pH Dependence of the Nitrotyrosine-248 and Arsanilazotyrosine-248 Carboxypeptidase A Catalyzed Hydrolysis of *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactate

Junghun Suh and Emil Thomas Kaiser*

Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received July 1, 1975*

Abstract: The kinetics of the nitroTyr-248 and arsanilazoTyr-248 carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (I) have been studied over the pH range 5.5–10.0 at 25°. The pH dependencies of $k_{cat.}/K_m$ and $k_{cat.}$ for these reactions were similar to the corresponding dependencies in the carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate (II). The similarities in the pH dependencies of $k_{cat.}/K_m$ for the hydrolysis of I and II catalyzed by the Tyr-248 modified and native carboxypeptidase A species lead us to conclude that the ionization of Tyr-248 exerts little or no effect on these enzymatic reactions. Furthermore, the pH dependencies of $k_{cat.}$ for the hydrolysis of I and II catalyzed by the modified and native enzymes can be explained in terms of the intermediacy of acylcarboxypeptidase A species.

The roles of Glu-270, Tyr-248, Arg-145, the active site zinc ion, and the zinc-bound water have all been discussed with regard to the mechanism of action of carboxypeptidase A (CPA).[1,2] The x-ray crystallographic studies suggest that Glu-270, Tyr-248, and the zinc ion are located near enough to the scissile bond of an enzyme-bound substrate to allow these groups to be directly involved in catalysis.[3] The only other residue within 3 Å of a functional group of the substrate in the productive binding mode appears to be Arg-145. Chemical modification studies have provided evidence for the essentiality of the zinc ion[4] and Glu-270[5-7] in catalysis. Studies on the chemical modification of Tyr-248,[8-14] however, have generally given mechanistically ambiguous results. This has been due in large part to the complicated kinetics involved in the conventional assay methods employed to determine the effects of modification of Tyr-248 and, also, frequently to the nonspecific nature of the modification reactions.

On the basis of a kinetic analysis of the carboxypeptidase A catalyzed hydrolysis of esters and peptides, arguments have been presented for the importance of the ionization of the carboxyl group of Glu-270 and that of the water bound to the active site zinc ion.[15] In the case of Tyr-248, the kinetic analysis suggested that for some substrates the presence of an un-ionized hydroxyl group in this residue is unimportant[16] whereas with others ionization to the phenolate form eliminates the enzyme's activity.[15]

Despite the relatively large body of experimental information on the action of CPA which is now available, a number of questions regarding the roles of each of the functional groups which appear to be implicated as active site residues interacting with substrate molecules remain to be clarified. While a mechanistic analysis of carboxypeptidase A action has supported the notion that the carboxylate group of Glu-270 acts as a nucleophile rather than as a general base catalyst,[15] further support for this hypothesis is needed. Although it seems reasonable to postulate that the active site zinc ion coordinates the carbonyl oxygen of the scissile bond of the substrate, aiding the polarization of the carbonyl function, another possibility which cannot be ruled out at the present time is that the metal ion merely serves as a template to bring the catalytically important groups to the right positions.[1] Finally, it remains to be shown whether with at least some substrates the phenolic hydroxyl group of Tyr-248 plays an important role in binding and in catalysis or not. Considerable attention has been given recently to conformational questions regarding the Tyr-248 residue.[17,18] Before assessing the effects of conformational changes involving Tyr-248, however, more must be known about the importance of this residue in binding and catalysis.

In the present article attention will be focused on the results of kinetic experiments designed to define the mechanistic role of Tyr-248. Additionally, kinetic evidence collected during our continued pursuit of the detailed mechanism of action of CPA will be presented for the existence of an acyl-enzyme intermediate.

Experimental Section

***O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactic Acid (I).** This compound was synthesized according to the procedure used earlier in the preparation of *O*-(*trans*-*p*-nitrocinnamoyl)-L-β-phenyllactic acid[19] and recrystallized from benzene–hexane (33% yield): mp 125–126.5° (uncor). Anal. Calcd for $C_{18}H_{15}ClO_4$: C, 65.34; H, 4.57. Found: C, 65.25; H, 4.57.

Because of its limited solubility in water, I was converted to the sodium salt by careful titration with sodium hydroxide solution using a Radiometer pH stat maintained below pH 8. The salt was isolated by evaporation of the resultant solution in vacuo.

Other Chemicals. The buffers, salts, and standard titrants used in these studies were all of the highest grade available and were obtained from the following sources: tris(2-amino-2-hydroxymethyl-1,3-propanediol) from Schwarz/Mann; 2-amino-2-ethyl-1,3-propanediol from Sigma; Mes (4-morpholinoethanesulfonic acid) from Aldrich; sodium chloride from Mallinckrodt; and standard sodium hydroxide, hydrochloric acid, and buffer solutions from Fisher Scientific Co. The water employed was distilled and then demineralized with a mixed-bed ion exchange column (Continental Demineralization Service of Chicago).

Enzyme Stock Solutions. Carboxypeptidase A, prepared from bovine pancreas by the method of Anson[20] as modified by Putnam and Neurath,[21] was purchased as a suspension of crystals in toluene–water from Worthington Biochemical Corp. (Lot No. COA 34E741). Nitration of carboxypeptidase A at the Tyr-248 residue (nitroCPA) was carried out by the reaction of CPA with tetranitromethane.[13,22] The nitrotyrosine content of the nitroCPA obtained was found spectroscopically to be 1.0 residue/molecule of enzyme.[13,23]

ArsanilazoTyr-248 carboxypeptidase A (azoCPA) was obtained by the treatment of CPA with *p*-diazoarsanilate[14] which was prepared by the reaction of arsanilic acid with nitrous acid.[24] This procedure is known to result in the modification of only Tyr-248.[25] The arsanilazoTyr content of the azoCPA which was prepared was

found spectroscopically to be 0.96 residue/molecule of the enzyme.[26] The concentrations of the stock solutions of the modified enzymes were measured by the Lowry method.[27]

Kinetic Measurements. These measurements were carried out on Cary 15 or Gilford spectrophotometers. The uv spectra of I and of the product mixture obtained upon complete hydrolysis of I to *trans-p*-chlorocinnamate and L-β-phenyllactate are illustrated in Figure 1. Kinetic runs were followed at wavelengths (>300 nm) which afforded a convenient absorbance change during the reaction. The absorbance due to the enzyme present was negligible at the concentrations employed. The kinetic data obtained were analyzed on an IBM 370-168 computer using a computer program provided by Dr. J. H. Smith.

Results

Kinetic Analysis. The kinetics of the nitroCPA- and azoCPA-catalyzed hydrolysis of I were found to be closely related to the kinetics of the CPA-catalyzed hydrolysis of II.[28] A simple Michaelis-Menten scheme was obeyed, complicated only by competitive product inhibition, as illustrated by eq 1.

$$\begin{gathered} E + S \rightleftharpoons ES \rightarrow E + P_1 + P_2 \\ E + P_1 \underset{K_p}{\rightleftharpoons} EP_1 \end{gathered} \tag{1}$$

The steady-state rate expression for this scheme when $S_0 \gg E_0$ is given by eq 2, where $k_{cat.}$ is the observed catalytic rate constant and $K_{m_{app}}$ is the observed Michaelis constant.

$$v = \frac{-d[S]}{dt} = \frac{k_{cat.}[E_0][S]}{\dfrac{K_{m_{app}} + [S] + K_{m_{app}}([S_0] - [S])}{K_p}} \tag{2}$$

