (*4*, *15*).

*pH−Rate Profiles of R11A, R39A, R39Q, and R61A Using **2**.* The pH dependence of the rate of ketonization of **2** to **3** by the four mutants of 4-OT was determined in 50 mM sodium phosphate buffer over the indicated pH range at 23 °C using the following modifications to a previously described procedure (*5*). For the R11A 4-OT, the final enzyme (monomer) concentration was either 7.1 μM (pH 4.4−5.8) or 1.4 μM (pH 6.0−9.0). For the R39A 4-OT, the final enzyme concentration was 0.4 μM (pH 4.9−10). For the R39Q 4-OT, the final enzyme concentration was 1.2 μM (pH 4.6−11). For the R61A 4-OT, the final enzyme concentration was 0.2 μM (pH 4.9−6.3 and 9.5−11) or 5.9 nM (pH 6.7−9.2). It was necessary to use different enzyme concentrations of the mutants in order to facilitate the measurement of the reduced rates at the extreme pH values. The reaction was initiated by the addition of a quantity of **2** (final concentration 30−600 μM) from stock solutions made

Table 1: Summary of the pH Dependences of Kinetic Parameters[a] and pK_a Values Determined by Direct ^{15}N NMR Titration for 4-OT and Arginine Mutants

enzyme	substrate	$(k_{cat})^{max}$ (s^{-1})	$(k_{cat}/K_m)^{max}$ (M^{-1} s^{-1})	pK_a of Pro-1[b]	pK_a[a]			
					pK_{H2E}	pK_{HE}	pK_{H2ES}	pK_{HES}
WT	**2**	3500 ± 500	1.9×10^7	6.4 ± 0.2[c] 6.0 ± 0.1[d] 8.2 ± 0.1[e]	5.2 ± 0.1[c]	10.3 ± 0.2[c]	6.5 ± 0.2[c]	9.6 ± 0.3[c]
R61A	**2**	3500 ± 500	$(1.8 \pm 0.3) \times 10^7$		6.5 ± 0.2	9.0 ± 0.2	7.1 ± 0.1	9.6 ± 0.1
R11A	**2**	186 ± 24[f] 15[g]	$(8.9 \pm 1.4) \times 10^5$ [f] 7.4×10^4 [g]	6.0 ± 0.1 5.6 ± 0.1[d]	6.4 ± 0.2	>9	6.3 ± 0.3	>9
R39A	**2**	25 ± 1	$(1.1 \pm 0.1) \times 10^5$		5.0 ± 0.2	9.8 ± 0.2	6.1 ± 0.2	>10
R39Q	**2**	9.4 ± 1	$(1.9 \pm 0.2) \times 10^4$	7.1 ± 0.1 7.6 ± 0.1[h]	4.6 ± 0.2	10.0 ± 0.1	5.8 ± 0.1	>10
WT	**5**	0.40 ± 0.02	3.6×10^2	6.4 ± 0.2[c]	6.2 ± 0.3[c]	9.0 ± 0.3[c]	7.7 ± 0.2[c]	8.5 ± 0.3[c]
R11A	**5**	1.1 ± 0.2	$(1.0 \pm 0.1) \times 10^3$	6.0 ± 0.1	5.8 ± 0.1	>9	5.8 ± 0.2	>9

[a] From pH−rate profiles performed at 23 °C. [b] From 1D ^{15}N NMR pH titration of the amino group of Pro-1 at 30 °C. [c] From ref *5*. [d] At 42 °C. [e] At 42 °C in the presence of 10 mM CCM. [f] For the reaction with the 6-COOH form of substrate **2**. [g] For the reaction with the 6-COO^- form of substrate **2**. [h] At 30 °C in the presence of 10 mM CCM.

up in ethanol. The pH dependence of the kinetic parameters was fitted and analyzed as described previously (*5*).

*pH−Rate Profiles of R11A Using Substrate **5**.* The pH dependence of the rate of ketonization of **5** to **6** by the R11A mutant was determined in 50 mM sodium phosphate buffer, pH 4.9−10.6, at 23 °C using the following modifications to a previously described procedure (*5*). The final concentration of enzyme monomers was 18 μM. The rate of formation of **6** was monitored at 232 nm. The assay was initiated by the addition of a small quantity (1−6 μL) of **5** from a stock solution (44 or 88 mM) made up in ethanol. The concentrations of **5** used ranged from 40 to 530 μM.

1D ^{15}N NMR Spectroscopy. The NMR data were collected on a Varian Unity Plus 600 NMR spectrometer operating at 60.783 MHz for ^{15}N. Spectra were acquired without proton decoupling using a 5 mm broad-band detection probe. The ^{15}N chemical shifts are measured with respect to external $^{15}NH_4Cl$ (2.9 mM in 1 M HCl) at 20 °C, which is 24.93 ppm downfield from liquid ammonia (*16*), and are reported with respect to liquid ammonia. The acquisition parameters were as follows: spectral width, 12 001.2 Hz; acquisition time, 0.683 s; relaxation delay, 0.1 s; total number of transients, 10 000−100 000.

