pH Dependence and Competitive Product Inhibition of the Carboxypeptidase A Catalyzed Hydrolysis of O-(*trans*-Cinnamoyl)-L-β-phenyllactate

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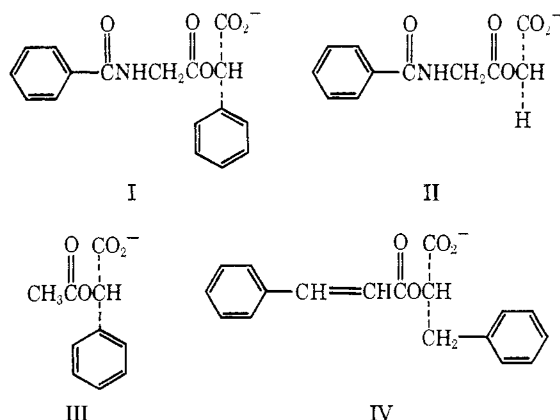
Abstract: The pH dependencies of the kinetic constants for the carboxypeptidase A catalyzed hydrolysis of O-(*trans*-cinnamoyl)-L-β-phenyllactate (IV) were studied over the range pH 5.5–10.2 at 25°. The value of k_{cat} was found to depend on a base of pK_a ~ 6.2 in the enzyme. This compares with the corresponding pK_a value of 7.2 determined earlier for O-acetyl-L-mandelate (III). The error limits on the k_{cat} data obtained in the high pH range were large and made analysis of those results difficult. The value of k_{cat}/K_m for IV depends on a base in carboxypeptidase of pK_a ~ 6.5 and on an acid of pK_a ~ 9.4. The corresponding dissociation constants for III are ~6.9 and 7.5, respectively. A plot of the pH dependence of the K_i values for the product, L-β-phenyllactate, which is a competitive inhibitor shows an inflection near pH 8.8. The interpretation of these results in terms of the ionization of specific groups at the active site of the enzyme has been considered.

In previous reports from these laboratories[2–7] we have presented and discussed a number of observations concerning the kinetics and mechanism of the esterolytic action of carboxypeptidase A (CPA). Perhaps the most striking aspect of our findings has been the intriguingly diverse behavior of this presumably simple proteolytic enzyme, which detailed kinetic studies with various ester substrates have brought to light. For example, the CPA-catalyzed hydrolysis of the ester O-(N-benzoylglycyl)-L-mandelate (I) at pH 7.5 and 25° has been found to be complicated by substrate inhibition as well as competitive inhibition by one of the products, L-mandelate.[3,6] On the other hand, if the ester substrate is O-(N-benzoylglycyl)glycolate (II), one observes substrate activation.[4] Observations of the phenomena of substrate inhibition and substrate activation in CPA-catalyzed hydrolyses are not limited to the cases cited here. Although few detailed kinetic studies carried out under ambiguous conditions have been published, it has been found that the most commonly employed "assay" substrates for CPA (the ester O-(N-benzoylglycyl)-L-β-phenyllactate[8–10] and the peptide N-(N-carbobenzoxyglycyl)-L-phenylalanine[9–11]) give rise to these complex kinetic effects as well. This behavior has been rationalized by a variety of more or less involved kinetic schemes.[3,4,6,8,11,12] Nevertheless, attempts at mechanistic speculation by weaving in the complex threads of available kinetic data on these assay substrates with observations concerning the effects of pH[13] and chemical modifications[13–15] on their CPA-catalyzed hydrolysis rates have proved frustrating.[9,10,16] More kinetic data, particularly with chemically modified CPA, will be required before the mechanisms of substrate inhibition and activation can begin to be elucidated.



Fortunately, not all conveniently studied CPA substrates give rise to complex kinetics. A large portion of our work with CPA esterase kinetics has been concerned with two substrates whose behavior seems adequately described by simple classical Michaelis–Menten rationale. These two substrates are O-acetyl-L-mandelate (III)[2,5] and O-(*trans*-cinnamoyl)-L-β-phenyllactate (IV).[6,7,17] Although the unusual behavior of CPA substrates such as I and II is of great interest and importance, we feel that the more straightforward kinetic behavior of substrates such as III and IV makes them more suitable for studies probing the effects of pH and chemical modification on CPA catalysis. In a previous paper[5] we examined in detail the pH dependence of the

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CPA-catalyzed hydrolysis of III. We now present a similar examination of the pH dependence of the CPA-catalyzed hydrolysis of IV with particular emphasis on the effect of pH on competitive product inhibition by β-phenyllactate. This is, to our knowledge, the first characterization of the pH dependence of competitive inhibition for carboxypeptidase A.

Experimental Section

O-(*trans*-Cinnamoyl)-DL-β-phenyllactic acid was obtained by the method described previously,[6,7] mp 129.5–131.5°. Due to its low solubility in water, it was converted to its sodium salt before use.[6] Stock solutions of the sodium salt, made up to an ionic strength of 0.5 *M* with sodium chloride, were standardized by following the enzymatic hydrolysis of the L component of the racemate on an automatic pH-Stat titrator at pH 7.5 and 25° using Fisher Certified standard sodium hydroxide solutions as the titrant.[5] Since only the L isomer of this substrate is subject to CPA-catalyzed hydrolysis, the concentrations given in this paper for substrate solutions refer always to the concentration of this isomer.

