nm (ε 26 300), 246 (ε 35 590), 284.5 (ε 15 880); mass spectrum $m/z$ 586, 568, 525 (see Table V): $^1$H NMR and $^{13}$C NMR (see Table I and II). Component $A_2$ (7.7 mg): UV ($CH_3OH$) 209 nm (ε 24 170), 246 (ε 32 870), 283 nm (ε 14 120); mass spectrum $m/z$ 734 ($M^+$), 586, 568, 525; $^1$H NMR and $^{13}$C NMR (see Tables I and II).

In another experiment crude L-681,110 (270 mg) was put on nine silica gel $HF_{254}$ plates (20 × 20 × 0.025 cm) and developed 3 times with chloroform–methanol (4:1) containing 2% ammonium hydroxide. Under these conditions the components running faster than $A_1$ and $A_2$ separated into several bands which were individually recovered. The major band with the second highest $R_f$ gave 41 mg of lypophilized component $B_1$: UV ($CH_3OH$) 246 nm (ε 38 200), 284.5 (ε 16 950); mass spectrum $m/z$ 618 ($M^+ - H_2O$), 600, 586, 568, 525; $^1$H NMR (see Table I and figure 2).

**Conversion of $A_1$ to $A_2$.** Component $A_1$ (50 mg) was dissolved in methanol (25 mL) and refluxed for 3.5 h. The solvent was removed under reduced pressure giving a glassy white solid (45 mg) indistinguishable by $^1$H NMR in $CD_3OD$ from component $A_2$.

**Methanolysis of $B_1$.** Component $B_1$ (2.5 mg) was recovered unchanged when refluxed in methanol (10 mL) for 3 h. Under the same conditions but in the presence of 5 drops of glacial acetic acid, an oil was obtained on evaporation of the solvent which by $^1$H NMR showed predominantly two methyl ketal isomers in the proportion of ca. 3:1. Well-resolved resonances of the major component are as follows: δ ($CDCl_3$) 1.41 (dd, 1, $J$ = 11, 12 Hz, H20α), 1.92 (br s, 3, C10–$CH_3$), 1.99 (br s, 3, C4-$CH_3$), 2.41 (m, 1, H20β), 2.54 (m, 1, H6), 3.04 (s, 3, C19-$OH_3$), 3.24 (s, 3, C14-$OCH_3$), 3.35 (s, 3, C21-$OCH_3$), 3.71 (s, 3, C2-$OCH_3$), 3.88 (t, 1, $J$ = 9.5 Hz, H14), 5.12 (br d, 1, $J$ = 7.5 Hz, H15), 5.22 (dd, 1, $J$ = 9, 15 Hz, H13), 5.78 (br d, 1, $J \simeq$ 10.5 Hz, H11), 5.83 (br d, 1, $J$ = 9 Hz, H5), 6.52 (dd, 1, $J$ = 10.5, 15 Hz, H12), 6.68 (s, 1, H3).

**Production of Labeled Material.** $^{13}$C-labeled product was produced by growing a *Streptomyces* species MA 5038 (Merck Culture Collection) in an inoculum medium which contains dextrose (1.0 g/L), soluble starch (10 g/L), beef extract (3.0 g/L), yeast autolysate (5.0 g/L), peptone (5.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.05 g/L), $KH_2PO_4$ (0.182 g/L), $Na_2HPO_4$ (0.190 g/L), and $CaCO_3$ (0.5 g/L) and was adjusted to pH 7.0–7.2 with NaOH. After 1 day of incubation at 28 °C with agitation, a portion of the culture broth (5% final concentration) is transferred into a production medium of the following composition: lactose (20.0 g/L), distillers solubles (10.0 g/L), and yeast autolysate (5.0 g/L) adjusted to pH 7.0 with NaOH. Following incubation at 28 °C for 3 days with agitation, a portion of this growth (5% final concentration) is transferred into flasks of fresh production media. After 18 h of incubation with agitation at 28 °C, a sterile solution of sodium [*methylene*-$^{13}$C]propionate (90 atom % $^{13}$C, MSD Isotopes) is added to the flasks to a final concentration of 100 μg/mL. Flasks are then incubated as before for another 54 h before product is isolated.

**Registry No.** L-681,110 component $A_1$, 82621-00-9; L-681,110 component $A_2$, 82623-58-3; L-681,110 component $B_1$, 82620-99-3; ATPase, 9000-83-3; GABA, 56-12-2.