When the kinetic scheme is modified to include a covalent intermediate as in eq 3 with $S_0 \gg E_0$, the steady-state rate expression becomes that shown in eq 4 where $K_m = (k_{-1} + k_2)/k_1$. The constants in eq 4 are related to those of eq 3 as illustrated in eq 5 and 6.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 + P_1 \tag{3}$$

$$v = \frac{-d[S]}{dt} = \frac{\dfrac{k_2k_3[E_0][S]}{k_2 + k_3}}{\dfrac{k_3K_m}{k_2 + k_3} + [S] + \dfrac{k_3K_m([S_0] - [S])}{(k_2 + k_3)K_p}} \tag{4}$$

$$k_{cat.} = k_2k_3/(k_2 + k_3) \tag{5}$$

$$K_{m_{app}} = k_3K_m/(k_2 + k_3) \tag{6}$$

Values of the kinetic parameters for individual runs at a given pH were estimated using either eq 7 or 8, the integrated forms of eq 2.

$$(1/k_{cat.}[E_0])[K_{m_{app}}(1 + [S_0]/K_p) \ln [S_0]/[S] + (1 - K_{m_{app}}/K_p)([S_0] - [S])] = t \tag{7}$$

$$(1/(k_{cat.}/K_{m_{app}})[E_0])[(1 + [S_0]/K_p) \ln [S_0]/[S] + (1/K_{m_{app}} - 1/K_p)([S_0] - [S])] = t \tag{8}$$

By repetitive iteration, computer-estimated values of $k_{cat.}$, $K_{m_{app}}$, and K_p for each run were obtained using eq 7. A similar analysis using eq 8 yielded values of $k_{cat.}/K_{m_{app}}$, $K_{m_{app}}$, and K_p. At least six runs were carried out at each pH, and the weighted averages and weighted standard deviations of the kinetic parameters at a given pH were calculated. The values of the kinetic parameters obtained for the nitroCPA- and azoCPA-catalyzed hydrolysis of I are presented in Tables I and II, respectively.

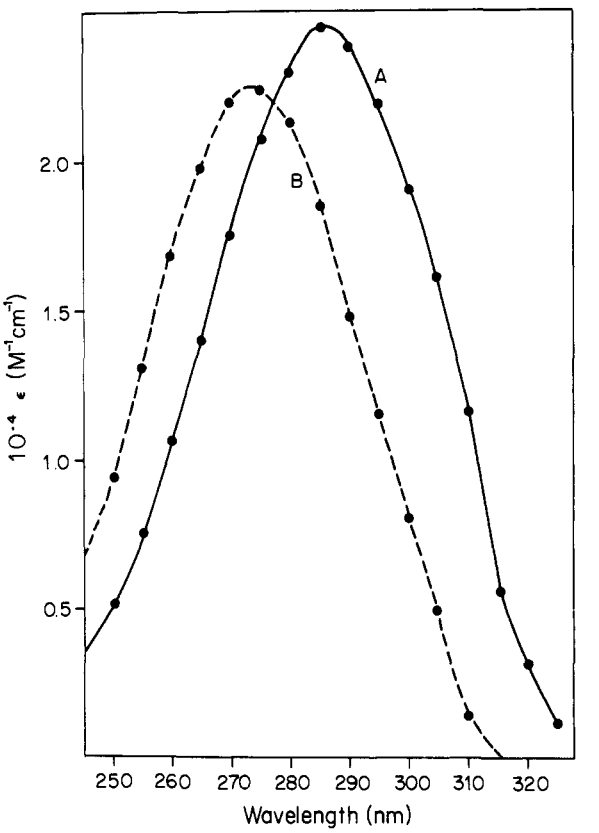


Figure 1. Substrate (curve A) and product (curve B) spectra for the hydrolysis of *O*-(*trans-p*-chlorocinnamoyl)-L-β-phenyllactate obtained in 0.5 M NaCl, 0.05 M Tris buffer at pH 7.5 and 25.0°.

Table I. Kinetic Parameters for the NitroTyr-248 Carboxypeptidase A Catalyzed Hydrolysis of *O*-(*trans-p*-Chlorocinnamoyl)-L-β-phenyllactate at Various pH Values at 25.0° [a,b]

pH	$k_{cat.}$, sec^{-1}	$10^{-4}\,k_{cat.}/K_{m_{app}}$, $M^{-1}\,sec^{-1}$	$10^4K_{m_{app}}$, M	10^4K_p, M
5.55	5.89 ± 0.01	6.04 ± 0.01	0.975 ± 0.004	1.42 ± 0.07
5.95	13.4 ± 0.01	12.3 ± 0.01	1.09 ± 0.002	1.50 ± 0.03
6.48	30.2 ± 0.07	46.6 ± 0.09	0.649 ± 0.003	0.507 ± 0.008
6.93	49.0 ± 0.08	51.4 ± 0.08	0.953 ± 0.003	0.650 ± 0.012
7.53[d]	64.5 ± 0.07	60.5 ± 0.06	1.07 ± 0.002	0.788 ± 0.007
7.96	65.5 ± 0.07	64.3 ± 0.06	1.02 ± 0.001	0.678 ± 0.007
8.50	64.8 ± 0.09	61.5 ± 0.09	1.05 ± 0.003	0.714 ± 0.009
9.00	64.3 ± 0.12	59.4 ± 0.09	1.08 ± 0.004	0.653 ± 0.013
9.48	85.3 ± 0.10	18.7 ± 0.02	4.56 ± 0.010	2.26 ± 0.03
9.92	107 ± 6[c]	6.68 ± 0.005	16.0 ± 0.91	4.45 ± 0.05

[a] The buffer solutions consisted of the following: 0.5 M NaCl, 0.05 M Mes in the pH range 5.55–6.93; 0.5 M NaCl, 0.05 M Tris in the pH range 7.53–9.00; 0.5 M NaCl, 0.05 M 2-amino-2-ethyl-1,3-propanediol in the pH range 9.48–9.92. [b] The values of $k_{cat.}$ for each run were obtained by analysis of the kinetic data using eq 7. Those of $k_{cat.}/K_{m_{app}}$ and K_p were determined using eq 8. The values of $K_{m_{app}}$ at each pH were calculated from the weighted average values of $k_{cat.}$ and $k_{cat.}/K_{m_{app}}$. The values of kinetic parameters calculated using eq 7 and 8 agreed well with each other. However, the standard deviations of $k_{cat.}$ obtained from eq 7 and $k_{cat.}/K_{m_{app}}$ obtained from eq 8 were much smaller than those of $k_{cat.}$ calculated from eq 8 and $k_{cat.}/K_{m_{app}}$ calculated from eq 7, respectively. [c] At this pH, $k_{cat.}$ could not be obtained by analysis of the kinetic data with eq 7. Therefore, this value was calculated using eq 8. [d] At pH 7.5, a study of the CPA-catalyzed hydrolysis of I gave the following kinetic parameters: $k_{cat.}$ = 144 sec^{-1}, $K_{m_{app}}$ = 1.36 × 10^{-4} M, $k_{cat.}/K_{m_{app}}$ = 1.01 × 10^6 M^{-1} sec^{-1}, and K_p = 0.585 × 10^{-4} M.[1]

pH Dependencies of Kinetic Parameters. If the concentration of the reactive form of the substrate is independent of pH over the pH range studied, the pH dependency of the kinetic parameter $k_{cat.}/K_{m_{app}}$ can be accounted for theoreti-