Sodium O-(*trans*-cinnamoyl)-L-β-phenyllactate was prepared by a procedure which we have described in an earlier publication.[7]

L-β-Phenyllactic acid was purchased from Ash Stevens, Inc., mp 124–125° (lit.[18] 124–125°), and was used in inhibition studies without further purification. The neutralization equivalent of this compound (phenolphthalein end point) was found to be 165.8 mg/mmol, corresponding closely to the calculated equivalent weight of 166.2 mg/mequiv.

Other Materials. Buffers, salts, and standard titrants used in these studies were all of the highest grade available and were obtained from the following sources: "Tris" (or 2-amino-2-hydroxymethyl-1,3-propanediol) and "Ammediol" (or 2-amino-2-methyl-1,3-propanediol) from Matheson Coleman and Bell; sodium chloride from Baker and Adamson (Allied Chemical Co.); standard sodium hydroxide solutions, hydrochloric acid solutions, and standard buffers from Fisher Scientific Co. The water used was distilled and then demineralized with a mixed-bed, ion-exchange column (Continental Demineralization Service).

Carboxypeptidase A was purchased as a suspension of crystals in toluene–water from either Sigma Chemical Co. (Lot No. 1233-1590) or Worthington Biochemical Corp. (Lots No. CoA-708 and CoA-6FB). All enzyme preparations used in this work were obtained from bovine pancreas by the method of Anson[19] as modified by Putnam and Neurath[20] and have been designated CPA_γ by Bargetzi, *et al.*[21] Stock solutions were prepared as described earlier[2,5] and stored in a refrigerator at 4°. These solutions contained approximately 3 mg of protein per milliliter, and hence had concentrations (based on a molecular weight estimate of 34,000[21,22]) of about 10^{-4} *M*. The stock solutions were indefinitely stable, and were used for up to 1 year with negligible loss of enzymic activity. Standard solutions of CPA for use in kinetic runs were prepared as needed by diluting 500-μl aliquots of stock solution to 5.00 ml. The exact concentrations of such solutions were then determined spectrophotometrically at 25.0° using the value of 6.42×10^4 l./mol cm as the molar extinction coefficient at 278 mμ.[23] Activity determinations were performed spectrophotometrically according to the procedure described below for a typical kinetic run. The assay was performed with 4.0×10^{-5} *M* O-(*trans*-cinnamoyl)-L-β-phenyllactate (IV) at pH 7.5 in 0.005 *M* Tris-HCl buffer, ionic strength 0.5 *M* (NaCl), at 25°. Under these conditions the initial velocity for the pseudo-first-order reaction, normalized to 1 *M* enzyme concentration ($v_0/[E]_0$), was 64.2 ± 4.2 sec^{-1}.

Kinetic Measurements. CPA-catalyzed hydrolyses of IV were followed spectrophotometrically on a Cary 14 recording spectrophotometer. The ultraviolet spectra of this substrate and of the product mixture obtained upon complete hydrolysis of the L isomer to cinnamic acid and L-β-phenyllactate are characterized in Figure 1 and Table I. Kinetic runs were followed at wavelengths which afforded a maximal difference between initial and final absorbance within the wavelength range yielding accessible absorbance readings at the substrate concentration employed (see Table I). It should be noted that although CPA itself is a powerful chromophore in the wavelength range employed for these studies, such low concentrations of enzyme were required that its contribution to the total observed absorbance was always far too small to be significant. Hence, no corrections for enzyme absorbance or reference cell "blanking" were ever required.

In a typical run in the absence of added L-β-phenyllactate, a quartz cuvette was filled with 3.00 ml of the appropriate buffer. Then with a microsyringe, a predetermined small volume of buffer was removed and replaced with substrate stock solution so that the total volume was still 3.00 ml. The cuvette was then placed in the thermostated cell compartment of the spectrophotometer and allowed to equilibrate. An initial absorbance reading was then taken at the appropriate wavelength. To initiate the reaction, a small measured volume of enzyme standard solution (5–25 μl) was intro-

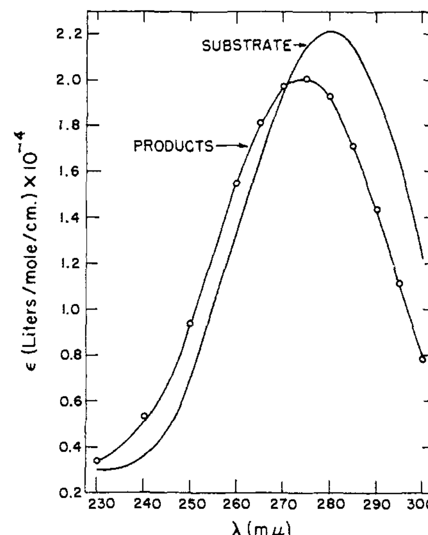

Figure 1. Substrate and product spectra for O-*trans*-cinnamoyl-DL-β-phenyllactate. The points on the observed product spectrum marked O are calculated extinction coefficients from the combined observed absorptivities of the three individual products at equal concentrations.