This 1D ^{15}N NMR procedure was used to determine the pK_a for the amino group of the general base, Pro-1, and lower limits to the pK_a values of all of the arginines in wild-type 4-OT and the R11A and R39Q mutants by monitoring the pH dependence of their ^{15}N chemical shifts. The titrations were performed using samples which were 3.0 mM in monomers of uniformly ^{15}N-labeled wild-type 4-OT, the R11A mutant, or the R39Q mutant in 8 mM potassium phosphate buffer at 30 °C (R11A and R39Q) or 42 °C (wild type and R11A) by addition of small amounts of 1 M HCl or 1 M NaOH to the sample. The titrations were found to be reversible in the range pH 4.7−10.0, and each mutant enzyme retained ≥85% of its initial activity at the conclusion of the experiment. The pK_a value for Pro-1 in wild-type 4-OT and the R39Q mutant in the absence and presence of CCM was determined from a nonlinear least-squares fit of the data to the equation:

$$\delta(\text{ppm})^{\text{app}} = \frac{\delta_1 + \delta_2(10^{\text{pH}-\text{p}K_a})^n}{(10^{\text{pH}-\text{p}K_a})^n + 1} \qquad (2)$$

where n is the Hill coefficient and δ_1 and δ_2 are the limiting chemical shifts at low and high pH values, respectively. The ^{15}N chemical shift of the secondary amino nitrogen resonance of Pro-1 in the R11A mutant was not detectable at pH <5.7 (30 °C) and pH <4.7 (42 °C), probably due to denaturation, so the limiting chemical shift at low pH could not be determined. A limiting pK_a value was therefore determined from a nonlinear least-squares fit of the data to eq 2, using $\delta_2 = 48$ ppm for the limiting chemical shift at high pH and differing trial values of $\delta_1 = 55.1-58.3$ ppm for the limiting chemical shift at low pH.

RESULTS AND DISCUSSION

pH Titration of Pro-1 in Wild-Type 4-OT and in the R11A Mutant of 4-OT by ^{15}N NMR. An important property of wild-type 4-OT is the unusually low pK_a of Pro-1, which serves as the general base. The kinetically determined pK_a value for k_{cat}/K_m of 6.2 ± 0.3 agreed with the value of 6.4 ± 0.2 obtained by direct ^{15}N titration of Pro-1 at 30 °C (Table 1) (*5*). These pK_a values are 3 units lower than that of proline amide, a model compound with a pK_a of 9.4 (*5*). The low pK_a value of Pro-1 was ascribed to both a low dielectric constant at the active site and the proximity of the cationic residues Arg-11 and Arg-39 (*1*, *5*). In support of this hypothesis, saturation of the wild-type enzyme with the dicarboxylic acid, CCM, which would be expected to neutralize the positive charge on these two residues, increases the pK_a of Pro-1 by 2.2 units from a value of 6.0 to 8.2 at 42 °C as determined by direct ^{15}N NMR titration (Figure 1A) (Table 1). The availability of mutants, uncharged at positions 11 and 39, permits an independent test of this hypothesis.

Over the pH range 4.7−9.6, the ^{15}N chemical shift of the amino nitrogen of Pro-1 in the R11A mutant ($^{15}N\delta = 47.5-57$ ppm) is upfield and well resolved from the other ^{15}N resonances of the enzyme (*4*, *5*). The ^{15}N resonance of Pro-1 was not detectable at pH <5.4 at 30 °C and pH <4.7 at 42 °C; therefore, the data were fitted to eq 2 to obtain the pK_a and Hill coefficient (n) at differing trial values for the limiting chemical shift at low pH ($\delta_1 = 56-59$ ppm) using the directly determined value of $\delta_2 = 47.5$ ppm for the limiting chemical shift at high pH. Figure 1B shows that the best fits to the data at 30 °C occur for δ_1 values between 56.5 and