Table II. Kinetic Parameters for the ArsanilazoTyr-248 Carboxypeptidase A Catalyzed Hydrolysis of *O*-(*trans*-*p*-Chlorocinnamoyl)-L-β-phenyllactate at Various pH Values at 25.0° [a,b]

pH	$k_{cat.}$, sec^{-1}	$10^{-4}\ k_{cat.}/K_{m_{app}}$, $M^{-1}\ sec^{-1}$	$10^4 K_{m_{app}}$, M	$10^4 K_p$, M
5.55	3.30 ± 0.07	2.63 ± 0.005	1.28 ± 0.02	1.18 ± 0.05
5.95	5.57 ± 0.07	6.17 ± 0.01	0.954 ± 0.006	0.773 ± 0.013
6.48	10.2 ± 0.1	12.7 ± 0.01	0.831 ± 0.006	0.807 ± 0.007
6.93	17.2 ± 0.1	19.1 ± 0.01	0.924 ± 0.005	0.673 ± 0.004
7.53	22.0 ± 0.1	22.0 ± 0.01	1.00 ± 0.005	0.915 ± 0.005
7.96	22.0 ± 0.1	21.6 ± 0.01	1.05 ± 0.004	0.900 ± 0.006
8.50	21.9 ± 0.1	19.7 ± 0.01	1.10 ± 0.004	0.894 ± 0.004
9.00	23.6 ± 0.1	14.2 ± 0.01	1.69 ± 0.009	1.36 ± 0.007
9.48	30.3 ± 0.3	7.30 ± 0.01	4.16 ± 0.04	2.60 ± 0.02
9.92	79.6 ± 0.5	1.91 ± 0.001	42.9 ± 0.2	12.8 ± 0.2

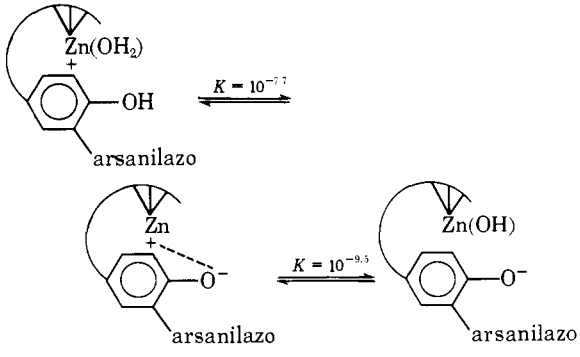
[a] See footnote *a* of Table I for a description of the buffer solutions employed here also. [b] The values of $k_{cat.}/K_{m_{app}}$, $K_{m_{app}}$, and K_p for each run were obtained by analysis of the kinetic data using eq 8. The $k_{cat.}$ values were calculated from the values of $k_{cat.}/K_{m_{app}}$ and $K_{m_{app}}$.

cally by consideration of the pH-dependent variations of the molar fractions and of the relative reactivities of the reactive forms of the enzyme. The simplest scheme for the ionization of the free enzyme which is consistent with the pH profiles of $k_{cat.}/K_{m_{app}}$ for the hydrolysis reactions described in this paper is shown in eq 9 where EH represents the only reactive form of the enzyme and EH_2 and E, the protonated and deprotonated forms, respectively, are unreactive. Making this assumption, the pH dependency of $k_{cat.}/K_{m_{app}}$ is given by eq 10 where $(k_{cat.}/K_{m_{app}})_{lim}$ is the limiting value of this parameter which would be obtained if all of the enzyme were in the reactive form. Analysis of the $k_{cat.}/K_{m_{app}}$ values shown in Tables I and II using a computer program based on eq 10 gave values of $pK_1 = 6.4$ and $pK_2 = 9.3$ for the action of nitroCPA on I and $pK_1 = 6.4$ and $pK_2 = 9.1$ for the action of azoCPA on I.

$$EH_2 \overset{K_1}{\rightleftharpoons} EH \overset{K_2}{\rightleftharpoons} E \tag{9}$$

$$k_{cat.}/K_{m_{app}} = \frac{(k_{cat.})/K_{m_{app}})_{lim}}{[H^+]/K_1 + 1 + K_2/[H]} \tag{10}$$

From spectrophotometric titrations, the p*K* value for the ionization of the phenolic hydroxyl of nitroTyr-248 in nitroCPA was found to be 6.3[13] and two p*K* values, 7.7 (7.8) and 9.5, were observed for the corresponding ionization in azoCPA.[17,29] The interpretation shown in eq 11 was proposed to account for the ionization behavior of the arsanilazoTyr-248 residue in azoCPA.[17,30]



(11)

If one were to assume that the deprotonation of Tyr-248 results in the conversion of the enzyme into an inactive form, the p*K* values for the basic limbs of the $k_{cat.}/K_{m_{app}}$ profiles for the action of nitroCPA and azoCPA should be related to the spectrophotometrically measured p*K* values of 6.3 and 7.7, respectively. However, as discussed above, the actual pK_2 values seen in the hydrolysis of I by nitroCPA and azoCPA were significantly higher than the spectrophotometrically determined values for the ionization of the modified Tyr-248 residues. This implies that the state of ionization of the modified Tyr-248 residues has little or no effect on $k_{cat.}/K_{m_{app}}$ in the hydrolysis of I catalyzed by nitroCPA or azoCPA.

To explore this point further, the action of nitroCPA on I was analyzed according to the scheme of eq 12. Here EH_2

$$\begin{array}{ccc} EH_3 & \overset{K_1}{\rightleftharpoons} & EH_2 \\ {\scriptstyle K_2}\downarrow\uparrow & & \uparrow\downarrow{\scriptstyle K_2} \\ EH_2' & \overset{K_1}{\rightleftharpoons} & EH \overset{K_3}{\rightleftharpoons} E \end{array} \tag{12}$$

and EH are forms of the enzyme in which the phenolic hydroxyl in nitroTyr-248 is un-ionized and ionized, respectively, and both of these species are assumed to be reactive. When pK_2 was taken to be 6.3, the expression of eq 13 was obtained, making the assumption of equal reactivity for EH_2 and EH.

$$\frac{k_{cat.}}{K_{m_{app}}} = \frac{(k_{cat.}/K_{m_{app}})_{lim}\left[1 + \frac{10^{-6.3}}{[H^+]}\right]}{\left[1 + \frac{[H^+]}{K_1} + \frac{10^{-6.3}}{K_1} + \frac{10^{-6.3}}{[H^+]} + \frac{10^{-6.3}K_3}{[H^+]^2}\right]} \tag{13}$$

Analysis of the pH dependency of $k_{cat.}/K_{m_{app}}$ for the action of nitroCPA on I using a computer program based on eq 13 gave values for pK_1, pK_2, and pK_3 of 6.4, 6.3 (fixed), and 9.3. In Figure 2 illustrations of the results found from the examination of the pH dependency of $k_{cat.}/K_{m_{app}}$ are presented. Curve A represents a theoretical curve based on the schemes of either eq 9 or 12 and the p*K* values which have been described. This curve also shows the pH dependency of the molar fraction of the species EH of eq 9 and the pH dependency of the sum of the molar fractions of the species H_2 and EH of eq 12. Curve B represents the pH dependency of the molar fraction of species EH_2 of eq 12, and it also illustrates the shape of the hypothetical pH profile of $k_{cat.}/K_{m_{app}}$ for the action of nitroCPA if the ionization of the phenolic hydroxyl of nitroTyr-248 were to result in an inactive form of the enzyme.