Table I. Spectral Data Employed in Kinetic Studies on O-*trans*-Cinnamoyl-DL-β-phenyllactate[a]

Species	Spectral feature	Wavelength, mμ	Extinction coefficient[g] ($\epsilon \times 10^{-4}$ l. mol^{-1} cm^{-1})
Substrate	λ_{max}	280	2.22
Substrate	$\lambda_{opt\ I}$[b]	290	1.95
Substrate	$\lambda_{opt\ II}$[c]	312.5	0.290
Substrate	$\lambda_{opt\ III}$[d]	315	0.181
Substrate	$\lambda_{opt\ IV}$[e]	325	0.0174
Products	λ_{max}	274	2.00
Products	$\lambda_{opt\ I}$[b]	290	1.44
Products	$\lambda_{opt\ II}$[c]	312.5	0.150
Products	$\lambda_{opt\ III}$[d]	315	0.0930
Products	$\lambda_{opt\ IV}$[e]	325	0.0096
Mixture	$\lambda_{isosbestic}$[f]	271	1.96

[a] Obtained in 0.005 *M* Tris–, 0.5 *M* sodium chloride, pH 7.5, buffer at 25.0° on a Cary Model 14 recording spectrophotometer. Found to be independent of pH or buffer composition (Tris, ammediol, Tris–acetate) between pH 5.5 and 10.0. [b] Wavelength employed for kinetic runs for $[S]_0 < 4 \times 10^{-5}$ *M*. [c] Wavelength employed for kinetic runs for 4×10^{-5} *M* $< [S]_0 < 3.2 \times 10^{-4}$ *M*. [d] Wavelength employed for kinetic runs for 3.2×10^{-4} *M* $< [S]_0 < 4.5 \times 10^{-4}$ *M*. [e] Wavelength employed for kinetic runs for 4.5×10^{-4} *M* $< [S]_0 < 5.5 \times 10^{-3}$ *M*. [f] Found to be invariant for any mixture of substrate and products. [g] Based upon the total concentration of racemic substrate.

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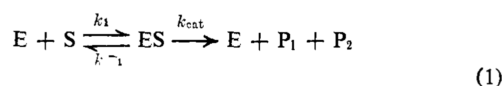
duced with a 1-μl pipet, and the solution in the cuvette was stirred. Exactly 20 sec after the introduction of enzyme, the instrument was activated, and the absorbance was monitored through at least two half-lives of the reaction. In random cases, infinity absorbance readings were taken and were found in all cases to agree within 1% with the values for complete hydrolysis of the L component of IV to cinnamic acid and L-β-phenyllactate. Some kinetic runs using pure sodium O-(*trans*-cinnamoyl)-L-β-phenyllactate were carried out spectrophotometrically, and it was apparent that under the conditions employed, $[S]_0 = 4 \times 10^{-5}$ *M* at pH 7.5 in 0.005 *M* Tris–0.5 *M* NaCl buffer at 25.0° with $[E]_0 = 1.6 \times 10^{-8}$ *M*, the hydrolysis rates were exactly those expected on the basis of kinetic data previously obtained with the racemic substrate. Thus, the nonreactive D component of the racemic substrate IV appears to have no effect on the enzymatic hydrolysis kinetics of the reactive L component. Furthermore, we have found that at the substrate concentrations employed in our kinetic studies the cinnamate produced does not affect the reaction rates.

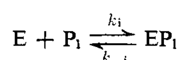
For the runs done in the presence of added L-phenyllactate inhibitor the following procedure was used. An enzyme solution buffered with 0.05 *M* Tris-HCl at pH 7.5 and containing 0.5 *M* NaCl was allowed to equilibrate at 25.0°. Then an aliquot of this solution was added to a buffer (having the same composition of Tris-HCl and NaCl) containing inhibitor and substrate in order to initiate the reaction. The hydrolysis of IV was monitored in the same way as described above.

Results

The kinetics of the CPA-catalyzed hydrolysis of IV were found to be closely related to the kinetics of the CPA-catalyzed hydrolysis of III which were presented in earlier papers from these laboratories.[2,5] That is, the kinetics seem to conform to a simple Michaelis–Menten scheme complicated only by competitive product inhibition, the inhibitory product in this case being L-β-phenyllactate (eq 1). The steady-state rate expression



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (1)$$



$$E + P_1 \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EP_1$$

for this scheme is given by

$$v = \frac{-d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_m + [S] + K_m([S]_0 - [S])/K_i} \qquad (2)$$

where $K_m = (k_{-1} + k_{cat})/k_1$, $K_i = k_{-i}/k_i$, and the quantity $([S]_0 - [S])$ represents the concentration of the inhibitory product P_1 at any time *t*.

In actual practice values for these kinetic parameters at any given pH are extracted from a set of data representing several (at least eight) kinetic runs representing at least a 20-fold range of initial substrate concentrations. A computer program has been written to accomplish this.[5,6,24] The computer first fits the raw data to each run (points representing the extent of reaction (y) at equidistant time intervals, the interval number being specified by s ($s = 0, 1, 2, \ldots, n$)) to orthogonal polynomials of the form[25,26]

$$y_s = a_0 + a_1 s + a_2 s + \ldots + a_m s^m \qquad (3)$$

where $y_s - a_0$ is a measure of the concentration of product formed (the *a*'s are constants). By the method of least squares, the best set of *a*'s is found so that the experimentally determined time course curve is expressed analytically. The derivative of this equation is then taken at each time interval and expressed in terms of the substrate concentration and reaction velocity at that time. After all the runs of a given set (*i.e.*, at a given pH) are thus analyzed, the *initial* rates of all of the runs are fitted by least squares to the Lineweaver–Burk equation[27]

$$[E]_0/v_0 = (K_m/k_{cat})[(1/[S]_0) + (1/k_{cat})] \qquad (4)$$

and the best values of K_m and k_{cat}, along with their standard deviation estimates, are obtained. With values of K_m and k_{cat} in hand, the time course of each run in the set is then fitted by least squares to the double-reciprocal form of eq 2 (eq 5) with K_i as the only adjustable parameter.