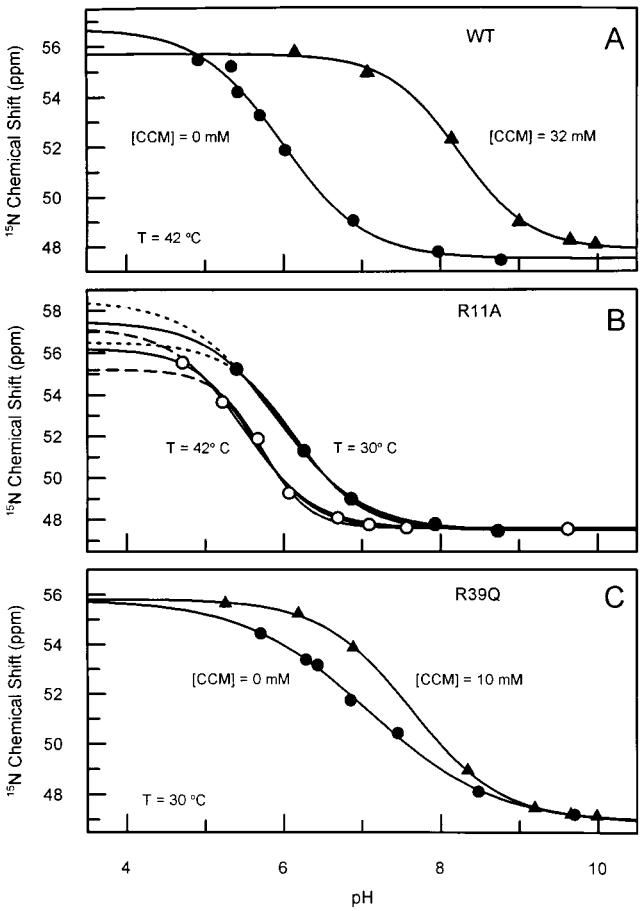


FIGURE 1: Determination of the pK_a for the amino proton of Pro-1 by ^{15}N NMR spectroscopy. (A) pH titration curves for the amino nitrogen atom of Pro-1 in wild-type 4-OT at 42 °C with 0 mM CCM (●) and with 32 mM CCM (▲). The curve for 0 mM CCM (●) is described by eq 2 using $\delta_1 = 56.7 \pm 0.9$ ppm, $\delta_2 = 47.5 \pm 0.3$ ppm, p$K_a = 6.0 \pm 0.1$, and Hill coefficient $n = 0.82 \pm 0.17$. The curve for 10 mM CCM (▲) is described by eq 2 using $\delta_1 = 55.7 \pm 0.2$ ppm, $\delta_2 = 47.9 \pm 0.2$ ppm, p$K_a = 8.2 \pm 0.1$, and $n = 0.95 \pm 0.14$. (B) pH titration curves for the amino nitrogen atom of Pro-1 in the R11A mutant of 4-OT at 30 °C (●) and at 42 °C (○). The curves for the data at 30 °C (●) are described by eq 2 using a limiting chemical shift at high pH (δ_2) of 47.5 ppm and p$K_a = 6.14$, 6.01, and 5.88 and $n = 1.01$, 0.87, and 0.79 for limiting chemical shifts at low pH (δ_1) of 56.5, 57.5, and 58.5 ppm, respectively. The complete solid line describes the best overall fit using p$K_a = 6.01 \pm 0.1$ and $n = 0.87 \pm 0.1$ for $\delta_1 = 57.5$ ppm. The curves for the data at 42 °C (○) are described by eq 2 using a limiting chemical shift at high pH (δ_2) of 47.6 ppm and pK_a = 5.72, 5.60, and 5.48 and Hill coefficient $n = 1.4$, 1.2, and 0.97 for limiting chemical shifts at low pH (δ_1) of 55.2, 56.2, and 57.2 ppm, respectively. The complete solid line describes the best overall fit using p$K_a = 5.61 \pm 0.07$ and $n = 1.2 \pm 0.2$ for $\delta_1 = 56.2$ ppm. (C) pH titration curves for the amino nitrogen atom of Pro-1 in the R39Q mutant of 4-OT at 30 °C with 0 mM CCM (●) and with 10 mM CCM (▲). The curve for 0 mM CCM (●) is described by eq 2 using $\delta_1 = 55.8 \pm 0.6$ ppm, $\delta_2 = 46.9 \pm 0.3$ ppm, p$K_a = 7.1 \pm 0.1$, and $n = 0.54 \pm 0.10$. The curve for 10 mM CCM (●) is described by eq 2 using $\delta_1 = 55.8 \pm 0.1$ ppm, $\delta_2 = 47.0 \pm 0.1$ ppm, p$K_a = 7.6 \pm 0.1$, and $n = 0.76 \pm 0.05$. At 10 mM CCM the tight sites were 71% occupied and the weak sites were 23% occupied when calculated as previously described (*9*).

58.5 ppm, yielding a value of p$K_a = 6.0 \pm 0.1$ and $n = 0.87 \pm 0.11$ for the R11A mutant as shown by the solid line. Thus, the enzymatic environment of the R11A mutant lowers the pK_a of Pro-1 by 3.4 units as compared to proline amide and by 0.4 unit as compared to wild-type 4-OT (Table 1), indicating that the low pK_a of Pro-1 in free 4-OT does not result from the proximity of Arg-11. From the X-ray structures of 4-OT (*2*), the distance between the ϵ-nitrogen of Arg-11 and the amino nitrogen of Pro-1 is 11.0 Å in the free enzyme and 8.3 Å in the affinity-labeled enzyme.

Similar analysis of the pH titration of Pro-1 in the R11A mutant at 42 °C yields a value of p$K_a = 5.6 \pm 0.1$ and $n = 1.15 \pm 0.10$ (Figure 1B) (Table 1). The 0.4 unit decrease in pK_a for the ammonium group of Pro-1 with increasing temperature from 30 to 42 °C is typical of nitrogen-containing bases that generally have high heats of ionization. Because of the low affinity of the R11A mutant for CCM (K_d = 21.7 mM) (*9*), this site could not be completely saturated. Hence, no attempt was made to determine the pK_a of Pro-1 in the R11A·CCM complex.

pH Titration of Pro-1 in the R39Q Mutant of 4-OT by ^{15}N NMR. From the X-ray structures of 4-OT (*2*), the distance between the ϵ-nitrogen of Arg-39 and the amino nitrogen of Pro-1 is 7.1 Å in the free enzyme and 6.5 Å in the affinity-labeled enzyme. Direct pH titration of the uniformly ^{15}N-labeled R39Q mutant enzyme was carried out at 30 °C using ^{15}N NMR (Figure 1C). The ^{15}N chemical shift of the amino nitrogen of Pro-1 in the R39Q mutant (^{15}Nδ = 47−56 ppm) over the pH range 5.7−10, in the absence and presence of 10 mM CCM, is upfield and well resolved from the other ^{15}N resonances of the enzyme similar to wild-type 4-OT (Figure 1A) (*4*, *5*) and the R11A mutant of 4-OT (Figure 1B). The titration data of R39Q were fitted to eq 2 to yield an apparent p$K_a = 7.1 \pm 0.1$ and $n = 0.54 \pm 0.1$ for Pro-1 in the free enzyme and an apparent p$K_a = 7.6 \pm 0.1$ and $n = 0.76 \pm 0.05$ for Pro-1 in the R39Q mutant of 4-OT complexed with 10 mM CCM, conditions under which the tight binding sites were 71% occupied and the weak binding sites were 23% occupied (*9*). The significant departure of the Hill coefficient of R39Q from 1.0 to lower values implies negative cooperativity in proton binding by the R39Q mutant. Hence, the pK_a value of the R39Q mutant is referred to as an apparent pK_a. While the pK_a of Pro-1 in wild-type 4-OT increases by 2.2 units to p$K_a = 8.2$ in the presence of saturating 32 mM CCM (Figure 1A), the apparent pK_a of Pro-1 in the R39Q mutant is only slightly increased by 0.5 unit upon complex formation with 10 mM CCM, presumably due to less than optimal binding of the analogue near Pro-1.