To explore the effect of the ionization of the arsanilazotyr-248 residue on azoCPA action, the pH dependency of $k_{cat.}/K_{m_{app}}$ for the hydrolysis of I catalyzed by azoCPA was analyzed according to eq 14. Here EH_2 and EH, forms of the free enzyme in which the phenolic hydroxyl groups of the modified Tyr residue are un-ionized and ionized, respectively, are proposed to be equally reactive, and EH_3 and E are inactive. Making these assumptions eq 15 can be derived.

$$EH_3 \overset{K_1}{\rightleftharpoons} EH_2 \overset{K_2}{\rightleftharpoons} EH \overset{K_3}{\rightleftharpoons} E \tag{14}$$

$$\frac{k_{cat.}}{K_{m_{app}}} = \frac{(k_{cat.}/K_{m_{app}})_{lim}\left[1 + \frac{K_2}{[H^+]}\right]}{\left[\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H]} + \frac{K_2K_3}{[H^+]^2}\right]} \tag{15}$$

The analysis of the pH dependency of $k_{cat.}/K_{m_{app}}$ for the action of azoCPA on I using a computer program based on eq 15 yielded values of $pK_1 = 6.4$, $pK_2 = 7.6$, and $pK_3 = 9.1$. In Figure 3 the results of the analysis of the pH dependency of $k_{cat.}/K_{m_{app}}$ for the azoCPA-catalyzed hydrolysis of I are illustrated. Curve A is a theoretical one based on either the schemes of eq 9 or 14 and the p*K* values which have been described. Curve B shows the shape of the hypothetical pH profile of $k_{cat.}/K_{m_{app}}$ for the action of azoCPA, assuming that only species EH_2 of eq 14 is reactive. In other words, this curve illustrates what would be seen if the ion-

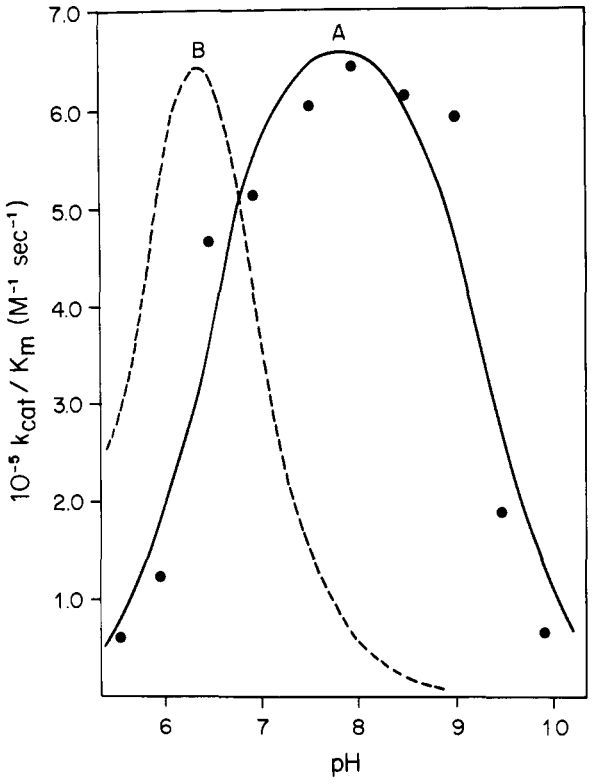


Figure 2. ●, experimentally determined values of $k_{cat.}/K_{m_{app}}$ at each pH for the nitroCPA-catalyzed hydrolysis of I. Curve A shows a theoretical plot based on the schemes of either eq 9 or 12 and the pK values given in the text. It also illustrates the pH dependency of the molar fraction of the species EH of eq 9 and the pH dependency of the sum of the molar fractions of the species EH_2 and EH of eq 12. Curve B illustrates the shape of the hypothetical pH profile of $k_{cat.}/K_{m_{app}}$ resulting if the ionization of the phenolic hydroxyl of nitroTyr-248 is assumed to produce an inactive form of the enzyme. It also represents the pH dependency of the molar fraction of species EH_2 of eq 12.

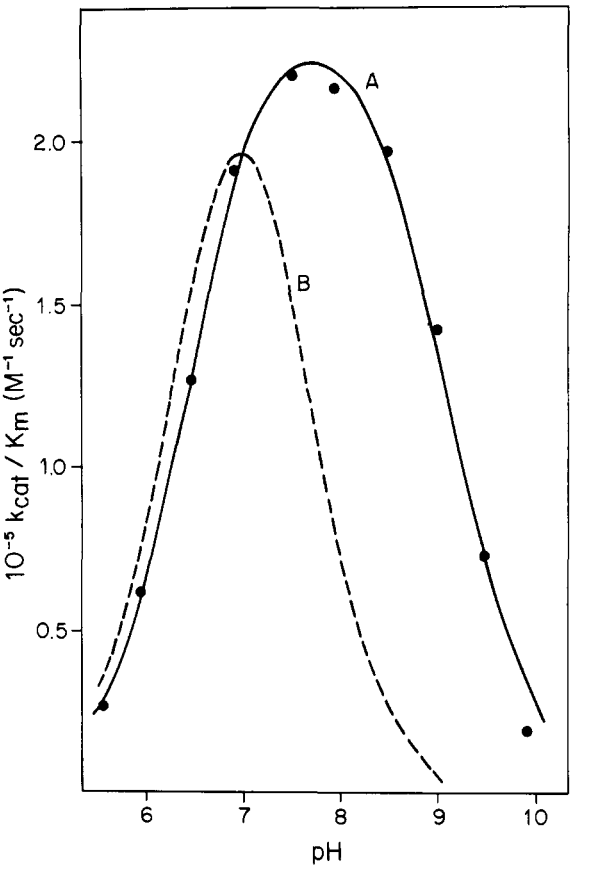


Figure 3. ●, experimentally determined values of $k_{cat.}/K_{m_{app}}$ at each pH for the azoCPA-catalyzed hydrolysis of I. Curve A shows a theoretical plot based on the schemes of eq 9 or 14 and the pK values given in the text. Curve B is a theoretical profile of $k_{cat.}/K_{m_{app}}$ based on the assumption that only species EH_2 of eq 14 is reactive.

ization of the phenolic hydroxyl of the arsanilazoTyr-248 group were to convert the enzyme into an unreactive form.

The pH dependency of $k_{cat.}/K_{m_{app}}$ in the hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate catalyzed by native CPA has been reported.[28] A comparison of $k_{cat.}/K_{m_{app}}$ and k_{OH^-} for the CPA and hydroxide ion catalyzed hydrolysis of several para-substituted *trans*-cinnamoyl derivatives of L-β-phenyllactate[1] has established that the substituents examined exert only electronic effects on the rates of reaction. Thus, results for the pH profile of the action of native CPA on I should be comparable to those which have been obtained with II already. Based on the assumption that the rate data for the hydrolysis of II catalyzed by CPA could be analyzed in terms of two ionization constants, values of pK_1 and pK_2 were calculated to be 6.5 and 9.4, respectively, by Hall et al.[28] Our reanalysis of their results using eq 14 gave values of pK_1, pK_2, and pK_3 of 6.6, 8.2, and 9.1, respectively, for the CPA-catalyzed hydrolysis of II. In Figure 4, the results of the reanalysis of the pH dependency of $k_{cat.}/K_{m_{app}}$ in the latter reaction are illustrated. Curve A is a theoretical one based on eq 14, representing the sum of the molar fractions of EH_2 and EH, and curves B, C, D, and E illustrate the molar fractions of the EH_2, EH, EH_3, and E species, respectively. A summary of the ionization constants derived from pH profiles of $k_{cat.}/K_{m_{app}}$ for the action of nitroCPA and azoCPA on I and of native CPA on II is presented in Table III.

The pH dependencies of $k_{cat.}$ for the action of nitroCPA and azoCPA on I are similar to that found for the action of native CPA on II.[28] The pH profiles seen appear to be a composite of a sigmoidal curve going up to a value of about

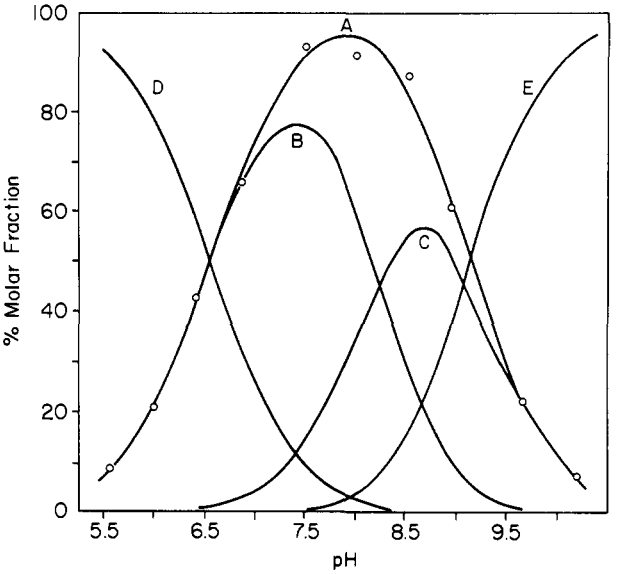


Figure 4. O, experimental values of $k_{cat.}/K_{m_{app}}$ at each pH for the CPA-catalyzed hydrolysis of II[28] divided by the value of $(k_{cat.}/K_{m_{app}})_{lim}$ obtained by a computer analysis of the data. Curve A is a theoretical one based on eq 14, representing the sum of the molar fractions of EH_2 and EH, and curves B, C, D, and E illustrate the molar fractions of the EH_2, EH, EH_3, and E species, respectively.