$$\frac{[E]_0}{v} = \frac{K_m}{k_{cat}}\left(1 + \frac{[S]_0}{K_i}\right)\frac{1}{[S]} + \frac{1}{k_{cat}}\left(1 - \frac{K_m}{K_i}\right) \qquad (5)$$

Initial rate data for the CPA-catalyzed hydrolysis of IV at pH 7.48 and 25° in 0.005 *M* Tris are presented in Table II and plotted in Figure 2. The kinetic parameters derived from these data are $k_{cat} = 67.2 \pm 1.7$ sec^{-1} and $K_m = 1.87 \times 10^{-4} \pm 0.07 \times 10^{-4}$ *M*. The value of K_i taken from the computer analysis of the time course data for these runs is $5.78 \pm 0.32 \times 10^{-5}$ *M*.

The variations of these kinetic parameters with pH at 25° for the CPA-catalyzed hydrolysis of IV over the pH range 5.5 to 10.2 are given in Table III. All of the data were analyzed by computer as described above, and

Table II. Initial Rate Data for the Hydrolysis of O-*trans*-Cinnamoyl-L-β-phenyllactate Catalyzed by CPA at pH 7.48 and 25° in 0.005 *M* Tris-HCl, 0.5 *M* NaCl Buffer

$10^5[S]_0$, *M*	$10^2[E]_0/v_0$, sec[a]	$10^{-4}/[S]_0$, M^{-1}
1.72	17.6	5.82
1.73	18.4	5.78
1.73	17.5	5.78
2.58	12.9	3.88
2.54	13.1	3.87
2.60	12.1	3.85
3.45	10.1	2.90
3.46	9.18	2.89
3.49	8.82	2.87
4.30	8.58	2.33
4.32	8.16	2.32
4.35	7.98	2.30
8.60	5.08	1.16
8.63	4.66	1.16
9.01	4.11	1.11
12.81	3.84	0.781
12.98	3.64	0.770
13.38	3.34	0.747
17.04	4.06	0.587
17.14	3.29	0.583
17.26	3.22	0.579
17.83	2.96	0.561
42.64	2.20	0.236
43.74	2.20	0.229
44.41	2.02	0.225
164.1	1.72	0.0609
344.7	1.72	0.0290
544.1	1.69	0.0184

[a] The values of $[E]_0/v_0$ in this table were calculated by a high-speed computer from fits of time course data for each run to orthogonal poynomials.

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(27) It will be apparent to the reader that if $[S]_t = [S]_0$, eq 2 is easily simplified to eq 4. Clearly the *initial* rate of the reaction is the rate in the *absence* of product, and no inhibition terms are included in the initial rate expression.

Table III. Variation of Kinetic Parameters with pH for the Hydrolysis of O-*trans*-Cinnamoyl-L-β-phenyllactate Catalyzed by Carboxypeptidase A at 25°

pH	Buffer	$10^5[S]_0$, *M*	k_{cat}, sec^{-1}	10^4K_m, *M*	$10^{-5}k_{cat}/K_m$, l./mol sec	10^4K_i, *M*
5.55	Tris–HOAc, 0.005 *M*, 0.5 *M* in NaCl	1.7–42.0, 8 runs	14.0 ± 1.6	3.35 ± 0.46	0.418	0.369 ± 0.070
6.00	Tris–HOAc, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 10 runs	25.2 ± 5.2	3.04 ± 0.77	0.827	0.365 ± 0.084
6.41	Tris-HOAc, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 8 runs	39.7 ± 0.5	2.33 ± 0.05	1.70	0.381 ± 0.036
6.86	Tris–HOAc, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 8 runs	55.7 ± 1.2	2.14 ± 0.06	2.60	0.427 ± 0.061
7.48	Tris-HCl or Tris-HOAc, 0.005 *M*, 0.5 *M* in NaCl	1.7–544, 28 runs	67.2 ± 1.7	1.87 ± 0.07	3.67	0.578 ± 0.032
8.00	Tris-HCl, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 11 runs	63.0 ± 2.0	1.74 ± 0.10	3.60	0.805 ± 0.075
8.51	Tris-HCl, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 21 runs	66.0 ± 1.2	1.93 ± 0.06	3.43	1.37 ± 0.04
8.93	Ammediol-HCl or Tris-HCl, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 17 runs	71.3 ± 2.3	2.96 ± 0.13	2.40	1.68 ± 0.06
9.66	Ammediol-HCl, 0.005 *M*, 0.5 *M* in NaCl	1.7–43.0, 9 runs	116 ± 30.8	13.10 ± 3.70	0.881	2.34 ± 0.56
10.18	Ammediol-HCl, 0.005 *M*, 0.5 *M* in NaCl	1.7–41.0, 9 runs	. . . .[a]	. . . .[a]	0.277	4.20 ± 0.59[a]

[a] The ordinate intercept of the Lineweaver–Burk plot for the pH 10.18 runs was very nearly zero. In fact, the least-squares fitting of the data to the Lineweaver–Burk equation by the computer gave a *negative* ordinate intercept. Thus k_{cat}, which is the reciprocal of this intercept, is a relatively large number which cannot be evaluated from the data at hand. The slope of the Lineweaver–Burk plot was well defined, yielding a reasonable value for the quantity k_{cat}/K_m, but a K_m value could not be obtained due to the fact that k_{cat} was unknown. The K_i value provided by the computer analysis is included in the table, but its meaning is questionable.