Effect of pH on the ^{15}Nϵ Chemical Shifts of Arginine Residues of 4-OT. The enzyme 4-OT has a total of six arginine residues per subunit (Arg-11, -21, -29, -39, -61, and -62). The Nϵ resonances of all these residues have been assigned (*9*). The chemical shifts of all of the Arg Nϵ resonances in wild-type 4-OT and in the R11A and R39Q mutants (*9*) were found to be independent of pH over the range 4.9−9.7 (data not shown), indicating that no arginine residue is responsible for the kinetically determined pK_a of 9.0 for an essential protonated group in free 4-OT (*5*).

pH Dependence of the Kinetic Parameters of Wild-Type 4-OT and of the R61A Mutant of 4-OT. The large values of k_{cat} and k_{cat}/K_m for ketonization of the dicarboxylate substrate **2** by the wild-type enzyme (Table 1) were interpreted to indicate that **2** is a sticky substrate (*5*). The R61A mutant shows very similar behavior to that of the wild-type enzyme with substrate **2** (Table 1). With sticky substrates, the expressions for k_{cat}/K_m and k_{cat} under steady-state conditions are complex, and a rigorous analysis of the pH dependence of the kinetic parameters would require knowledge of the

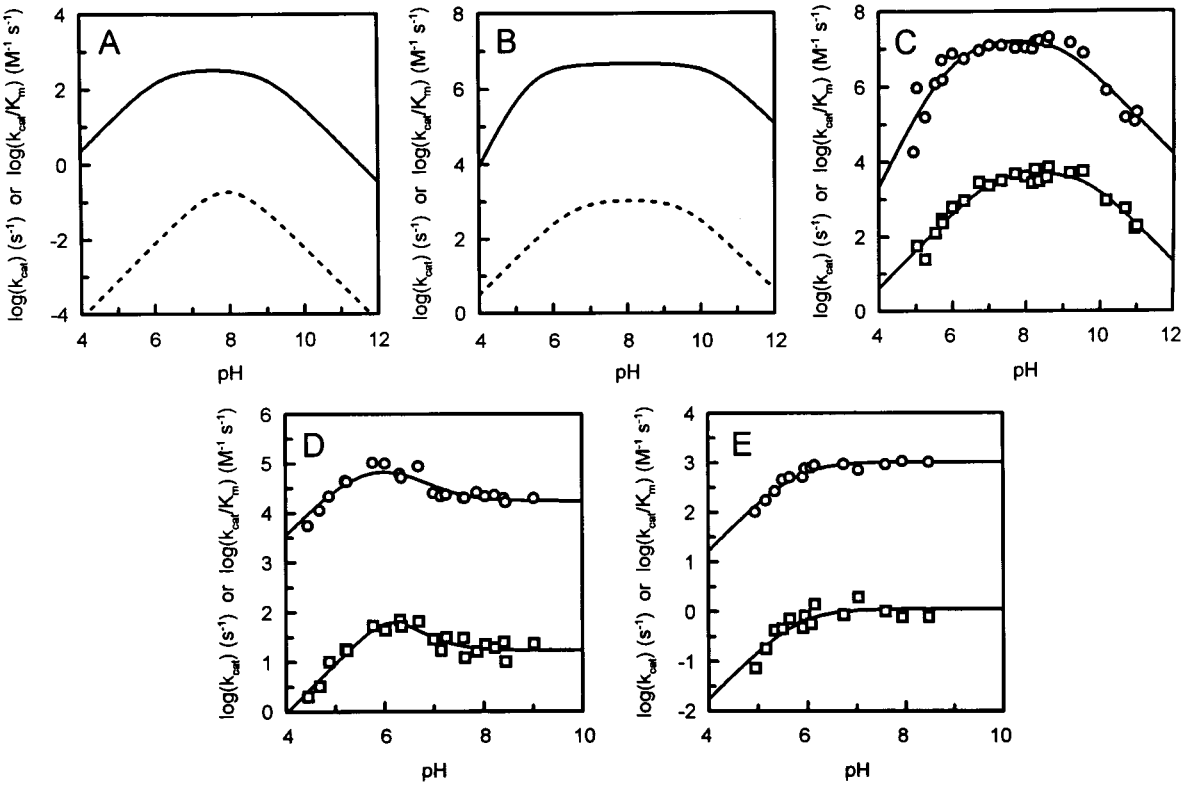


FIGURE 2: pH dependence of the kinetic parameters of wild-type 4-OT and its mutants R61A and R11A. Diagram summarizing the effect of pH on k_{cat}/K_m (solid curve) and k_{cat} (dashed curve) for the reaction of wild-type 4-OT with (A) the monocarboxylate substrate **5** and (B) the dicarboxylate substrate **2** (*5*). In ref *5* the values of $(k_{cat}/K_m)^{max}$ and $(k_{cat})^{max}$ were 3-fold lower than those currently measured (Table 1). Such variability in the activity of homogeneous 4-OT is commonly seen. pH dependence of the kinetic parameters k_{cat}/K_m (○) and k_{cat} (□) for the reaction of (C) the R61A mutant with **2**, (D) the R11A mutant with **2**, and (E) the R11A mutant with **5**. The curves were computed from a nonlinear least-squares fit of the data to the equations described in the text, and the results are summarized in Table 1.

relative stickiness of the substrate and of protons to the enzyme (*17*), which are unknown. In the absence of this information, it is reasonable to model the data using equilibrium assumptions and then qualitatively compare the results obtained for the R61A mutant with those obtained for wild-type 4-OT. For such comparisons, the curves used to fit the data for wild-type 4-OT are summarized in Figure 2A,B (*5*).