pH 9 and a steeply rising line beyond pH 9. The sigmoidal portions of the pH profiles observed for $k_{cat.}$ were analyzed using two different schemes. In the first scheme, shown in eq 16, one ionization of the Michaelis complex was consid-

Table III. Values of Ionization Constants Derived from the pH Dependence of $k_{cat.}/K_{m_{app}}$

Rxn studied	Eq used for analysis	K_1, M	K_2, M	K_3, M	pK_1	pK_2	pK_3
NitroCPA with I	10	$(3.70 \pm 1.71) \times 10^{-7}$	$(5.36 \pm 2.43) \times 10^{-10}$		6.4	9.3	
NitroCPA with I	13	$(3.80 \pm 1.71) \times 10^{-7}$		$(5.36 \pm 2.42) \times 10^{-10}$	6.4	6.3[a]	9.3
AzoCPA with I	10	$(3.76 \pm 0.43) \times 10^{-7}$	$(7.86 \pm 0.89) \times 10^{-10}$		6.4	9.1	
AzoCPA with I	15	$(3.83 \pm 0.63) \times 10^{-7}$	$(2.65 \pm 3.98) \times 10^{-8}$	$(7.40 \pm 10.64) \times 10^{-10}$	6.4	7.6	9.1
CPA with II	c	$(2.85 \pm 0.27) \times 10^{-7}$	4.26×10^{-10}		6.5	9.4	
CPA with II[b]	15	$(2.78 \pm 0.31) \times 10^{-7}$	$(5.88 \pm 5.78) \times 10^{-9}$	$(8.35 \pm 7.29) \times 10^{-10}$	6.6	8.2	9.1

[a] This number was fixed in the computer analysis. [b] The ionization constants were calculated from the data given in ref 28. [c] See ref 28.

Table IV. Values of Parameters Derived from the pH Dependence of $k_{cat.}$[a]

Rxn studied	Eq used for analysis	K, M	pK	$(k_{cat.})_{lim}$, sec^{-1}	$(k_2)_{lim}$, sec^{-1}	k_3, sec^{-1}
NitroCPA with I	17	$(2.74 \pm 0.1) \times 10^{-7}$	6.6	67.6 ± 1.2		
NitroCPA with I	20	$(5.87 \pm 0.66) \times 10^{-8}$	7.2		339 ± 67	83.5 ± 4.7
AzoCPA with I	17	$(4.37 \pm 0.50) \times 10^{-7}$	6.4	22.0 ± 1.1		
AzoCPA with I	20	$(1.98 \pm 0.27) \times 10^{-7}$	6.7		61.2 ± 22.2	37.1 ± 7.5
CPA[b] with II	17	$(6.90 \pm 5.2) \times 10^{-7}$	6.2	66.7 ± 1.9		
CPA[b] with II	20	$(1.22 \pm 0.16) \times 10^{-7}$	6.9		339 ± 67	85.0 ± 5.8

[a] The data points up to pH 9 were analyzed with the aid of a computer program. [b] The data analyzed were obtained from ref 28.

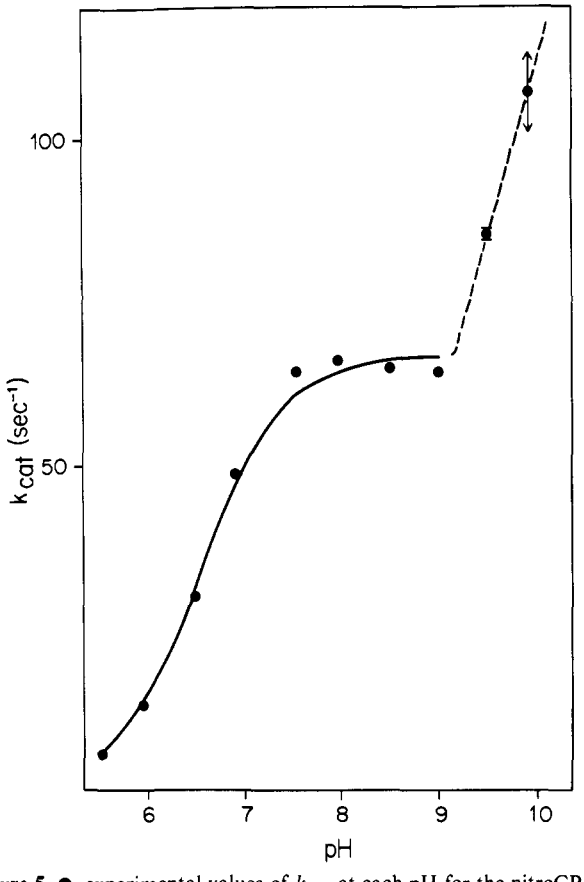


Figure 5. ●, experimental values of $k_{cat.}$ at each pH for the nitroCPA-catalyzed hydrolysis of I. The solid line represents a theoretical plot of $k_{cat.}$ based on eq 17 or 20 and the pK values given in Table IV.

ered, and the observed value of $k_{cat.}$ was taken to reflect the molar fraction of ES, the productive form of the complex. According to this scheme the relationship of eq 17 should hold.

$$\mathrm{ESH} \overset{K}{\rightleftharpoons} \mathrm{ES} \xrightarrow{(k_{cat.})_{lim}} \mathrm{E} + \mathrm{P_1} + \mathrm{P_2} \qquad (16)$$

$$k_{cat.} = (k_{cat.})_{lim}/(1 + [\mathrm{H^+}]/K) \qquad (17)$$

In the second scheme, shown in eq 19, the existence of a covalent intermediate was assumed. The relationship between the observed $k_{cat.}$ values and the values of k_2 and k_3 has been given already in eq 5 and is written in another form in eq 18. Employing the scheme of eq 19, k_3 was assumed to be constant over the pH range where the pH dependence of $k_{cat.}$ is sigmoidal, and k_2 was assumed to depend on the ionization of the Michaelis complex. With these assumptions, eq 20 can be obtained. In Table IV the analyses of the pH dependency of $k_{cat.}$ for the action of nitroCPA and azoCPA on I and of CPA on II using eq 17 and 20 are presented.

$$1/k_{cat.} = 1/k_2 + 1/k_3 \qquad (18)$$

$$\mathrm{ESH} \overset{K}{\rightleftharpoons} \mathrm{ES} \xrightarrow{(k_2)_{lim}} \mathrm{ES'} \xrightarrow[+\,\mathrm{P_1}]{k_3} \mathrm{E} + \mathrm{P_2} \qquad (19)$$

$$1/k_{cat.} = 1/k_3 + (1 + [\mathrm{H^+}]/K)/(k_2)_{lim} \qquad (20)$$

In Figures 5-7, the pH profiles of $k_{cat.}$ for the action of nitroCPA and azoCPA on I and of CPA on II are illustrated. The solid lines drawn are theoretical ones calculated with our computer analysis. In Figures 5 and 7 the use of eq 17 and 20 resulted in nearly identical curves. In Figure 6 line A was obtained by calculation with eq 20 and line B from eq 17.

