Interaction of Carboxypeptidase A with Carbamate and Carbonate Esters†

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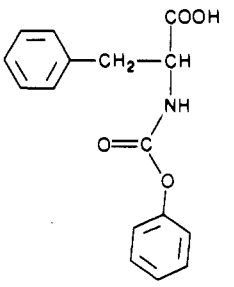
ABSTRACT: The carbamate ester *N*-(phenoxycarbonyl)-L-phenylalanine binds well to carboxypeptidase A in the manner of peptide substrates. The ester exhibits linear competitive inhibition toward carboxypeptidase A catalyzed hydrolysis of the amide hippuryl-L-phenylalanine (K_i = 1.0 × 10^{-3} M at pH 7.5) and linear noncompetitive inhibition toward hydrolysis of the specific ester substrate *O*-hippuryl-L-β-phenyllactate (K_i = 1.4 × 10^{-3} M at pH 7.5). Linear inhibition shows that only one molecule of inhibitor is bound per active site at pH 7.5. The hydrolysis of the carbamate ester is not affected by the presence of 10^{-8}–10^{-9} M enzyme (the concentrations employed in inhibition experiments), but at an enzyme concentration of 3 × 10^{-6} M catalysis can be detected. The value of k_{cat} at 30 °C, μ = 0.5 M, and pH 7.45 is 0.25 s^{-1}, and K_m is 1.5 × 10^{-3} M. The near identity of K_m and K_i shows that K_m is a dissociation constant. Substrate inhibition can be detected at pH less than 7 but not at pH values above 7, which suggests that a conformational change is occurring near that pH. The analogous carbonate ester *O*-(phenoxycarbonyl)-L-β-phenyllactic acid is also a substrate for the enzyme. The K_m is pH independent from pH 6.5 to 9 and has the value of 7.6 × 10^{-5} M in that pH region. The rate constant k_{cat} is pH independent from pH 8 to 10 at 30 °C (μ = 0.5 M) with a limiting value of 1.60 s^{-1}. Modification of the carboxyl group of glutamic acid-270 to the methoxyamide strongly inhibits the hydrolysis of *O*-(phenoxycarbonyl)-L-β-phenyllactic acid. Binding of β-phenyllactate esters and phenylalanine amides must occur in different subsites, but the ratios of k_{cat} and k_{cat}/K_m for the structural change from hippuryl to phenoxy in each series are closely similar, which suggests that the rate-determining steps are mechanistically similar.

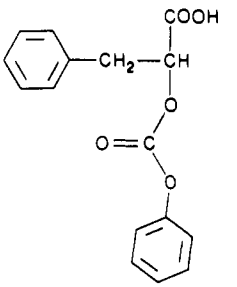
Carboxypeptidase A (peptidyl L-amino acid hydrolase, EC 3.4.12.2) is a Zn(II) metalloenzyme that catalyzes the hydrolysis of peptides and *O*-acyl derivatives of α-hydroxycarboxylic acids (Hartsuck & Lipscomb, 1971). From X-ray crystallographic studies of the enzyme in the presence of poor peptide substrates it has been presumed that the metal ion complexes the substrate carbonyl oxygen. Glutamic acid-270 has also been implicated in the catalytic reaction, and both nucleophilic and general base mechanisms have been suggested involving carboxyl group participation (Lipscomb, 1970; Ludwig & Lipscomb, 1973; Kaiser & Kaiser, 1972).

Kinetic evidence (biphasic kinetics) has been presented for the formation of an intermediate in the hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate and the corresponding *p*-dimethylamino-substituted ester at subzero temperature in a mixed aqueous–organic solvent system (Makinen et al., 1976; Hoffman et al., 1983). However, there does not appear to be any spectral evidence for buildup of an anhydride even at low temperature (Hoffman et al., 1983). Experiments designed to detect the presence of an anhydride intermediate in ester or peptide hydrolysis in H_2O at normal temperatures have failed to do so (Kaiser & Kaiser, 1972; Breslow & Wernick, 1977; Breslow et al., 1983; Galdes et al., 1983). Rapid breakdown of a reactive anhydride through reversal of the acylation reaction and hydrolysis would preclude direct observation of the intermediate under normal conditions of temperature and solvent with conventional types of ester and amide substrates. Furthermore, experiments with added alcohol nucleophiles, in which evidence for transesterification is sought, have an inherent ambiguity when specific substrates for carboxypeptidase A are employed in that the leaving group will bind strongly in the active site and might not depart until the anhydride hydrolyzes (Hoffman et al., 1983). Thus, it is not known whether an anhydride is a general intermediate in ester and peptide hydrolysis, and as a consequence, it has often not been clear which step in the reaction was being monitored in kinetic studies.