# pH Dependency of the Zinc and Cobalt Carboxypeptidase Catalyzed Enolization of (*R*)-2-Benzyl-3-(*p*-methoxybenzoyl)propionic Acid

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**Abstract:** The pH dependency of the kinetics of the CPA-catalyzed enolization of the ketonic substrate (*R*)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid ((−)-**1**) has been determined. The study of this relatively simple reaction allows us to examine the catalytic properties and ionization behavior of the enzyme-bound active-site bases and acids without the complications that would be encountered for peptides and esters where the formation and breakdown of a multiplicity of intermediates along the reaction pathway must be considered. The p$K_a$ values of 6.03 ± 0.35 and 6.04 ± 0.31 measured from the pH dependency of $k_{cat}$ for the Zn(II) and Co(II) CPA catalyzed enolization of (−)-**1**, respectively, correspond to the p$K_a$ for the ionization of the γ-carboxyl of Glu-270. The binding of (−)-**1** to CPA was investigated by examining the inhibition by this compound of the enzyme-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate. The $K_I$–pH dependencies for the inhibitory activity of (−)-**1** show that in alkaline solution one ionization of an enzyme-bound group occurs with p$K_a$ = 7.56 ± 0.15 for the Zn(II) enzyme and p$K_a$ = 8.29 ± 0.32 for Co(II) CPA while the other occurs above pH 9. The p$K_a$ values in the vicinity of 8 represent the ionization of the phenolic hydroxyl of Tyr-248, in good agreement with earlier assignments of this p$K$. Finally, a reasonable interpretation of the pH dependency on a group with p$K_a$ <9 is that it corresponds to the ionization of the active-site metal ion bound water.

The action of many acyl transfer enzymes is known to involve the participation of highly reactive nucleophiles present in their active sites. Although kinetic and chemical modification studies on the hydrolytic processes provide powerful techniques, a multiplicity of reaction intermediates, the possibility of changes in rate-determining step with changes in pH, and other factors often conspire to make the precise nature of the participation of the enzyme-bound nucleophile obscure. For example, in the instance of the well-known enzyme pepsin, although many studies have been made on the chemistry of the active site and the X-ray structures of related acid proteases are known, the interpretation of the wealth of kinetic and chemical modification data available in terms of a detailed reaction mechanism remains highly controversial.[1] With these problems in mind, in our laboratory an investigation was undertaken on the zinc-containing enzyme carboxypeptidase A (CPA) to establish whether we could find a simple reaction where the problem of the existence of multiple intermediates would not occur and that could be used to probe the catalytic activity of the presumed nucleophile in the active site of CPA, the γ-carboxylate group of the essential Glu-270 residue.[2] Indeed, we have demonstrated that the active site of the hydrolytic enzyme CPA is capable of catalyzing stereospecifically an enolization reaction.[3] In particular, the enzyme catalyzes stereospecifically[3,4] the exchange of hydrogens present in an activated methylene group of a ketonic substrate. More recently, it has been shown that CPA catalyzes the α,β-elimination

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reaction of a ketonic substrate containing in addition to the activated $\alpha$-methylene group a good leaving group $\beta$ to the ketone function.[5] From stereochemical studies on the CPA-catalyzed hydrogen–deuterium exchange at the activated methylene group of (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid ((−)-**1**) a ketonic analogue of ester and and peptide substrates, the *pro-R* hydrogen of the 3-methylene group of (−)-**1** was found to be the one undergoing exchange.[4] This finding was in accord with the postulate that (−)-**1** binds to CPA in a fashion analogous to that proposed for hydrolytic substrates on the basis of X-ray crystallographic studies on peptide–enzyme complexes.[6] Following these observations, an X-ray structure determination by the Lipscomb group[7,8] on the complex of CPA with (−)-**1** showed that the arrangement of **1** at the enzyme's active site corresponded to the binding picture deduced from our stereochemical studies.

Since the CPA-catalyzed hydrogen–deuterium exchange at the 3-methylene group of (−)-**1** provides a unique example of a relatively uncomplicated reaction of a substrate having an arrangement at the active site that has been established both by solution studies[3,4] and by X-ray crystallographic structure determination,[7,8] the interpretation of the pH dependencies of the kinetic parameters for this process should be considerably less ambiguous than would be the case for the reactions of hydrolytic substrates. In the present paper we report the pH dependencies of the kinetic parameters $k_{cat}$ and $K_m$ for the native Zn(II) and Co(II) CPA catalyzed enolization of (−)-**1** and our interpretation of the results in terms of the active-site chemistry of the enzyme.

## Experimental Section

NMR spectra were recorded on a University of Chicago built 500-MHz DS-1000 $^1$H FT-NMR spectrometer. Atomic absorption spectral measurements were carried out on a Perkin-Elmer 306 instrument. Ultraviolet spectral measurements were carried out on a Cary 219 or a Beckman Acta MVI spectrophotometer.

Tris (tris(hydroxymethyl)aminomethane) was purchased from Aldrich as the Gold Label brand. MES (morpholinoethanesulfonic acid) and bis(tris)propane (1,3-bis[tris(hydroxymethyl)methylamino]propane) were purchased from Sigma. Ultrapure concentrated HCl and ultrapure NaOH, 30% in $H_2O$, were purchased from Alfa.

***O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactic Acid.** This compound was synthesized as described by Suh and Kaiser.[9] A 30% yield of product with mp 125–126 °C (uncorrected) (lit.[9] mp 125–126.5 °C) was obtained.