Discussion

The pK_1 values measured from the acidic limbs of the pH profiles of $k_{cat.}/K_{m_{app}}$ for the action of the various CPA species listed in Table III are all about 6.5. In a considerable number of published studies[31] of the pH dependency of $k_{cat.}/K_{m_{app}}$ in CPA-catalyzed hydrolysis reactions similar pK_1 values have been obtained. From a mechanistic analysis of the action of CPA Kaiser and Kaiser[15] assigned the free carboxyl group of Glu-270 as the ionizing residue responsible for this pK. Support for this assignment has come from the agreement between the pK_1 values determined in hydrolysis reactions with the pK values observed in studies of the chemical modification of Glu-270[5,7] and also with the results of a ^{35}Cl NMR study of the rate of exchange of Cl^- from the zinc ion in CPA.[32] The unusually high pK of 6.5 observed for the carboxylate group may be caused by the high hydrophobicity of its environment which may also be responsible for its unusually high nucleophilicity as observed, for example, in its reaction with *N*-α-bromoacetyl-*N*-methyl-L-phenylalanine.[7]

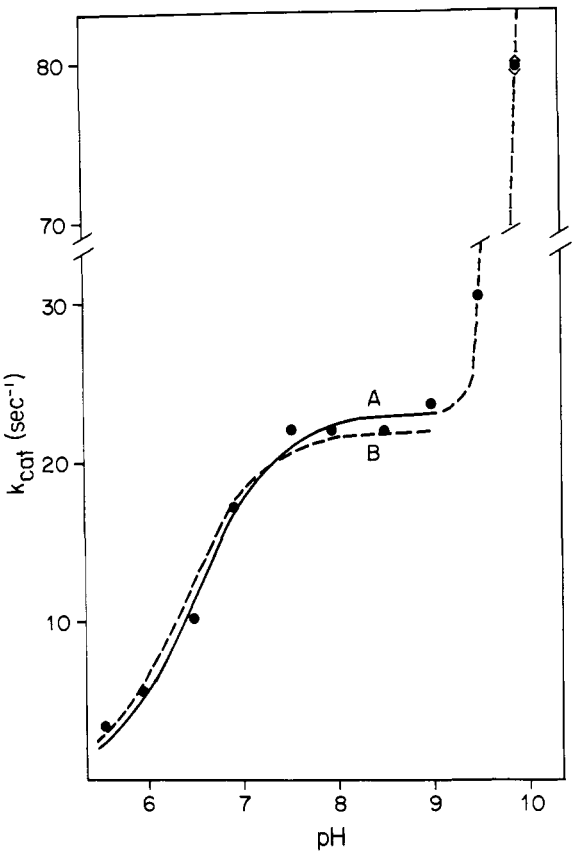


Figure 6. ●, experimental values of $k_{cat.}$ at each pH for the azoCPA-catalyzed hydrolysis of I. Curve A represents a theoretical plot of $k_{cat.}$ based on eq 20 and curve B shows the plot calculated from eq 17.

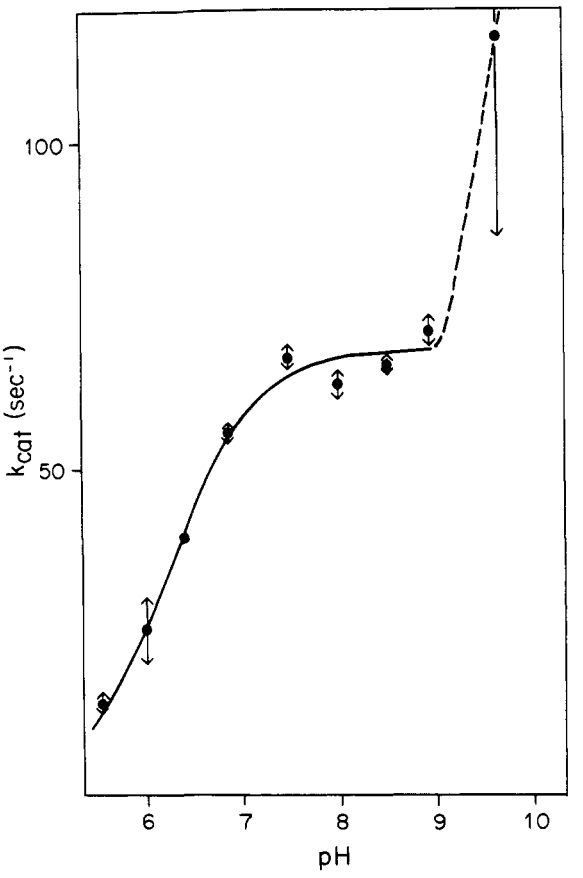


Figure 7. ●, experimental values of $k_{cat.}$ at each pH for the CPA-catalyzed hydrolysis of II. The solid line represents a theoretical plot of $k_{cat.}$ based on eq 17 and 20 and the pK values given in Table IV.

When the basic limbs of the pH profiles of $k_{cat.}/K_{m_{app}}$ for the hydrolysis reactions listed in Table III were analyzed using the two-proton ionization scheme of eq 9, pK_2 values near 9 were found. Although the known pK values for the ionization of the phenolic hydroxyl groups in the modified Tyr-248 residues in nitroCPA and azoCPA lie well below the pK_2 values observed for the basic limbs of the $k_{cat.}/K_{m_{app}}$ profiles, they are in a pH region where CPA is very active. Therefore, forms of the enzyme in which Tyr-248 is in different states of ionization should be considered in analyzing the pH dependency for the action of CPA or modified CPA species. When the pH profile of $k_{cat.}/K_{m_{app}}$ for the nitroCPA-catalyzed hydrolysis of I was examined, the pK observed for the acidic limb was seen to be close to the known pK for the ionization of nitroTyr-248, and, thus, the scheme of eq 12 was employed to analyze the kinetic data. Assuming that the ionization of the carboxyl group of Glu-270 affects the acidic limb of the $k_{cat.}/K_{m_{app}}$ profile, EH_2 in eq 12 refers to the form of the enzyme in which this group is in the carboxylate state, EH_2' to that with nitroTyr-248 ionized, EH to that with both Glu-270 and nitroTyr-248 ionized, and E to the one with Glu-270, nitroTyr-248, and an additional functional group responsible for the basic limb ionized. Because the data points for the pH dependency of $k_{cat.}/K_{m_{app}}$ in the hydrolysis of nitroCPA were somewhat scattered, the pK values for the ionizations $EH_3 \rightleftharpoons EH_2'$ and $EH_2 \rightleftharpoons EH$ were assumed to be identical as were the pK values for the ionizations $EH_3 \rightleftharpoons EH_2$ and $EH_2' \rightleftharpoons EH$, instead of assigning independent pK values to each ionization. In other words, the ionization of Glu-270 and that of nitroTyr-248 was assumed to be independent of each other. Because of computational difficulties in our kinetic analysis the pK value for the ionization of nitroTyr-248 was fixed as 6.3, the figure which was obtained by spectrophotometric titration.[13] As mentioned above, curve B of Figure 2 shows that the shape of the hypothetical curve obtained by assuming that Glu-270 should be ionized and nitroTyr-248 un-ionized for the nitroCPA to be in its active form differs markedly from the shape of the experimentally determined curve, supporting the argument that the ionization of nitroTyr-248 has no effect on the action of nitroCPA on I.