The pH−rate profiles of k_{cat}/K_m and k_{cat} for the reaction of wild-type 4-OT with the nonsticky monocarboxylate substrate **5**, in which rapid equilibrium kinetics apply, have previously been shown to be bell-shaped with limiting slopes of unity for both the ascending and descending limbs (Figure 2A), yielding pK_a values of 6.2 ± 0.3 for the general base and 9.0 ± 0.3 for an essential protonated group (*5*). The pK_a value for the general base agreed with that of Pro-1 measured by direct ^{15}N NMR titration (pK_a = 6.4 ± 0.2) as expected for a reaction occurring under rapid equilibrium conditions (*17*). With the sticky dicarboxylate substrate **2**, the slope of the ascending limb of log(k_{cat}/K_m) vs pH (Figure 2B) increased to 2, resulting from the simultaneous deprotonation of both Pro-1 and the 6-carboxyl group of substrate **2** (pK_a = 5.4 ± 0.1) (*5*). With a sticky substrate in which steady-state rather than rapid equilibrium kinetics apply, the pK_a values of the ionizing groups of the free enzyme are perturbed (*17*) such that the apparent pK_a of the basic group is lowered and that of the acidic group is raised (Table 1).

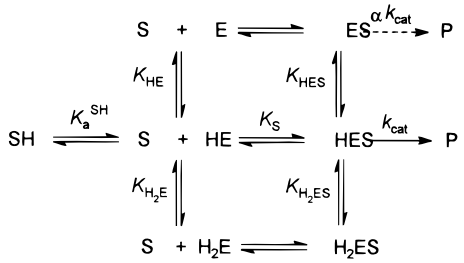
For the reaction of the fully active R61A mutant with **2**, a plot of log(k_{cat}/K_m) vs pH shows a bell-shaped dependence on pH with limiting slopes of 2 and 1 on the ascending and descending limbs, respectively (Figure 2C), similar to that observed for wild-type 4-OT (Figure 2B) (*5*). A simple model which best fits these observations is shown in Scheme 2 where α = 0, K_a^{SH} is the ionization constant of the 6-carboxylic acid group of the free substrate **2** (pK_a^{SH} = 5.4 ± 0.1), K_S is the dissociation constant of the dianionic form of the substrate (S) from the enzyme, K_{H2E} and K_{H2ES} are the ionization constants for the base catalyst (Pro-1) in the free enzyme and in the enzyme−substrate complex, respectively, K_{HE} and K_{HES} are the ionization constants for a required acid group in the free enzyme and in the enzyme−substrate complex, respectively, and α is a proportionality constant relating k_{cat} of the reactions of the two different ionic forms of the enzyme−substrate complex, ES and HES. Assuming rapid equilibrium, the pH dependences of k_{cat}/K_m and k_{cat} are given by eqs 3 and 4, respectively (*18*). For both

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

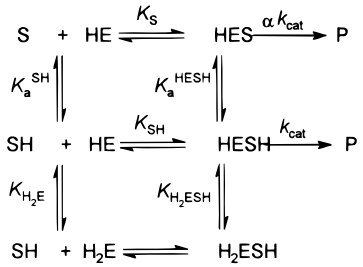
$$k_{cat} = \frac{(k_{cat})^{max}(1 + \alpha K_{HES}/[H^+])}{1 + [H^+]/K_{H_2ES} + K_{HES}/[H^+]} \quad (4)$$

the wild-type enzyme and the R61A mutant α = 0 since ES is catalytically inactive. Nonlinear least-squares fits of the pH dependence of k_{cat}/K_m to eq 3 and the pH dependence of k_{cat} to eq 4 using pK_a^{SH} = 5.4 and α = 0 (Figure 2C) gave the pK_a values listed in Table 1. While the R61A mutation had little effect on k_{cat}/K_m or k_{cat} at pH 7.3, it increased the

Scheme 2



Scheme 3



apparent pK_a values for the base catalyst in both the free and substrate-bound enzyme and decreased the apparent pK_a of the required protonated group in the free enzyme to values obtained with wild-type 4-OT using the nonsticky substrate **5** (*5*), suggesting substrate **2** to be less sticky in the R61A mutant as reflected in a small 1.6-fold increase in K_m (*9*). Since k_{cat} is unaltered and the increase in K_m is small in the R61A mutant, the decrease in substrate stickiness could have resulted from comparable increases in both k_{on} and k_{off} of the dicarboxylate substrate **2** upon decreasing the size of Arg-61 to Ala. If equilibrium kinetics are applicable, then the pK_a values derived from the effect of pH on k_{cat} indicate that the binding of the dianionic substrate to the R61A mutant has increased the pK_a values of both the general base and acid catalysts by 0.6 unit (Table 1).

*pH Dependence of the Kinetic Parameters of the R11A Mutant with Substrate **2**.* With the kinetically damaged arginine mutants (Table 1), the relatively low values of k_{cat}/K_m and k_{cat} for ketonization of substrate **5** by R11A and of substrate **2** by the R11A, R39A, and R39Q mutants suggest that the enzyme−substrate complex is indeed in rapid equilibrium with the free enzyme and substrate. Hence, the pH dependence of the steady-state kinetic parameters k_{cat}/K_m and k_{cat} were analyzed using rapid equilibrium assumptions as described previously for the reaction of wild-type 4-OT with substrate **5** (*5*).