The carbamate and carbonate esters I and II possess a terminal aromatic side chain and carboxyl group that are



I



II

necessary for binding (Hartsuck & Lipscomb, 1971), and the phenolic leaving groups would permit the enzyme reaction to be easily monitored spectrophotometrically. Carbonate diesters have not been previously investigated as possible substrates for carboxypeptidase A. Carbamate esters have been extensively studied as inhibitors of bovine pancreatic carboxypeptidase A (CPA)[1] (Awazu et al., 1967; Auld & Vallee, 1970a; Kaiser et al., 1965), but compounds with phenolic leaving groups of this type have not been previously employed

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[1] Abbreviations: CPA, bovine pancreatic carboxypeptidase A; HPL, *O*-hippuryl-L-β-phenyllactic acid; HPA, hippuryl-L-phenylalanine; Cbz-Gly, carbobenzoxyglycine; CPL, *O*-(*trans*-cinnamoyl)-L-β-phenyllactic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

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in kinetic studies. Expulsion of phenol would be expected to occur rapidly in an intracomplex nucleophilic reaction of these compounds. Neighboring alkoxide (Hutchins & Fife, 1973a), phenoxide (Hutchins & Fife, 1973b), amino, sulfhydryl (Fife et al., 1975), and carboxylate (Hegarty et al., 1974) groups have been shown to be highly effective nucleophiles in intramolecular reactions of carbamate esters, and various intramolecular nucleophilic groups are effective in reactions of carbonate diesters (Fife & Hutchins, 1981). The phenol group of I and II should also depart readily from the active site, thereby minimizing reversibility of the reaction in comparison with reactions of corresponding carboxylate derivatives. Therefore, in view of the hydrolytic lability of anhydrides (Fife & Przystas, 1983), nucleophilic attack on such compounds should be the rate-determining step and might be studied without complication from the possible anhydride breakdown. Consequently, compounds of this general type could have considerable mechanistic utility.

Experimental Procedures

Materials. *N-(Phenoxycarbonyl)-L-phenylalanine* (*I*) was prepared by refluxing 2 equiv of L-phenylalanine (Sigma) and 1 equiv of phenyl chloroformate (Eastman) for 48 h in chloroform that was washed and dried by the method of Perrin et al. (1966). The mixture was then filtered. The chloroform was removed by rotary evaporation, and the remaining solid was recrystallized from dry benzene. The white needles were subsequently vacuum dried over calcium sulfate. The compound had mp 62–63 °C. Anal. Calcd for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 66.97; H, 5.42; N, 4.73.