Although the pK_2 value obtained from the basic limb of the pH profile for the action of azoCPA on I, assuming the two-proton ionization scheme of eq 9, differs markedly from the pK value for the ionization of the phenolic hydroxyl in azoTyr-248 obtained from spectrophotometric titration, it agrees reasonably well with the pK value assigned to the formation of the zinc-bound hydroxide species in the latter experiment.[17] Ionization of the zinc-bound water appears to retard productive binding of the substrate to the enzyme. The unusually low pK of azoTyr-248 is probably due to the coordination of the phenolate form of this residue to the zinc ion, and the formation of the active site zinc-hydroxide complex appears to involve replacement of the phenolate species bound to the zinc ion by hydroxide ion. Because the known pK value of azoTyr-248 lies in the pH range where azoCPA is active, we analyzed the $k_{cat.}/K_{m_{app}}$ data for the action of this enzyme species on I further, using the scheme of eq 14. This scheme takes into consideration the fact that the phenolic hydroxyl group of azoTyr-248 can be either ionized or un-ionized in the pH region where azoCPA shows activity. The pK_2 and pK_3 values of 7.6 and 9.1 derived from the analysis of the pH dependency of $k_{cat.}/K_{m_{app}}$ with eq 14 agree quite well with the corresponding pK values obtained from the spectrophotometric titration of azoTyr-248, 7.7 and 9.15.[17] This agreement looks particu-

larly good when one considers the relatively large standard deviations of the ionization constants derived from the kinetic data and also that the assumption of equal reactivity for the EH_2 and EH forms might not be exactly correct. The results obtained clearly establish that the ionization of azoTyr-248 has little or no effect on the azoCPA-catalyzed hydrolysis of I.

As an extrapolation of our analysis of the action of nitroCPA and azoCPA, we reanalyzed earlier data on the action of CPA on II[28] similarly in order to achieve a better understanding of the effects of the ionization of Tyr-248 on the catalytic activity of the native enzyme. Assuming that the Tyr-248 phenolate group can coordinate to the zinc ion in native CPA in a manner related to that postulated for the modified Tyr-248 residue in azoCPA, the three-proton ionization scheme of eq 14 was employed to analyze the pH dependency for the action of CPA on II. On the basis of this scheme, values of $pK_2 = 8.2$ and $pK_3 = 9.1$ were obtained. As illustrated in Figure 4, with these pK values it can be estimated that the percent molar fraction of the form of the enzyme (species EH of eq 14) in which the Tyr-248 phenolate group is coordinated to the zinc ion is about 15% at pH 7.5 and 25.0°. This percentage can be compared with the percent molar fraction of 15–25% for the corresponding species in the crystalline state estimated to be present at pH 7.4 and 4° on the basis of a reexamination of the original x-ray data for CPA.[18] From our kinetic analysis we conclude, therefore, that the ionization of Tyr-248 seems to exert little or no effect on the action of CPA on II.

In summary, our results are consistent with the assignment of the ionizing group with a pK value of approximately 6.5 detected in the acidic limbs of the $k_{cat.}/K_{m_{app}}$ profiles for the CPA-catalyzed hydrolysis of a variety of substrates as the free carboxyl group of Glu-270 and that detected on the alkaline side with a pK value near 9 as the zinc-bound water. These assignments are in accord with the mechanism we have proposed previously for the action of CPA as an esterase and a peptidase.[1,15]

The kinetic analysis given in the present article indicates that ionization of the phenolic hydroxyl group of Tyr-248 is not important to the action of CPA on substrates for which the pH profiles of $k_{cat.}/K_{m_{app}}$ are characterized by basic limbs exhibiting pK values of about 9, i.e., *O*-hippuryl-L-β-phenyllactate,[33] depsipeptide esters,[34] Cbz-Gly-Gly-L-Leu,[31] Cbz-Gly-Gly-L-Val,[31] Bz-Gly-Gly-L-Leu,[31] Cbz-Gly-Gly-Gly-L-Phe,[31] chloroacetyl-L-Phe,[31] Bz-Gly-L-Phe,[31] and (−)-*S*-(*trans*-cinnamoyl)-α-mercapto-β-phenylpropionate,[1] in addition to I and II. However, we believe that ionization of Tyr-248 to the phenoxide form results in the inactivation of the enzyme toward substrates for which the basic limbs of the pH profiles of $k_{cat.}/K_{m_{app}}$ exhibit pK values of 7.5–8, i.e., *O*-acetyl-L-mandelate[35] and *O*-(*p*-nitrobenzoyl)-L-mandelate.[33] Toward the substrates in the latter class, coordination of the Tyr-248 phenolate group to the active site zinc appears to result in effective intramolecular competitive inhibition. It is noteworthy that the substrates falling in this class ("nonspecific" substrates[15]) are generally much more slowly hydrolyzed by CPA than those for which pK values of 9 are seen in the $k_{cat.}/K_{m_{app}}$ profiles on the alkaline side. Our model building studies based on the x-ray data of Lipscomb et al.[36] indicate that the fits of the "nonspecific" substrates in the binding pocket of CPA in the productive mode are far less snug than those seen with the better substrates of the enzyme. Apparently, thus, the effectiveness of the phenoxide form of Tyr-248 as an intramolecular competitive inhibitor shows a marked dependence on the nature of the fit between the enzyme's binding pocket and the substrate molecule. Similar observations on intramolecular competitive inhibition by an enzyme-bound group have already been made in studies of the acylation of δ-chymotrypsin by specific[37] and nonspecific substrates.[38] A further investigation of the relationship between the proposed intramolecular competitive inhibition by the ionized Tyr-248 in CPA and substrate structure is in progress in this laboratory.

Turning now to the parameter $k_{cat.}$, its pH dependency in the action of CPA on several rapidly hydrolyzed substrates such as oligopeptides, ester II, and *O*-hippuryl-L-β-phenyllactate has been reported. In contrast to the pH profiles of $k_{cat.}/K_{m_{app}}$ for the hydrolysis of these substrates which were quite similar to each other, the pH dependency of $k_{cat.}$ differed from one substrate to the next. Thus, over the pH ranges studied the pH profiles of $k_{cat.}$ for the action of CPA on the oligopeptides examined had simple sigmoidal shapes,[31] that in the case of II was sigmoidal up to a pH value of about 9 and then rose sharply[28] while $k_{cat.}$ was observed to be essentially pH independent in the hydrolysis of *O*-hippuryl-L-β-phenyllactate.[33] The suggestion has been made that the different shapes of the pH profiles of $k_{cat.}$ for the various substrates reflect mechanistic differences in the action of CPA on them.

The pH profiles of $k_{cat.}$ for the nitroCPA- and azoCPA-catalyzed hydrolysis of I (Figures 5 and 6) which have similar shapes to that for the reaction of CPA with II (Figure 7) were analyzed using two kinetic schemes. If the scheme of eq 19 which involves the postulated existence of a covalent intermediate is examined, it can be seen that the steeply rising portion of the pH dependency of $k_{cat.}$ above pH 9 can be understood while this is not the case if the scheme of eq 16 is employed. Specifically, if the acylation step represented by k_2 in eq 19 involves attack of the carboxylate group of Glu-270 on the carbonyl group of the substrate, the deacylation step represented by k_3 corresponds to the breakdown of the anhydride formed. Anhydride breakdown at the lower pH values would be expected to involve reaction with water but at higher pH values reaction with hydroxide ion should prevail.[39] Thus, analysis of the data of Figures 5–7 using eq 19 gave pK values near 7 for the pH dependence of k_2 and a constant k_3 value to pH 9. Although it has not been feasible to determine the precise shapes of the steeply rising portions of the pH profiles shown in Figures 5–7, this feature of the pH dependencies of $k_{cat.}$ is expected according to the scheme of eq 19 if ES′ is an anhydride and if the hydroxide ion catalyzed breakdown of ES′ is rate controlling in the alkaline region.