For the reaction of the R11A mutant with the dicarboxylate substrate **2**, plots of both log(k_{cat}/K_m) and log(k_{cat}) vs pH show humps, i.e., sharply defined maxima near pH 6 followed by plateaus (Figure 2D),[2] indicating that more than one ionic form of the enzyme−substrate complex undergoes catalysis (*18*). In principle, the ionizing group in the enzyme−substrate complex could be provided by the enzyme (Scheme 2) or by the enzyme-bound substrate (Scheme 3) with $0 < \alpha < 1$ in both cases. It will be shown below that the kinetic and thermodynamic data are consistent with Scheme 3 and rule out Scheme 2 for the reaction of the R11A mutant with substrate **2**.

[2] Above pH 9, the R11A mutant becomes unstable with a very high K_m such that it becomes difficult to distinguish the enzymatic rate from the base-catalyzed nonenzymatic rate.

In Scheme 3, K_{SH} and K_S are the dissociation constants from the enzyme of the monoanionic (SH) and dianionic (S) forms of the substrate, respectively, K_{H2E} and K_{H2ESH} are the ionization constants of the base catalyst (Pro-1) in the free enzyme and in the enzyme−substrate complex, respectively, K_a^{SH} and K_a^{HESH} are the ionization constants of the 6-carboxylic acid group of **2** free in solution (p$K_a = 5.4 \pm 0.1$) and in the enzyme−substrate complex, respectively, and α is a proportionality constant relating k_{cat} for the reaction of the enzyme with the two different ionic forms of the substrate. With the rapid equilibrium assumption, the pH dependences of k_{cat}/K_m and k_{cat} are given by eqs 5 and 6 (*18*). From eqs 5 and 6 (with $0 < \alpha < 1$), maximum values

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)^{max}(1 + \alpha K_a^{HESH}/[H^+])}{(1 + K_a^{SH}/[H^+])(1 + [H^+]/K_{H_2E})} \quad (5)$$

$$k_{cat} = \frac{(k_{cat})^{max}(1 + \alpha K_a^{HESH}/[H^+])}{1 + [H^+]/K_{H_2ESH} + K_a^{HESH}/[H^+]} \quad (6)$$

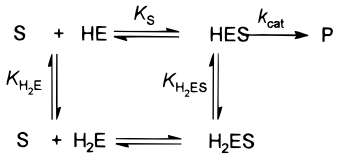
of k_{cat}/K_m and k_{cat} would occur for the reaction of the R11A mutant with the monoanionic form of the substrate, and values of k_{cat}/K_m and k_{cat} in the plateau region (pH >7) would occur for the slower reaction with the dianionic form of the substrate, resulting in a hump. With Scheme 3, the lack of a descending limb at high pH indicates that if there is an essential acidic group on the enzyme, its pK_a must be greater than 9.[2]

Experimentally (Figure 2D), with the measured pK_a of the 6-carboxyl group of substrate **2** (5.4 ± 0.1) as an input parameter, both Scheme 2 and Scheme 3 provide reasonable fits to the data on k_{cat}/K_m and k_{cat} with similar error values. However, in fitting k_{cat}/K_m, Scheme 2 predicts a pK_a for Pro-1 (pK_{H2E}) of 4.9 while Scheme 3 predicts this pK_a to be 6.4, in excellent agreement with the independently measured value of 6.3 ± 0.1 obtained by NMR titrations at 42 and 30 °C and extrapolation to 23 °C (Figure 1B) (Table 1). Moreover, to generate the descending limb of the hump, Scheme 2 requires the pK_a of the required acidic group of the enzyme (pK_{HE}) to have decreased from 9.0 in the wild-type enzyme to a value of 5.9 in the R11A mutant. Setting the pK_a of Pro-1 in the free R11A mutant enzyme to 6.3, using Scheme 2, further decreases pK_{HE} to 4.6. In the next section, evidence establishing Scheme 3 is presented, using substrate **5**, which lacks a 6-carboxyl group and shows no hump in its pH−rate profile.

Using Scheme 3, the value of the proportionality constant $\alpha = 0.083$, determined from analysis of both k_{cat} and k_{cat}/K_m with eqs 5 and 6, indicates that the R11A mutant converts the monoanionic form of the substrate to product 12-fold faster than the dianionic form. This change in the preferred ionization state of the 6-carboxyl group of substrate **2** from anionic to neutral, which occurs upon mutation of Arg-11 to Ala, provides strong evidence that Arg-11 interacts with the 6-carboxylate of the substrate. Protonation of the 6-carboxylate group of the substrate on the R11A mutant would restore some of the inductive and resonance effects of a hydrogen-bonded 6-carboxylate group on k_{cat} as on the wild-type enzyme (Table 1) (*5*, *9*, *19*).