**(−)-2-Benzyl-3-(*p*-methoxybenzoyl)propionic-*3,3*-$d_2$ (1-$d_2$) Acid.** This compound was synthesized as described by Sugimoto and Kaiser,[3] with the exception that the resolution was carried out with a 5% solution of anisole in acetonitrile instead of diethyl ether, resulting in a substantial reduction in the number of recrystallizations that had to be performed. The material obtained had mp 91–92 °C (uncorrected) (lit.[3] mp 91–92.5 °C) and $[\alpha]^{22}_D$ −21.4° (*c* 10.0, ethyl acetate).

**Enzyme Stock Solutions.** Carboxypeptidase $A_\alpha$ was puchased from Sigma as the type I enzyme with lot numbers 99C-8075 and 119C-8030. First, the crystals were washed with deionized water and the suspension was centrifuged three times. Subsequently, the CPA crystals were dissolved in 0.05 M Tris, 0.50 M NaCl, pH 7.50. This solution was centrifuged, and the supernatant was employed as the enzyme stock solution.

Apocarboxypeptidase $A_\alpha$ and the cobalt enzyme were prepared as described by Davies et al.[10] Enzyme concentrations were determined spectrophotometrically for both the zinc and cobalt enzymes by using the value of $6.42 \times 10^4$ L/mol·cm as the extinction coefficient at 278 nm.

**Enzymatic Assays.** The zinc and cobalt enzymes were assayed in 0.05 M Tris, 0.50 M NaCl, pH 7.50. An aliquot of the enzyme stock solution was added, and the pseudo-first-order rate constant was divided by the enzyme concentration to gain an assessment of the activity of enzyme. The $k_{obsd}$/[E] value for the native zinc CPA was $8.40 \times 10^5$ $M^{-1}$ $s^{-1}$ with a substrate concentration of $8.20 \times 10^{-5}$ M. Under similar conditions, the corresponding value measured for the apoenzyme was $2.03 \times 10^4$ $M^{-1}$ $s^{-1}$, indicating that there was 3.1% residual activity. Regeneration of zinc CPA from apo-CPA gave an enzyme for which the $k_{obsd}$/[E] value was $8.05 \times 10^5$ $M^{-1}$ $s^{-1}$. In the case of the cobalt CPA, a $k_{obsd}$ value of $1.56 \times 10^6$ $M^{-1}$ $s^{-1}$ was obtained. Atomic absorption measurements performed on a stock solution of cobalt CPA (enzyme concentration = $2.34 \times 10^{-5}$ M) gave a value of 0.98 equiv of cobalt and 0.024 equiv of zinc per 1 equiv of CPA.

**Kinetic Measurements.** Deuterium–hydrogen exchange of (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic-*3,3*-$d_2$ acid (**1**-$d_2$) was studied by adding an aliquot of an aqueous solution of the substrate which was dissolved in 0.50 M NaCl to the CPA solution containing 0.05 M buffer, 0.50 M NaCl, at the appropriate pH at a temperature of 25.0 °C. Six aliquots were removed at various times, acidified with 2 drops of 0.01 M HCl and extracted with ether. The ether solution was dried over $Na_2SO_4$ and evaporated in vacuo. As described in the work of Sugimoto and Kaiser,[3] kinetic measurements on deuterium–hydrogen exchange were performed by following the appearance of the NMR peak for the proton at 2.98 ppm ($H_a$).[3]

The binding of **1**-$d_2$ to CPA was examined by determining the inhibition constant for the inhibition by **1**-$d_2$ of the CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate.[2] At each pH the hydrolysis of the ester was monitored by UV spectrometry following the decrease in absorbance at a wavelength in the range 310–325 nm, depending on the particular initial absorbance of the solution employed. At each pH a series of runs were done at various substrate concentrations. (Typically the range of concentrations was $2.50 \times 10^{-4}$ to $5.00 \times 10^{-5}$ M.) In the Co(II) CPA experiments at pH 6.5 and below, $CoCl_2$ was added at a concentration equal to the enzyme concentration. Subsequently, runs were performed at single substrate concentrations by employing various inhibitor concentrations (typical inhibitor concentration ranges were $1.00 \times 10^{-5}$ to $5.00 \times 10^{-4}$ M). From the rate data obtained, pseudo-first-order rate constants were calculated by the least-squares method. The rate constants vs. pH curves were catalyzed by using an iterative curve-fitting program (written by B. Blumenstein of Emory University) and employing a Dec-20 computer.