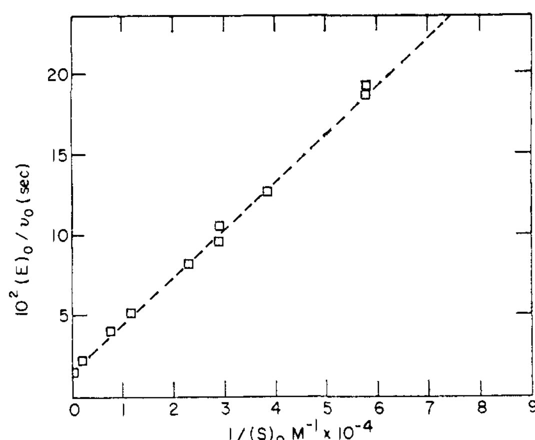
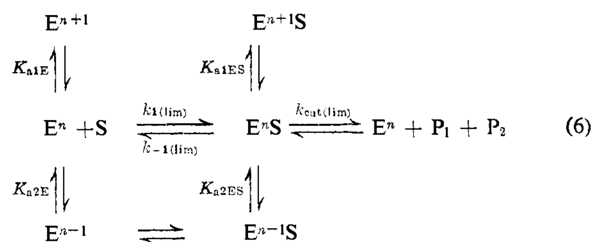


Figure 2. Lineweaver–Burk plot of initial rates of uninhibited CPA-catalyzed hydrolyses of O-*trans*-cinnamoyl-L-β-phenyllactate at pH 7.48, 25°. See Table II.

initial rates at any pH were found to give linear Lineweaver–Burk plots over the substrate concentration range indicated in the table.

In earlier work on the carboxypeptidase-catalyzed hydrolysis of O-acetyl-L-mandelate (III)[5] we found that the reaction scheme shown below (eq 6) fits the observed pH dependence where *n* represents the net charge on the active form of the enzyme and (lim) refers to the limiting values which would be obtained if all of the enzyme were in the active form. The K_a values are acid dissociation constants for the ionizing groups affecting catalytic activity.



Figures 3 through 6 show the plotted pH profiles of k_{cat}, k_{cat}/K_m, K_m, and K_i, respectively. Although the profile for k_{cat}/K_m (Figure 4) is bell-shaped, the bell is broader than that observed for O-acetyl-L-mandelate (III). Analysis according to the reaction scheme in eq 6 yields values for K_{a1E} and K_{a2E} of $2.85 \pm 0.27 \times 10^{-7}$ and 4.26×10^{-10} compared to the values of 1.25×10^{-7} and 3.34×10^{-8} which we reported for the corresponding parameters in the case of III.[5]

The pH dependence of k_{cat} (Figure 3) appears to be complex. A major difficulty in interpreting this pH profile is that the error limit at pH 9.66 is very large and at pH 10.2 we could not obtain a value for k_{cat}. If, however, an analysis of the data for k_{cat} in the alkaline range shown in Figure 3 is attempted, then it would seem that the pH profile for k_{cat} could be a

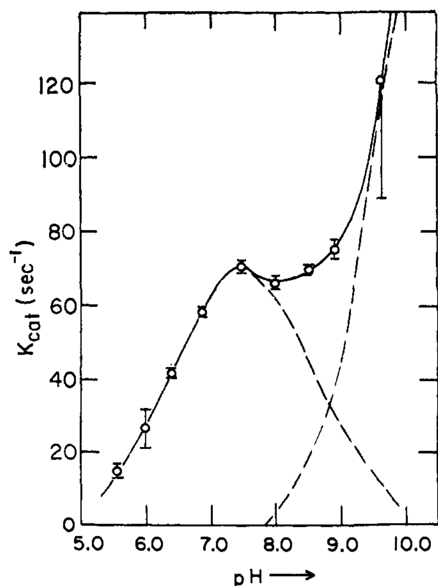


Figure 3. k_{cat} *vs.* pH for the carboxypeptidase A catalyzed hydrolysis of O-*trans*-cinnamoyl-L-β-phenyllactate at 25.0°. Broken lines indicate hypothetical pH profiles for two contributing mechanisms (see text). Standard deviation estimates are indicated for each point.