*pH Dependence of the Kinetic Parameters of the R11A Mutant with Substrate **5**.* To further distinguish between the

Scheme 4



mechanisms of Schemes 2 and 3, the pH dependences of the kinetic parameters were determined for the reaction of the R11A mutant with the monocarboxylate substrate **5** which does not contain the 6-carboxylic acid group and in which the 1-carboxylic acid group ($pK_a^{COOH} \sim 2.4$) (*5*) does not titrate in the pH range studied. Plots of both $\log(k_{cat}/K_m)$ and $\log(k_{cat})$ vs pH show ascending limbs with slopes of unity followed by plateaus (Figure 2E). The disappearance of the hump in the pH profiles with the monocarboxylate substrate **5** confirms that the 6-carboxylic acid group of the dicarboxylic acid substrate **2** is responsible for the descending limb of the hump (Figure 2D) in accord with Scheme 3 and eqs 5 and 6. For substrate **5** which is not ionizable in the pH range studied, Scheme 3 simplifies to Scheme 4 in which Pro-1 is the only ionizable group with dissociation constants K_{H2E} in the free enzyme and K_{H2ES} in the enzyme−substrate complex. The assumption of rapid equilibrium leads to eqs 7 and 8 which were fit to the kinetic data for the reaction of

$$\frac{k_{cat}}{K_m} = \frac{(k_{cat}/K_m)^{max}}{1 + [H^+]/K_{H_2E}} \quad (7)$$

$$k_{cat} = \frac{(k_{cat})^{max}}{1 + [H^+]/K_{H_2ES}} \quad (8)$$

the R11A mutant with substrate **5** (Figure 2E). This analysis gives a pK_a value of 5.8 ± 0.1 for the base catalyst in both the free enzyme and enzyme−substrate complex. This value is comparable to the value of 6.3 ± 0.1 (23 °C) obtained by direct ^{15}N NMR titration and extrapolation (Figure 1B, Table 1) and 6.4 ± 0.2 obtained from the pH dependence of k_{cat}/K_m for the reaction of R11A with **2** (Figure 2D, Table 1). Analysis of the effects of pH on k_{cat} of R11A with both substrate **2** and substrate **5** indicates that the pK_a of Pro-1 is not affected by the binding of either substrate, consistent with substrate **2** functioning as a monoanion.

If, in addition, there is a pK_a for an essential acidic group in the free or substrate-bound R11A, it must be greater than 9.[2] Although the descending limb at high pH could not be reached with the R11A mutant, it is unlikely that Arg-11 is responsible for the pK_a of the acid group in wild-type 4-OT (9.0 ± 0.3, free enzyme; 8.5 ± 0.3, enzyme−substrate complex) since the ^{15}N chemical shifts for the side chain ϵ-nitrogen of Arg-11 and of all other arginine residues were independent of pH over the range 4.9−9.7 (see above).

pH Dependence of the Kinetic Parameters of the R39A and R39Q Mutants of 4-OT. For both the R39A and R39Q mutants, plots of $\log(k_{cat}/K_m)$ for the dicarboxylate substrate **2** (Figure 3) show bell-shaped dependences on pH with limiting slopes of 2 and 1 on the ascending and descending limbs, respectively, although only limited data could be obtained at the extremes of pH. This pH behavior indicates the importance of three ionizable groups in the free enzyme or substrate. If it is assumed that the dianionic form of **2** binds to both the R39A and R39Q mutants, an assumption which fit the data for the wild-type enzyme (Figure 2B) (*5*), then one of the two pK_a values responsible for the ascending limb corresponds to the ionization of the 6-carboxylic acid group of substrate **2** ($pK_a^{SH} = 5.4 \pm 0.1$). A simple rapid equilibrium model which best describes these observations is shown in Scheme 2 with $\alpha = 0$ so that product formation occurs only from the HES complex. A nonlinear least-squares fit of the pH dependence of k_{cat}/K_m to eq 3 using $\alpha = 0$ and $pK_a^{SH} = 5.4$ gave pK_a values for the free enzyme of 5.0 ± 0.2 and 9.8 ± 0.2 for the R39A mutant and 4.6 ± 0.2 and 10.0 ± 0.1 for the R39Q mutant (Table 1).

The kinetically determined pK_a of 4.6 ± 0.2 for the R39Q mutant differs greatly from the apparent pK_a value of 7.1 ± 0.1 for Pro-1 determined by direct NMR titration (Figure 1C). Three possible explanations for the 2.5 unit difference in pK_a have been considered. First, a change in mechanism in the R39Q mutant to one in which either His-6 or His-49 takes over the role of general base may be excluded. Although His-6 and His-49 have appropriately low pK_a values of ≤5 and 5.1, respectively, in the wild-type enzyme (*5*), their nearest imidazole nitrogens are 9.0 and 14.7 Å from the Pro-1 nitrogen (*2*). Second, a departure of the kinetic scheme from rapid equilibrium would result in a lower apparent pK_a as is observed (*17*). However, the 372-fold decrease in $(k_{cat})^{max}$ of the R39Q mutant together with a 2.7-fold increase in K_m (Table 1) renders this explanation highly unlikely but does not rule it out. Third, as a result of negative cooperativity in the deprotonation of Pro-1 (Figure 1C), the apparent pK_a of 7.1 determined by direct titration actually represents an average of the pK_a values of the six Pro-1 residues of the homohexamer. From the Hill coefficient of 0.54, it can be shown that the apparent pK_a value of 7.1 in the titration could result most simply from the rapid averaging of two limiting pK_a values of 4.6 and 8.2 at a rate $> 2\pi\Delta\delta$ or 3400 s^{-1} (Figure 1C).[3] The pK_a value of 4.6 would agree with the kinetic data, and the pK_a value of 8.2 is not unreasonable for a Pro-1 which is partially exposed to solvent since the model compound proline amide has a pK_a of 9.4 in aqueous solution (*5*). From the X-ray structure of 4-OT (*2*), Arg-39″ is in molecular contact with the β-hairpin (residues 50′ to 57′) which covers the active site, and the backbone ^{15}N and NH resonances of residues in this region show changes in chemical shift as a result of the R39Q mutation (*9*). Hence mutation of Arg-39, by altering the structure of the β-hairpin, could result in an increase in the solvent exposure of Pro-1, raising its upper limit pK_a value. If in the R39Q-catalyzed reaction only the sites with the lower limit pK_a values were operative, the discrepancy in pK_a values would be resolved. The R39Q mutant also shows negative cooperativity in binding the substrate analogue CCM with K_d values ranging from 3.5 to 29 mM (*9*). This observation and the low K_m of substrate **2** with the R39Q mutant (0.5 mM) indicate that the tight substrate binding sites are likely to be the ones with the lower limit pK_a values for Pro-1.