## Results

**pH Dependency of the Kinetics of the CPA-Catalyzed Enolization of (−)-1-$d_2$.** As previously described,[3] deuterium–hydrogen exchange was initiated by adding the deuterium-labeled substrate (−)-**1**-$d_2$ to aqueous solutions of CPA. The incorporation of protons from the solvent into the $H_a$ portion[3] of the methylene group was monitored by observing the increase of the signal intensity at 2.98 ppm in the NMR spectrum of the substrate. Under conditions where the substrate concentration was much greater than that of the enzyme, pseudo-first-order kinetics were seen for the deuterium–hydrogen exchange process. The exchange reaction can be treated as illustrated in eq 1.[3] Assuming that the extent of deuteration of the methylene group of (−)-**1** does not affect the $K_m$ value ($K_m = K_p$), the rate expression for the exchange process can be shown to be that given in eq 2.[3] As already reported in our earlier study of pH 7.5 with the Zn(II) enzyme, for both Zn(II) and Co(II) CPA, the observed first-order rate constant ($k_{obsd}$) shows the expected inverse dependence on the initial substrate concentration over the entire pH range utilized in the present study. From plots of $[\mathbf{1}\text{-}d_2]_0$ vs. $[E]_0/k_{obsd}$, the values of $k_{cat}$ were calculated for the pH region employed. As shown in Figures 1 and 2, , the $k_{cat}$ vs. pH profiles can be fit to sigmoidal curves for both the Zn(II) and Co(II) enzymes, consistent with the dependency of the value of $k_{cat}$ on an ionizing group calculated to have p$K_a$ values of 6.03 ± 0.35 and 6.04 ± 0.31, respectively. Because of the denaturation of the Zn(II) and Co(II) enzymes at low pH over the long time periods required for the kinetic experiments under these conditions, the pH range over which measurements could be made in acidic solutions was limited.

$$\mathrm{E} + (-)\text{-}\mathbf{1}\text{-}d_2 \underset{K_m}{\rightleftharpoons} \mathrm{E}\cdot(-)\text{-}\mathbf{1}\text{-}d_2 \xrightarrow{k_{cat}} \mathrm{E} + (-)\text{-}\mathbf{1}\text{-}d \qquad (1)$$
$$\mathrm{E} + (-)\text{-}\mathbf{1}\text{-}d \underset{K_p}{\rightleftharpoons} \mathrm{E}\cdot(-)\text{-}\mathbf{1}\text{-}d$$

$$v = \frac{k_{cat}[E][\mathbf{1}\text{-}d_2]}{[\mathbf{1}\text{-}d_2]_0 + K_m} = k_{obsd}[\mathbf{1}\text{-}d_2] \qquad (2)$$

where $[E]_0$ and $[\mathbf{1}\text{-}d_2]_0$ are the initial concentrations of the enzyme and substrate respectively.

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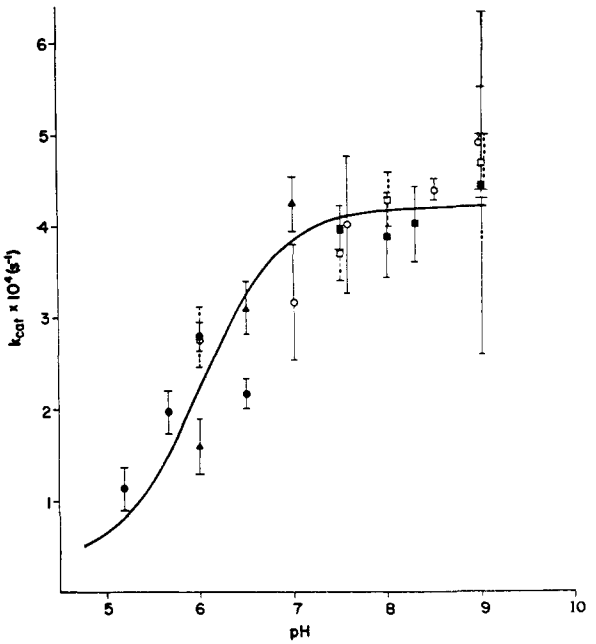


**Figure 1.** $k_{cat}$ vs. pH profile for the CPA-catalyzed deuterium–hydrogen exchange of (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic-3,3-$d_2$ acid at 25.0 °C. The buffer concentrations were 0.05 M, and the solutions contained 0.5 M NaCl. The various buffers are indicated by the following symbols: (●) MES; (▲) cacodylate; (○) bis(tris)propane; (■ and □) Tris. The two different symbols for Tris buffer differentiate between experiments that were performed with different enzyme preparations at different times. Error bars are shown to illustrate the standard deviation for each point. When two sets of error bars cross, the dashed error bars correspond to the point represented by the open symbol. At pH 9 the solid error bars correspond to the ■ point, the dashed error bars to the □ point, and the dotted errors bar to the ○ point. The enzyme concentration varied from $9.80 \times 10^{-5}$ to $1.58 \times 10^{-4}$ M, and the substrate concentration varied from $1.56 \times 10^{-3}$ to $8.17 \times 10^{-3}$ M. The solid line is a theoretical sigmoidal curve calculated from the experimental points by employing an iterative curve-fitting program (see Experimental Section).

**pH Dependency of the Binding of (−)-1-$d_2$ to CPA.** The binding of **1**-$d_2$ to Zn(II) and Co(II) CPA was investigated by examining the inhibition by **1**-$d_2$ of the enzyme-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (**2**). This approach was used because errors in the ordinate intercepts of plots of $[\mathbf{1}\text{-}d_2]_0$ vs. $[E]_0/k_{obsd}$ were too large for us to obtain meaningful $K_m$ values for **1**-$d_2$ directly.