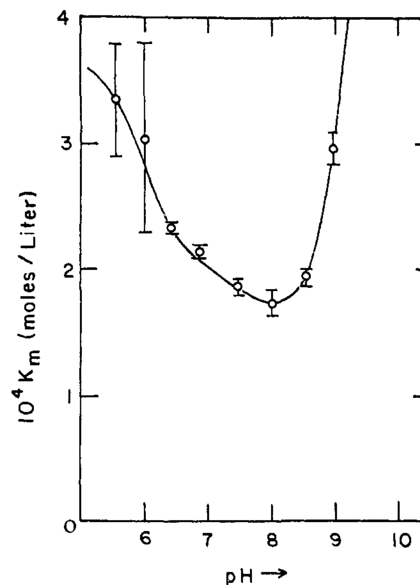


Figure 5. K_m *vs.* pH for the carboxypeptidase A catalyzed hydrolysis of O-*trans*-cinnamoyl-L-β-phenyllactate at 25.0°. Standard deviation estimates are indicated for each point.

composite of two simpler profiles, one bell shaped and the other of undetermined shape but rising steeply with pH above pH ~8. The low pH limb of the k_{cat}–pH profile appears to implicate an ionizing group in the enzyme–substrate complex with $K_{a1ES} = 6.05 \pm 0.8 \times 10^{-7}$ which is probably the same group which gave a K_{a1E} of $2.85 \pm 0.27 \times 10^{-7}$ in the k_{cat}/K_m profile. The value of K_{a1ES} found for the carboxypeptidase-catalyzed hydrolysis of O-acetyl-L-mandelate was 6.2×10^{-8}.[5]

The pH profile for K_m (Figure 5) is also complex and does not appear to be interpretable at the present time in a straightforward way.

The interpretation of the apparent discrepancies in the pH dependence of the kinetic parameters for the hydrolyses of the two ester substrates, O-(*trans*-cinnamoyl)-L-β-phenyllactate (IV) and O-acetyl-L-mandelate (III), must await further study.[28] However, the pH dependence of K_i is apparently simpler than that of the other parameters. Meaningful data could not be gathered above pH 10, and the apparent sigmoidal profile for K_i is thus not complete. But if one disregards the questionable value of K_i at pH 10.2 (Table III) it appears that K_i may depend upon a single dissociating group at the enzyme active site with a pK_a between 8 and 9 (Figure 6).

In order to provide a crosscheck on the mode of inhibition by L-β-phenyllactate and on the validity of

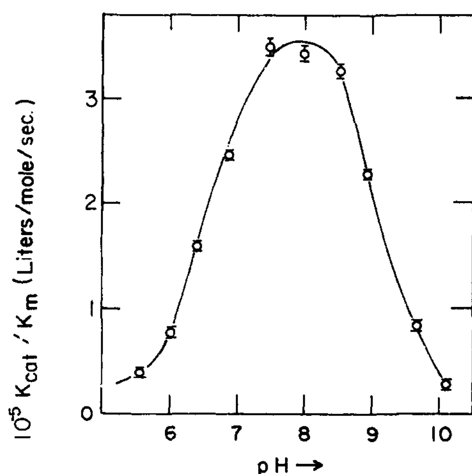


Figure 4. k_{cat}/K_m *vs.* pH for the carboxypeptidase A catalyzed hydrolysis of O-*trans*-cinnamoyl-L-β-phenyllactate at 25.0°. Standard deviation estimates are indicated for each point.

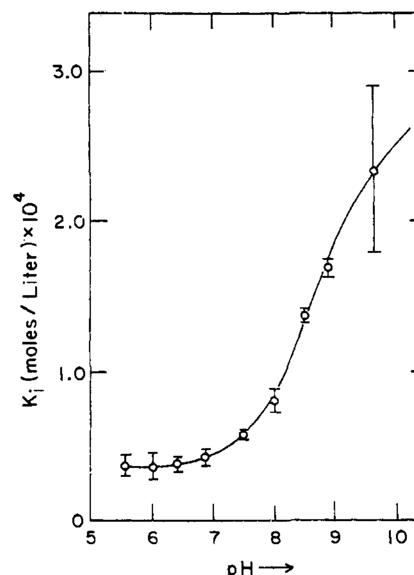


Figure 6. K_i *vs.* pH for competitive inhibition by the product L-β-phenyllactate in the carboxypeptidase A catalyzed hydrolysis of O-*trans*-cinnamoyl-L-β-phenyllactate at 25.0°.

(28) An investigation of the hydrolyses of ester substrates at high enzyme concentrations using rapid kinetic methods appears to be the next logical step in developing an understanding of the kinetics of the esterase action of carboxypeptidase. A study of this sort is currently in progress in our laboratory (G. Tomalin, unpublished results).

K_i values obtained in secondary fashion by computer analysis of time-course data, we have done a series of runs at pH 7.5 and 25° in which the inhibitor was present in roughly 100-fold excess over substrate. In all of these runs, the reaction time course was observed to follow pseudo-first-order kinetics. This is just what one would expect for a system obeying the competitive inhibition rate expression (eq 7) so long as $(K_m + [S]) \ll (K_m/K_i)[I]$. In such a case eq 7 simplifies to eq 8. The competitive inhibition constant,

$$v = \frac{k_{cat}[E]_0[S]}{(K_m + [S]) + (K_m/K_i)[I]} \quad (7)$$

$$v = k_{obsd}[S]$$

$$k_{obsd} = \frac{(k_{cat}/K_m)[E]_0}{[I]/K_i} \quad (8)$$

K_i, is here seen to be directly proportional to the experimentally determined first-order rate constant k_{obsd}. The components of the proportionality constant (k_{cat}/K_m, $[E]_0$, $[I]$) are all known quantities. The values of K_i obtained in this manner were reproducible to within about 10%, and a mean value for eight runs (9.77×10^{-5} M) agreed satisfactorily with a value ($8.44 \times 10^{-5} \pm 0.55 \times 10^{-5}$ M) obtained under identical conditions[29] using time-course analyses of 13 runs which had been carried out with no initially added inhibitor.