While over most of the pH range studied it appears that the magnitudes of k_2 and k_3 are not very different from each other (Table IV) for the action of nitroCPA and azoCPA on I and of CPA on II, if the deacylation step of the scheme of eq 19 is much faster than the acylation step, $k_{cat.}$ would be approximately equal to k_2. In such a case the pH profile of $k_{cat.}$ is expected to be the same as that of k_2, a sigmoid. This situation corresponds to what has been observed in the action of CPA on oligopeptides.[31]

On the other hand, assuming the scheme of eq 19, if the deacylation (k_3) step is much slower than the acylation (k_2) step over the pH range examined, $k_{cat.}$ will be equal to k_3, and the magnitude of $k_{cat.}$ is expected to be constant at pH values where the hydroxide ion path is not significant. This corresponds to the situation seen in the CPA-catalyzed hydrolysis of *O*-hippuryl-L-β-phenyllactate.[33]

The seemingly different shapes of the pH profiles seen for $k_{cat.}$ in the action of CPA or of nitroCPA and azoCPA on rapidly hydrolyzed substrates, thus, can be explained with the assumption of the existence of an acyl-enzyme (anhydride) intermediate. None of these $k_{cat.}$ profiles seems to reflect the ionization of the phenolic hydroxyl of Tyr-248 although the possibility cannot be precluded that the pK of

this group is shifted to a value higher than the highest pH examined in the profiles. In contrast, in the CPA-catalyzed hydrolysis of *O*-acetyl-L-mandelate[35] or *O*-(*p*-nitrobenzoyl)-L-mandelate[33] an ionizing group with a pK near 8 is reflected in the descending limbs of the $k_{cat.}$ profiles in the alkaline range, suggesting strongly the influence of the ionization of the Tyr-248 hydroxyl function.

Acknowledgment. Support of this research by grants from the National Institute of Arthritis, Metabolic and Digestive Diseases is gratefully acknowledged.

References and Notes

(1) E. T. Kaiser, T. W. Chan, and J. Suh in "Protein–Metal Interactions", M. Friedman, Ed., Plenum Press, New York, N.Y., 1974, p 59.
(2) W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974).
(3) W. N. Lipscomb, *Acc. Chem. Res.*, **3**, 81 (1970).
(4) J. P. Felber, T. L. Coombs, and B. L. Vallee, *Biochemistry*, **1**, 231 (1962), and references cited therein.
(5) P. H. Petra, *Biochemistry*, **10**, 3163 (1971).
(6) P. H. Petra and H. Neurath, *Biochemistry*, **10**, 3171 (1971).
(7) G. M. Hass and H. Neurath, *Biochemistry*, **10**, 3535, 3541 (1971).
(8) R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).
(9) J. R. Whitaker, F. Menger, and M. L. Bender, *Biochemistry*, **5**, 386 (1966).
(10) J. F. Riordan and B. L. Vallee, *Biochemistry*, **2**, 1460 (1963).
(11) R. T. Simpson and B. L. Vallee, *Biochemistry*, **5**, 1760 (1966).
(12) M. Sokolovsky and B. L. Vallee, *Biochemistry*, **6**, 700 (1967).
(13) J. F. Riordan, M. Sokolovsky, and B. L. Vallee, *Biochemistry*, **6**, 358 (1967).
(14) J. T. Johansen and B. L. Vallee, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2532 (1971), and references cited therein.
(15) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
(16) J. Glovsky, P. L. Hall, and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **47**, 244 (1972).
(17) J. T. Johansen and B. L. Vallee, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2006 (1973), and references cited therein.
(18) W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3797 (1973), and references cited therein.
(19) G. Tomalin, B. L. Kaiser, and E. T. Kaiser, *J. Am. Chem. Soc.*, **92**, 6046 (1970).
(20) M. L. Anson, *J. Gen. Physiol.*, **20**, 663 (1937).
(21) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **166**, 603 (1946).
(22) G. Muszynska and J. F. Riordan, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **32**, 466 (1973).
(23) M. Sokolovsky, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **5**, 3582 (1966).
(24) H. M. Kagan and B. L. Vallee, *Biochem. Biophys. Res. Commun.*, **34**, 654 (1969).
(25) J. T. Johansen, D. M. Livingston, and B. L. Vallee, *Biochemistry*, **11**, 2584 (1972).
(26) H. M. Kagan and B. L. Vallee, *Biochemistry*, **8**, 4223 (1969).
(27) O. H. Lowry, N. J. Roseborough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(28) P. L. Hall, B. L. Kaiser, and E. T. Kaiser, *J. Am. Chem. Soc.*, **91**, 485 (1969).
(29) F. A. Quiocho, C. H. McMurray, and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2850 (1972).
(30) The spectral titration of nitroCPA has not revealed any evidence for the coordination of the phenolate residue of nitroTyr-248 to the active site zinc ion. This result does not necessarily exclude the possibility of such coordination, however.
(31) D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970), and earlier references cited therein.
(32) R. S. Stephens, J. E. Jentoft, and R. C. Bryant, *J. Am. Chem. Soc.*, **96**, 8041 (1974).
(33) J. W. Bunting, J. Murphy, C. D. Myers, and G. G. Cross, *Can. J. Chem.*, **52**, 2648 (1974).
(34) D. S. Auld and B. Holmquist, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 435 (1972).
(35) F. W. Carson and E. T. Kaiser, *J. Am. Chem. Soc.*, **88**, 1212 (1966).
(36) F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971).
(37) P. Valenzuela and M. L. Bender, *Proc. Natl. Acad. Sci. U.S.A.*, **63**, 1214 (1969); *Biochemistry*, **9**, 2440 (1970).
(38) S. F. Bosen and E. T. Kaiser, *J. Am. Chem. Soc.*, **93**, 1038 (1971).
(39) W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, N.Y., 1969, pp 508–517.

Conformation and Reactivity of the Macrocyclic Tumor-Inhibitory Alkaloid Tetrandrine[1]

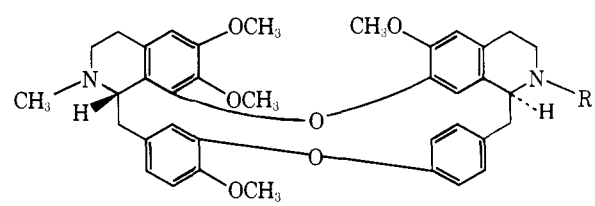
Christopher J. Gilmore,[2] Robert F. Bryan,* and S. Morris Kupchan

Contribution from the Departments of Chemistry, University of Virginia, Charlottesville, Virginia 22901, and University of Glasgow, Glasgow G12 8QQ, Scotland. *Received July 22, 1975*

Abstract: The crystal structure of the tumor-inhibitory bisbenzylisoquinoline alkaloid tetrandrine has been determined by direct x-ray analysis. Crystals are orthorhombic, space group $P2_12_12_1$, with a = 38.368 (6), b = 7.230 (2), c = 12.046 (2) Å, and Z = 4. All atoms, including hydrogen, were located and least-squares refinement gave R = 0.041 for 2633 nonzero reflections measured by diffractometry. The absolute configuration determined by taking account of anomalous dispersion agrees with that assigned chemically. The molecule is shaped as a rough equilateral triangle. The base is defined by one benzylisoquinoline residue in an extended conformation, the other two sides by the second benzylisoquinoline residue in a folded conformation. The marked difference in reactivity of the two chemically equivalent tertiary nitrogen atoms is explained in terms of the observed conformation. One N-methyl group occupies a pseudoequatorial site with unrestricted access to the lone pair, the other is in a pseudoaxial site with access to the lone pair sterically restricted at medium and long range.

The bisbenzylisoquinoline alkaloid *dl*-tetrandrine was found to have a significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 in rats, over a wide dosage range.[3,4] Subsequent studies revealed that the dextrorotatory enantiomer, tetrandrine (**1**), was equally active.[5] Tetrandrine has undergone extensive preclinical toxicological studies and is now in clinical trial, under the auspices of the National Cancer Institute.

During studies directed toward interrelation of tetrandrine with co-occurring alkaloids from *Cyclea peltata*, a markedly greater reactivity of N(2′) over N(2) was observed.[5] Thus, treatment of tetrandrine with an excess of



1, R = CH_3
2, R = $COOCH_3$

methyl chloroformate led to selective demethylation at N(2′) and formation of the monocarbamate **2**. Further-