The rate expression for the CPA-catalyzed hydrolysis of **2** can be fit approximately to pseudo-first-order kinetics because the $K_m$ value for **2** is similar to the competitive inhibition constant for the product, L-β-phenyllactate.[9] This is illustrated by eq 3 for the hydrolysis of **2**, where $K_p$ represents the inhibition constant for L-β-phenyllactate; when $K_m = K_p$, eq 4 results.

$$v = \frac{k_{cat}[E]_0[S]}{\frac{K_m[S]_0}{K_p} + K_m + [S]\left(1 - \frac{K_m}{K_p}\right)} \quad (3)$$

$$v = \frac{k_{cat}[E]_0[S]}{[S]_0 + K_m} \quad (4)$$

When eq 4 holds, in a plot of the reciprocals of the pseudo-first-order rate constants for the CPA-catalyzed hydrolysis of **2** vs. the initial substrate concentration, the slope is $1/k_{cat}[E]_0$ and the intercept is $K_m/k_{cat}[E]_0$. When the competitive inhibitor (−)-**1** is added to the reaction mixture, the rate expression for the enzyme-catalyzed hydrolysis of **2** becomes that shown in eq 5 (where

$$v = \frac{k_{cat}[E]_0[S]}{[S]_0 + K_m + \frac{[I]K_m}{K_I}} \quad (5)$$

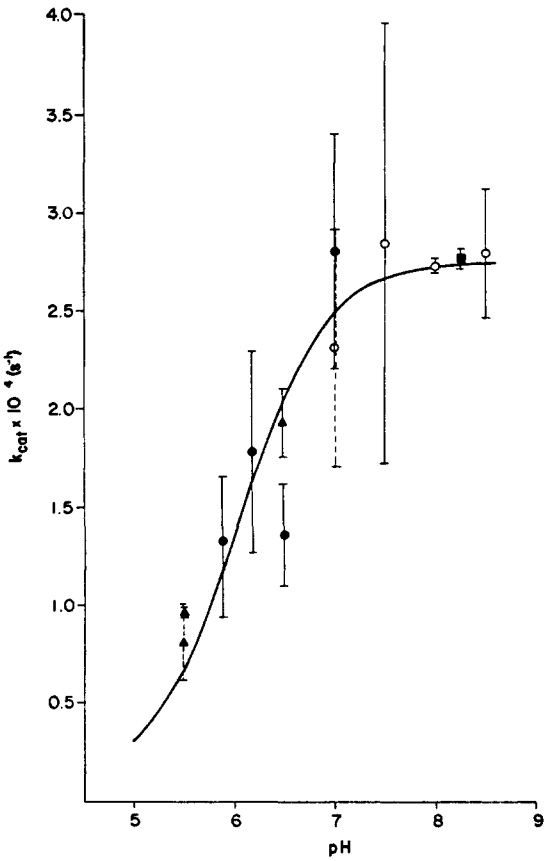


**Figure 2.** $k_{cat}$ vs. pH for the Co(II) CPA-catalyzed deuterium–hydrogen exchange of (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid at 25.0 °C. The buffer concentrations were 0.05 M, and the solutions contained 0.5 M NaCl. The buffers are indicated by the following symbols: (▲) cacodylate; (●) MES; (■) Tris; (○) bis(tris)propane. Standard deviations are indicated for each point by the error bars. When there is overlap between two sets of error bars, the error bars for the point with the lower $k_{cat}$ value are the dashed lines. The enzyme concentration varied from $6.00 \times 10^{-5}$ to $1.28 \times 10^{-4}$ M, and the substrate concentration varied from $1.88 \times 10^{-3}$ to $8.31 \times 10^{-3}$ M. The solid line corresponds to a theoretical sigmoidal curve calculated as in Figure 1.

[I] is the concentration of (−)-**1** and $[I]_0 \gg [E]_0$). The slope of a plot of the reciprocal of the pseudo-first-order rate constants measured vs. the inhibitor concentration yields $K_m/(K_I k_{cat}[E]_0)$. By dividing this slope by the intercept of the plot of the reciprocals of the pseudo-first-order rate constants vs. $1/[S]_0$ measured for **2** in the absence of (−)-**1**, one can calculate $K_I$ values for (−)-**1**.

In Figures 3 and 4, plots of $K_I$ vs. pH for the binding of (−)-**1** to CPA are shown for the Zn(II) and Co(II) enzymes, respectively. Below pH 9 the data points fit theoretical sigmoidal curves ascending in alkaline solution and corresponding to $pK_a$ values of 7.56 ± 0.15 for Zn(II) CPA and 8.29 ± 0.32 for Co(II) CPA. Above pH 9 the $K_I$ values rise again, but exact pH dependencies could not be determined in the very alkaline range.