In a previous paper from these laboratories,[2] competitive product inhibition by L-mandelate in the CPA-catalyzed hydrolysis of III was described in terms of the integrated form of eq 2 (eq 9 below). In the case

$$\frac{[S]_0 - [S]}{t} = 2.3\left(\frac{K_mK_i + K_m[S]_0}{K_m - K_i}\right)\left(\frac{\log [S]_0/[S]}{t}\right) + \left(\frac{k_{cat}[E]_0}{1 - K_m/K_i}\right) \quad (9)$$

of substrate III, K_m and K_i (for product L-mandelate) differ by almost two orders of magnitude at pH 7.5 and 25°.[2] However, in the case of the hydrolysis of IV at pH 7.5 the ratio K_m/K_i is only 3.22. Since the K_m/K_i ratio is small it can be seen from eq 2 that at the lower substrate concentrations the kinetics for a given run should be very nearly first order. In order to successfully apply eq 9 to the analysis of the kinetic data obtained it is necessary that the kinetics differ very significantly from first order. With a low K_m/K_i ratio this condition occurs at high substrate concentrations where $[S]_0 \gg K_m$. For example, when the data obtained at the two highest substrate concentrations listed in Table II were plotted according to eq 9 a fairly good fit to this equation was found. At pH 6.41 the ratio of K_m/K_i although still rather small appears to be large enough that eq 9 can be applied over a wide range of substrate concentrations. This is clearly illustrated in Figure 7 where some time-course data for pH 6.41 runs are plotted according to eq 9. The lines of Figure 7 were drawn with the slopes and the intercept calculated from eq 9 using the values of K_m, K_{cat}, and K_i from Table III. Although the data points do not fit these calculated lines perfectly, the fits are good enough to establish that eq 9 provides a valid description of the data for kinetic runs at pH 6.41. Figure 7 also provides another crosscheck on our assumption that the kinetic parameters K_m, k_{cat}, and K_i furnished by the computer analysis described earlier have real meaning in terms of eq 1 and rate eq 2.

(29) These runs were conducted at pH 7.52 ± 0.02 and 24.9 ± 0.1° in 0.05 M Tris-HCl buffer, 0.5 M in NaCl. A previous set of runs at the same pH, temperature, and salt concentration, but in tenfold more dilute Tris buffer, produced a value for K_i of $5.78 \pm 0.32 \times 10^{-5}$ M (see Table III). This "Tris effect" on K_i is not large, but it appears to be real.

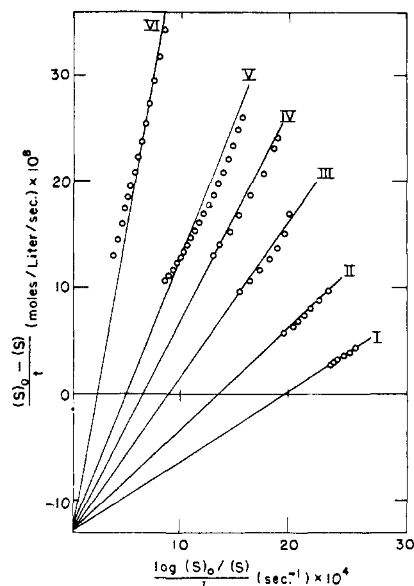


Figure 7. Integrated Michaelis–Menten equation plots for competitive inhibition by L-β-phenyllactate in the hydrolysis of O-*trans*-cinnamoyl-L-β-phenyllactate catalyzed by carboxypeptidase A at pH 6.41 and 25.0° (curve, $10^5[S]_0$, M): I, 1.72; II, 4.30; III, 8.44; IV, 12.84; V, 17.18; VI, 43.49.

Discussion

Rationalization of the dependence on pH of the k_{cat} and K_m functions for the CPA-catalyzed hydrolysis of O-(*trans*-cinnamoyl)-L-β-phenyllactate appears to be complex particularly in view of the uncertainties in the experimental quantities measured in the high pH range. Because of these uncertainties it is difficult to make quantitative comparisons in the alkaline range between the findings of this investigation and those reported earlier[5,24] for the pH dependence of the enzymatic hydrolysis of O-acetyl-L-mandelate.

A comparison of the dependence on pH of the k_{cat} functions for the hydrolysis of III and IV in the lower portion of the pH range studied indicates a reasonable correspondence between the pH_{a1ES} values observed. That measured for the former compound was 7.2, while the value in the case of the latter compound is 6.2 (see the Results). Furthermore, the pK_{a1E} values obtained from measurements of the k_{cat}/K_m functions in the hydrolyses of III and IV are 6.9 and 6.5, respectively. As discussed in a previous paper,[5] an obvious possibility for the group on the enzyme responsible for the ionizations represented by K_{a1E} and K_{a1ES} might be a histidine residue. X-Ray crystallographic studies on the structure of carboxypeptidase by Lipscomb and his coworkers indicate that a histidine

residue is one of the ligands bound to the zinc at the active site of the enzyme.[30,31]

On the basis of their structure determination Lipscomb and his coworkers have suggested that a carboxylate group of a glutamate residue in the enzyme might be catalytically important, acting as either a nucleophile or a general base.[30,31] Although this suggestion is attractive from the structural standpoint, it would require that the glutamic acid residue ionize at an unusually high pH if it is the species which is responsible for the pK_{a1E} and pK_{a1ES} ionizations.