O-(Phenoxycarbonyl)-L-β-phenyllactic Acid (*II*). Phenyl chloroformate (2.69 g, 0.017 mol), in 100 mL of diethyl ether was added to a solution of L-β-phenyllactic acid (2.8 g, 0.017 mol) in 150 mL of diethyl ether. The mixture was stirred in an ice bath for 15 min. Pyridine (1.4 mL, 0.017 mol) in 150 mL of diethyl ether was then added dropwise over a period of 3 h. The reaction mixture was allowed to stir for 16 h. The mixture was then filtered to remove pyridine hydrochloride, and the filtrate was rotary evaporated. The residual oil was dissolved in 10 mL of dichloromethane and applied to a 2.5 × 30 cm silica gel column equilibrated with 99% dichloromethane–1% methanol. Fifty-milliliter fractions were collected at a flow rate of 3 mL/min. Column fractions were analyzed by silica gel thin-layer chromatography, and the components were identified by comparison with standards. With this column technique, the carbonate diester II was successfully separated from contaminating phenol, L-β-phenyllactic acid, and other possible products (e.g., the anhydride formed upon reaction of phenyl chloroformate with the carboxyl group of L-β-phenyllactic acid). Column fractions containing II were combined, rotary evaporated, placed under a vacuum for 16 h, and stored at −20 °C in a desiccator. The final product was a clear oil at room temperature that only solidified at −20 °C. Attempted distillation of the oil resulted in decomposition. The TLC of II showed only one spot. Attempts to prepare the sodium salt and dicyclohexylammonium salt of II were unsuccessful. Complete hydrolysis of II gave a quantitative release of 1 equiv of phenol. Titration of the carboxyl group of II and phenol release in hydrolysis gave quantitatively equivalent results; i.e., the equivalents of OH^- consumed in titration equaled the equivalents of phenol released in hydrolysis. Therefore, the chromatographically pure material is free of all evident impurities.

O-Hippuryl-L-β-phenyllactic acid (HPL) was obtained as the sodium salt from Vega-Fox Biochemicals, and hippuryl-L-phenylalanine (HPA) was purchased from Sigma Chemical Co. *O-(trans*-Cinnamoyl)-L-β-phenyllactate (CPL) was prepared by reaction of distilled cinnamoyl chloride with β-phenyllactic acid as described by Hall et al. (1969). Formation of the sodium salt is described by King and Fife (1983). Carboxypeptidase A was obtained from Sigma (carboxypeptidase A–DFP from bovine pancreas). The buffers employed were all of reagent grade. Reagent sodium chloride (Mallinckrodt) was used to maintain ionic strength. Deionized water was used throughout.

Table I: Spectral Data Employed in Kinetic Studies[a]

compd	pH	wavelength (nm)	ε (M^{-1} cm^{-1})
hippurate	7.50	254	2600
	5.53	254	2750
I	6.0–10	270	180
phenol + phenylalanine	7.50	270	1300
	5.63	270	1250
II	6.0–10	270	202
phenol + phenyllactate	6.0–10	270	1230

[a] T = 30 °C (μ = 0.5 M, NaCl).

Stock solutions of HPL sodium salt were prepared by using deionized water. Spectroscopic grade (Matheson) acetonitrile was used to prepare the stock solutions of substrate esters I and II. The HPA stock solutions were also made with acetonitrile. The concentration of these solutions was adjusted so that the added acetonitrile represented 1% or less of the total reaction solution volume (3 mL per cuvette). Initial velocity measurements were the same in the absence or presence of this amount of acetonitrile. Enzyme stock solutions were prepared in a cold room at 4 °C as described previously (Kaiser, 1970). The commercial enzyme suspension was added to an appropriate amount of cold Tris buffer (0.05 M, 0.5 M NaCl, pH 7.50) and dialyzed against three successive changes of fresh buffer solution. After centrifugation the supernatant solution was stored at 4 °C. The enzyme concentration was determined spectrophotometrically, $\epsilon_{278} = 6.42 \times 10^4$ M^{-1} cm^{-1} (Simpson et al., 1963).

Kinetic Methods. Initial velocity measurements were carried out with a Beckman Model 25 or Pye Unicam SP8-100 recording spectrophotometer according to the procedure of Hall et al. (1969). Temperature was maintained at 30 ± 0.1 °C. An appropriate aliquot of enzyme stock solution was added to a cuvette containing 2–3 mL of buffer (0.05 M, μ = 0.5 M with NaCl) and allowed to thermally equilibrate. A predetermined aliquot of inhibitor and/or substrate was then added, and the reaction was monitored at a selected wavelength. The absorbance due to the enzyme was negligible at the concentrations employed (<3.0 μM). The molar extinction coefficients used to monitor the enzymatic reactions are presented in Table I. Initial velocity determinations in the reaction of 2×10^{-9} M CPA with 1×10^{-4} M HPL and/or CPL at pH 7.5 were reproducible and were conducted at the beginning and end of each set of experiments. The enzyme catalyzed hydrolysis of I and II was monitored by following an increase in absorbance at 270 nm due to phenol. Initial rate data were always corrected for spontaneous hydrolysis, but such correction was of negligible significance in the reactions of I at pH <9. Reaction solution pH values were measured with a Radiometer Model 22 or a Beckman Model 3500 pH meter that had been standardized with Mallinckrodt standard buffer solutions. The buffers employed were Tris–acetate (pH 6.50–7.0), Tris-HCl (pH 7.0–8.5), and Ammediol–HCl (pH 9.0–9.5).