## Discussion

For a variety of reasons, (*R*)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid ((−)-**1**) is a particularly useful compound with which the $pK_a$ values of the ionizing groups in the active site of CPA can be probed. First, it is the only reactive substrate for the enzyme for which the X-ray structure of a complex with CPA has been determined.[7,8] Second, the stereochemistry of the CPA-catalyzed reaction of (−)-**1** has been elucidated and the results obtained are consistent both with the postulate that (−)-**1** binds in a fashion analogous to that already proposed for reactive peptide and ester substrates[6,11] and with the X-ray structure found for the CPA–(−)-**1** complex.[7,8] Third, as compared to CPA-catalyzed hydrolysis reactions that may involve the formation of

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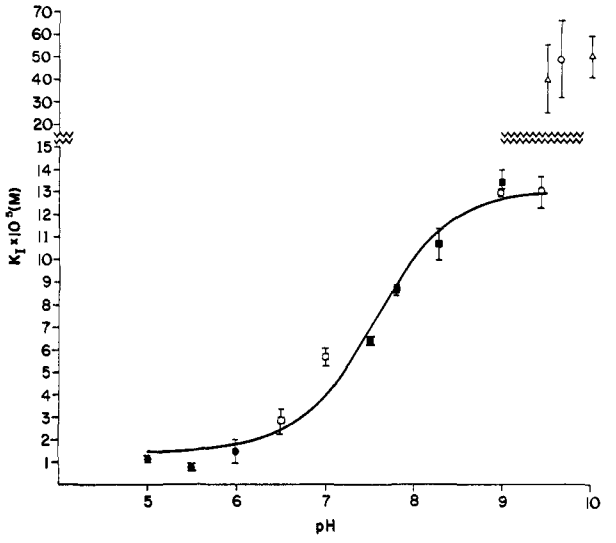


**Figure 3.** $K_I$ vs. pH profile for the inhibition of the CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid by (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid at 25.0 °C. The buffer concentrations were 0.05 M, and the solutions contained 0.5 M NaCl. The buffers are indicated by the following symbols: (●) MES; (□) MOPS; (■) Tris; (○) bis(tris)propane; (△) carbonate. Standard deviations for each point are indicated by the error bars. In these experiments the enzyme concentrations varied from $1.50 \times 10^{-8}$ to $9.50 \times 10^{-8}$ M. The substrate (*O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid) concentration varied from $1 \times 10^{-5}$ to $4 \times 10^{-4}$ M while the inhibitor ((−)-2-benzoyl-3-(*p*-methoxybenzoyl)propionic acid) concentration varied from $1 \times 10^{-5}$ to $2 \times 10^{-3}$ M. The solid line corresponds to a theoretical sigmoidal curve obtained by analysis of the experimental points using an iterative curve-fitting computer program.

multiple intermediates such as tetrahedral adducts and anhydrides, the enolization of the ketonic substrate (−)-**1**, appears to be a relatively simple process. A study of the pH dependency of this enolization reaction at the active site of CPA allows us to examine the catalytic properties and ionization behavior of enzyme-bound active-site bases and acids without having to consider the complications of the formation and breakdown of a multiplicity of intermediates along the reaction pathway.[12]

The stereochemical results obtained earlier[4] indicated that it is the *pro-R* hydrogen of the 3-methylene group of (−)-**1** that undergoes hydrogen–deuterium exchange at the site of CPA. When (−)-**1** is placed in the active site of CPA analogously with the proposed binding mode of reactive peptide and ester substrates,[6,11] and in accord with the X-ray structure determined for the CPA–(−)-**1** complex, it is clear that the γ-carboxylate group of Glu-270 is the functional moiety in the enzyme responsible for hydrogen abstraction and that the corresponding carboxyl group is the hydrogen donor for the exchange reaction. It can be concluded, therefore, that the $pK_a$ values of 6.03 ± 0.35 and 6.04 ± 0.31 measured from the pH dependency of $k_{cat}$ for the Zn(II) and Co(II) CPA catalyzed enolization of (−)-**1**, respectively, correspond to the p$K$ for the ionization of the γ-carboxyl of Glu-270. Considering that the $pK_a$ values assigned to the γ-carboxyl of Glu-270 in the present work are derived from the pH dependencies of $k_{cat}$ values and presumably reflect the ionization of this residue in the enzyme–substrate complex, there is a good correspondence with the $pK_a$ values calculated for this functional group from $k_{cat}/K_m$–pH profiles in CPA-catalyzed peptide and ester hydrolysis.[9] Recently, it has been suggested[13] that the $pK_a$ values measured from the acidic limbs of the $k_{cat}/K_m$–pH profiles correspond to the $pK_a$ for the ionization of the active-site metal ion-bound water molecule involved in the hydrolysis of the enzymatic mixed anhydride intermediates and that the anhydride breakdown process (step $k_3$ of eq 6) is rate controlling for peptides and esters. For several reasons such an analysis is implausible. First, it is difficult to understand how the parameter $k_{cat}/K_m$ for the CPA-catalyzed hydrolysis of peptides and esters would reduce to $k_3$ (see 6). Second, if CPA-catalyzed hydrolysis of peptides proceed through anhydride intermediates, it would be very surprising for these reactions if the $k_3$ step were rate determining. Third, both the X-ray structure of the CPA–(−)-**1** complex[7,8] and the stereochemistry of the CPA-catalyzed enolization of (−)-**1**[4] are not consistent with the involvement of a metal ion–hydroxide complex as the crucial catalytic moiety for this reaction. Only the γ-carboxylate of Glu-270 is positioned properly to be the attacking species