Alternatively, it may be that the catalytically active species to which the pK_{a1E} and pK_{a1ES} ionizations can be ascribed is a zinc hydroxide complex or possibly an ε-amino group of a lysine residue.[32]

Although there is a close correspondence between the pK_{a1E} values found in the hydrolyses of III and IV, there is a great difference between the pK_{a2E} values obtained from measurements on the k_{cat}/K_m functions for these compounds in the more alkaline range. In the case of III the pK_{a2E} value observed was 7.5 and for IV the pK_{a2E} value found is 9.4. The origin of this difference is unclear at the present time.

Because of its close structural similarity to the most widely studied CPA competitive inhibitor, β-phenylpropionate,[33] we were not surprised to find that L-β-phenyllactate is a competitive inhibitor for CPA. The same kind of parallelism in inhibitory properties toward CPA has been found between L-mandelate and phenylacetate.[24] The competitive inhibition constants for these four inhibitors are given for comparison in Table IV. It is quite clear from the table that substitution of a hydroxyl group for a hydrogen on the α-carbon atom of the inhibitor has little effect on the inhibitory properties of the molecule.

Finally, we should note that a plot of the pH dependence of the competitive inhibition constants, K_i, for L-β-phenyllactate (Figure 6) is qualitatively similar in shape to that of the pH dependence of K_m for the ester substrate, O-acetyl-L-mandelate (III).[5,24] Because of uncertainties in the values of K_i for L-β-phenyllactate at high pH a precise value cannot be assigned to the inflection point in the plot of K_i *vs.* pH. We estimate, however, that the inflection point occurs near pH 8.8. There are several possible explanations for this finding. One is that the carboxylate group of the inhibitor displaces water coordinated to the zinc in the enzyme in the binding process and that when this water is ionized and a zinc hydroxide complex is present, the inhibitor does not displace the hydroxide.

On the other hand it has been shown that there are tyrosine and arginine residues in the vicinity of the active site.[31] It would be reasonable thus to postulate either that the inhibitor will not bind to the enzyme if the hydroxyl substituent of the tyrosine is in the negatively charged phenolate form or if the arginine is not protonated. The usual pK values for the hydroxyl of tyrosine are 8.5–10.9 and those for the guanidinium of arginine are 11.6–13.3.[5]

From the foregoing discussion it is clear that despite the structural and kinetic information available, we cannot as yet formulate a stepwise mechanism for carboxypeptidase. One of the most critical aspects of carboxypeptidase action which needs elucidation is the establishment of the precise nature of the catalytically active groups.

Acknowledgments. This research was supported in part by grants from the National Institute of Arthritis and Metabolic Diseases.

Table IV. Comparison of Competitive Inhibition Constants for Some Phenyl-Substituted Carboxylic Acids and Their α-Hydroxylated Analogs

Inhibitor	Formula	10^4K_i, *M*
β-Phenylpropionate	$C_6H_5CH_2CH_2COOH$	0.62[a]
L-β-Phenyllactate	$C_6H_5CH_2CH(OH)COOH$	0.58[b]
Phenylacetate	$C_6H_5CH_2COOH$	4.1[c]
L-Mandelate	$C_6H_5CH(OH)COOH$	4.4[c]

[a] Determined by Elkins-Kaufman and Neurath (E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **175**, 893 (1948); **178**, 645 (1949)) and reported by Neurath and Schwert[33] for solutions containing about 0.01 to 0.05 *M* carbobenzoxyglycyl-L-phenylalanine, 0.1 *M* lithium chloride, 0.04 *M* phosphate buffer, and enough CPA to cause more than 60% hydrolysis within 120 min at 25° and pH 7.5. [b] Determined for aqueous solutions containing 0.000017 to 0.005 *M* O-(*trans*-cinnamoyl)-L-β-phenyllactate, 0.50 *M* sodium chloride, 1.66×10^{-8} *M* CPA, 0.005 *M* Tris buffer at pH 7.5 and 25°. [c] Determined for aqueous solutions containing 0.01 *M* O-(N-benzoylglycyl)-L-mandelate, 0.50 *M* sodium chloride, 9.35×10^{-8} *M* CPA, 2.50×10^{-4} *M* Tris, 0.77% (v/v) dimethylformamide at pH 7.5 and 25°.

(30) See G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, T. A. Steitz, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U. S.*, **58**, 2220 (1967), and earlier papers in this series.

(31) W. N. Lipscomb, J. A. Hartsuck, G. N. Reeke, Jr., F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, paper to be published in the Proceedings of the Brookhaven Symposium on "Structure, Function and Evolution in Proteins," 1968. We wish to express our appreciation to Professor W. N. Lipscomb for making a preprint of this paper available to us and for allowing us to quote the results contained therein.

(32) These hypotheses have also been considered in ref 31.

(33) H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).