The pK_{HES} values of 9.8 and 10 for the required protonated group observed in $\log(k_{cat}/K_m)$ versus pH for the R39A and

[3] This may be shown by solving the equation $pK_{obs} = (pK_1 \times pK_2)^n$. In the present case $7.1 = (4.6 \times 8.2)^{0.54}$.

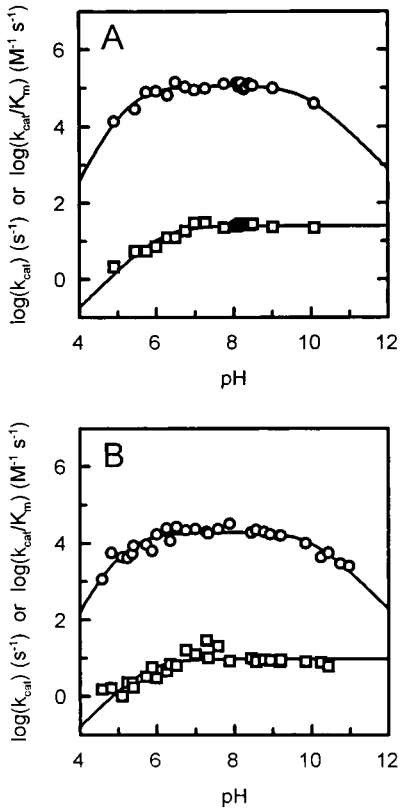


FIGURE 3: pH dependence of the kinetic parameters k_{cat}/K_m (○) and k_{cat} (□) for the reaction of (A) the R39A mutant and (B) the R39Q mutant with substrate **2**. The curves were computed from a nonlinear least-squares fit of the data to eq 3 for k_{cat}/K_m with $\alpha = 0$ and to eq 8 for k_{cat} as discussed in the text, and the results are summarized in Table 1.

R39Q mutants, respectively (Figure 3), were not greatly altered from that found with the wild-type enzyme (Table 1). This observation, together with the finding that the pK_a values of all of the arginine residues of 4-OT exceed 10, indicates that Arg-39 is not responsible for these pK_a values detected kinetically. The enzyme contains three lysine residues, K16, K47, and K59, surrounding the entrance to the active site with ϵ-NH_2 nitrogens 12.9−13.8 Å from the amino nitrogen of Pro-1 (*2*). One of these lysines begins to titrate at pH 9.2 in ^{15}N NMR spectra of wild-type 4-OT and may be responsible for the descending limb of the pH−rate profile. This is currently under investigation.

For both the R39A and R39Q mutants, a plot of log(k_{cat}) vs pH for **2** shows a single ascending limb with a slope of 1 (Figure 3). A fit of the pH dependence of k_{cat} to the logarithmic form of eq 8 gives pK_{H2ES} values for the enzyme−substrate complex of 6.1 ± 0.1 and 5.8 ± 0.1 for the R39A and R39Q mutants, respectively (Table 1). If there is a pK_a for a required protonated group in the enzyme−substrate complex, it must be greater than 10. Thus the binding of the dicarboxylate substrate has increased the kinetically determined pK_a values of both the general base and acid catalysts.

CONCLUSIONS

The pK_a values of the active site Pro-1 and the limiting pK_a values of the arginine residues of the active site arginine mutants of 4-OT were determined by direct ^{15}N NMR pH titrations and compared with those of wild-type 4-OT (*5*). In addition, pH−rate profiles were carried out with the arginine mutants to provide independent determinations of the pK_a values of Pro-1 and of the essential acidic group in catalysis. In the R11A mutant, the pK_a of Pro-1 is unaltered, but now the protonated 6-COOH form of the substrate is preferred over the unprotonated 6-COO^- form by a factor of ∼12. This reversal of substrate preference establishes a functional interaction between the 6-COO^- group of the substrate with Arg-11, in accord with the results of the preceding paper (*9*).

The R39Q mutation introduces negative cooperativity in the deprotonation of Pro-1 and in the binding of the substrate analogue CCM (*9*). Both of these effects may result from increased exposure to solvent due to an altered structure of the β-hairpin which covers the active site. These observations, together with the low K_m of substrate **2** with the R39Q mutant and the effects of pH on k_{cat}/K_m, indicate that only the sites with low pK_a and tight substrate binding are kinetically operative in the R39Q mutant. The results from these studies indicate that no arginine residue is responsible for the kinetically determined pK_a of 9.0 for the essential acidic group in free 4-OT and that proximities of the cationic residues Arg-39 and Arg-11, which interact with the 1- and 6-carboxylate groups of the substrate, respectively, contribute little to the unusually low pK_a value of the general base catalyst Pro-1 (pK_a = 6.4) in 4-OT, which likely results from a low local dielectric constant of ∼16. The 2.2 unit increase in pK_a of Pro-1 upon binding the dianionic substrate analogue CCM (Figure 1A) may result from an increase in the local dielectric constant or from the proximity to Pro-1 of the two negative charges of the ligand.

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