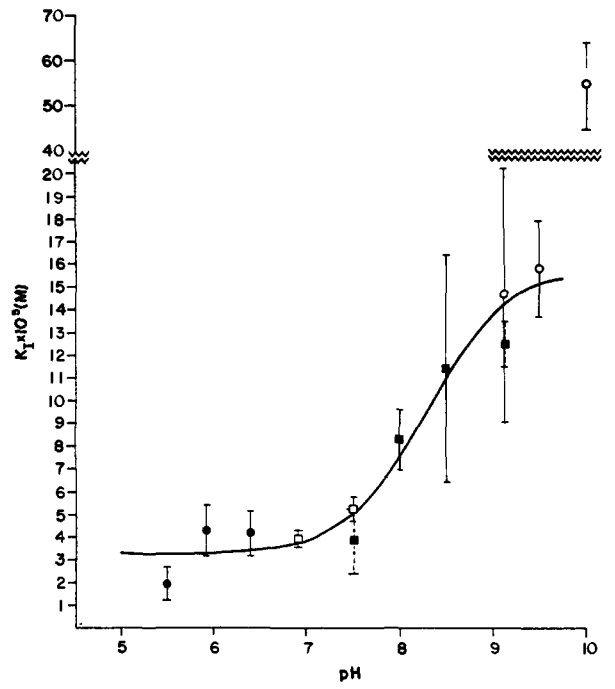


**Figure 4.** $K_I$ vs. pH profile for the inhibition of the Co(II)CPA-catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid by (−)-2-benzyl-3-(*p*-methoxybenzoyl)propionic acid at 25.0 °C. The buffer concentrations were 0.05 M, and the solutions contained 0.5 M NaCl. The buffers are indicated by the following symbols: (●) MES; (□) MOPS; (■) Tris; (○) bis(tris)propane. Standard deviations are indicated for each point by the error bars. Where there is overlap between two sets of error bars, the error bars for the solid symbol are the dashed lines. The enzyme concentration varied from $2.83 \times 10^{-8}$ to $1.67 \times 10^{-7}$ M. The substrate (*O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid) concentration varied from $1.71 \times 10^{-5}$ to $1.17 \times 10^{-3}$ M while the inhibitor concentration varied from $3.00 \times 10^{-6}$ to $6.03 \times 10^{-4}$ M. The solid line corresponds to a theoretical sigmoidal curve calculated as in Figure 3.

$$E + S \underset{K_1}{\rightleftharpoons} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3} E + P_1 + P_2 \qquad (6)$$

where ES′ represents the anhydride intermediate, $P_1$ is the α-amino acid product or the α-hydroxycarboxylic acid product in peptide or ester hydrolysis, respectively, and $P_2$ corresponds to the acyl portion of the substrate.

When the $K_I$–pH dependencies shown in Figures 3 and 4 are examined, there appear to be two ionizations that must be considered. One ionization occurs with $pK_a = 7.56 \pm 0.15$ for Zn(II) CPA and with $pK_a = 8.29 \pm 0.32$ for Co(II) CPA. The other ionization seems to occur above pH 9. An analysis of the pH dependency of $k_{cat}/K_m$ for the catalysis by arsanilazo Tyr-248 CPA[14] of the hydrolysis of **2** yielded a p$K$ value of 7.6[9] for the

(12) A possible alternative to a simple proton-abstraction–proton-donation mechanism for the CPA-catalyzed hydrogen-deuterium exchange reaction of (−)-**1** would involve enzyme-catalyzed hydration of the ketone carbonyl, followed by the elimination of water from the substrate to give the enol (or enolate) species and then reversal of this process. While such a mechanism cannot be ruled out at the present time, it would probably not affect appreciably the interpretation of the $pK_a$ values obtained in this work.

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ionization of the modified Tyr, in excellent agreement with the value of 7.7 measured spectrophotometrically.[15] A similar analysis of kinetic data for the CPA-catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate gave an estimated value of $pK_a$ = 8.2 for the ionization of unmodified Tyr-248. It seems probable, therefore, that the $pK_a$ values in the vicinity of 8 measured for the binding of (−)-**1** to Zn(II) and Co(II) CPA represent the ionization of the phenolic hydroxyl of Tyr-248. While the stereochemical studies[4] on the enolization of (−)-**1** catalyzed by CPA as well as the pH dependency of $k_{cat}$ for this process argue against the involvement of the phenol or phenoxide forms of Tyr-248 in a catalytic step, the X-ray study of the CPA–(−)-**1** complex[7,8] shows that the Tyr-248 residue is in close proximity to the bound substrate. As has been proposed for some ester substrates ("nonspecific" substrates[11,16]) , coordination of the Tyr-248 phenolate group to the active-site zinc may result in effective intramolecular competitive inhibition, causing poorer binding of (−)-**1** to CPA as the pH is raised in the vicinity of pH 8.

In addition to Glu-270 and Tyr-248, the two enzyme-bound moieties in close proximity to the substrate (−)-**1** in its complex with CPA are the zinc ion and the Arg-145 residue. Kinetic studies on the arsanilazo Tyr-248 CPA-catalyzed hydrolysis of **2** have been interpreted to indicate that the p*K* value of 9.1 calculated for the descending limb of the pH profile of $k_{cat}/K_m$ corresponds to the ionization of the zinc-bound water at the active site,[9] a conclusion that is in agreement with the results of the spectrophotometric titration of this modified enzyme.[15] In view of the present observations that the pH dependency of $K_I$ for the binding of (−)-**1** to both Zn(II) and Co(II) CPA appears to depend not only on an enzyme-bound group with $pK_a \simeq 8$ but also on one with $pK_a > 9$, a reasonable interpretation of our findings with (−)-**1** is that ionization of the zinc- (or cobalt-) bound water at the active site retards the formation of the CPA–(−)-**1** complex.

In summary, from measurements on the pH dependency of the parameter $k_{cat}$ for the Zn(II) and Co(II) CPA catalyzed deuterium–hydrogen exchange of (−)-**1**-$d_2$, we have obtained a $pK_a$ value of approximately 6 for the ionization of the catalytically important γ-carboxyl of Glu-270.[17] From a study of the pH dependency of $K_I$, the inhibition constant for (−)-**1** acting as an inhibitor in the Zn(II) and Co(II) CPA catalyzed hydrolysis of **2**, we assign the observed $pK_a$ values of $\simeq 8$ and $>9$ to the phenolic hydroxyl of Tyr-248 and to the active-site metal ion bound water.[18]

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**Registry No.** (−)-**1**, 68973-52-4; (−)-**1**-$d_2$, 84712-44-7; CPA, 11075-17-5; *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate, 61556-61-4; Glu, 56-86-0; Tyr, 60-18-4.

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(17) The observation that the $K_I$ ($K_m$) value for the complex of CPA with (−)-**1**-$d_2$ is nearly independent of pH between pH 5 and 6.5, together with the p*K* value of approximately 6 determined from the pH dependency of $k_{cat}$, suggest that a p*K* of about 6 also controls the pH dependency of $k_{cat}/K_m$ for (−)-**1**-$d_2$ in acidic solution. It appears, therefore, that the binding of (−)-**1**-$d_2$ does not cause a perturbation of the p*K* of Glu-270.
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# Out-of-Plane Deformation Modes in the Resonance Raman Spectra of Metalloporphyrins and Heme Proteins

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**Abstract:** The systematics of low-frequency (<1000 $cm^{-1}$) resonance Raman (RR) spectra have been explored for a series of iron–porphyrin complexes and myoglobin derivatives, with the aid of $^{54}Fe$ substitution and deuteration at the methine and vinyl (for protoporphyrins) carbon atoms. Evidence is presented for activation of several out-of-plane deformation modes. These include modes assigned to methine hydrogen (~840 $cm^{-1}$) and carbon (~300 $cm^{-1}$) motions, pyrrole folding (425–510 $cm^{-1}$), and two $^{54}Fe$-sensitive modes at ~250 and ~380 $cm^{-1}$, suggested to be coupled pyrrole tilting and substituent deformation. These are superimposed on the in-plane skeletal deformation Raman modes. Protoporphyrin complexes also show RR activation of in-plane IR modes and a pair of modes (~412 and ~290 $cm^{-1}$) associated with deformations of the vinyl groups. Activation mechanisms for the out-of-plane modes are discussed. The myoglobin spectra suggest that these modes are sensitive to heme–globin interactions.

Metalloporphyrins and heme proteins display Raman bands in the 1000–1700-$cm^{-1}$ region that are strongly enhanced in resonance with the dominant Q (α and β) and B (Soret) electronic transitions.[1] These arise from in-plane ring modes which are coupled to the π–π* excitations. They have been extensively analyzed and correlated with porphyrin structure.[1-5] Enhancements are less marked for modes below 1000 $cm^{-1}$ since these involve ring deformations and stretching of bonds to the central metal ion and are not as strongly coupled to the π–π* excitations; in addition there is a natural falloff of Raman intensity with decreasing vibrational frequency, if all other factors are equal.[6] This region is nevertheless of much interest because the bonds to the central metal ion are directly affected by chemical changes at the heme group. Under some conditions, good quality spectra

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