Formation of Methoxyamide Carboxypeptidase A. The method of modification of glutamic acid-270 in carboxy-

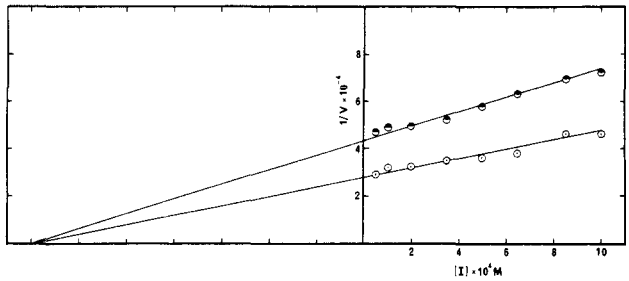


FIGURE 1: Plots of $1/V$ vs. *N*-(phenoxycarbonyl)-L-phenylalanine (I) concentration for reaction of 1×10^{-4} (◓) or 5×10^{-4} M (◎) hippuryl-L-β-phenyllactate with carboxypeptidase A at 30 °C. Velocity is expressed as the change in concentration of product per minute measured at 254 nm. The pH was 7.5 (0.05 M Tris and μ = 0.5 M with NaCl).

peptidase A by *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodwards reagent K) and subsequent formation of the methoxyamide derivative was that of Petra (1971) with the modifications described in King and Fife (1983). In kinetic studies with the modified enzyme, assay buffers were 0.05 M with an ionic strength of 1.0 M with NaCl. Enzyme concentrations were varied from 4×10^{-9} M to 8×10^{-6} M depending on the substrate.

RESULTS

The nonenzymatic rates of hydrolysis of *N*-(phenoxycarbonyl)-L-phenylalanine (I) were too slow to be accurately measured at 30 °C at pH values close to neutrality. Rate constants were obtained at pH >10. The reactions are OH^- catalyzed with $k_{OH} = 2.68$ M^{-1} s^{-1}. The nonenzymatic release of phenol from *O*-(phenoxycarbonyl)-L-β-phenyllactic acid (II) in 50% dioxane–H_2O (v/v) at pH <10 exhibits a k_{obsd} vs. pH profile at 30 °C that is sigmoidal with $pK_{app} = 4.3$. Titration of II in the same solvent yielded a pK_a value of 4.4. The pH-independent portion of the profile has a limiting rate constant of 9.01×10^{-4} s^{-1}. The D_2O solvent isotope effect in this region (k_{H_2O}/k_{D_2O}) is 1.03.

Plots of $1/V$ vs. $1/(S)_0$ for carboxypeptidase A catalyzed hydrolysis of *O*-hippuryl-L-β-phenyllactic acid (HPL) in the presence of three constant concentrations of I and in the absence of I at pH 7.5 and 30 °C were linear and intersected on the $1/(S)_0$ axis. In the absence of I, K_m has the value of 8.1×10^{-5} M and k_{cat} is 460 s^{-1}. The K_m is in reasonable agreement with the value of 1×10^{-4} M obtained from the data of Bunting et al. (1974). A plot of $1/V$ vs. the concentration of I is shown in Figure 1. Linear noncompetitive inhibition is indicated with a K_i value of 1.4×10^{-3} M. Carboxypeptidase A catalyzed hydrolysis of HPL is known to be subject to substrate inhibition (Bunting et al., 1974). In all experiments substrate concentrations less than 0.001 M were therefore employed.

Plots of $1/V$ vs. $1/(S)_0$ for carboxypeptidase A catalyzed hydrolysis of hippuryl-L-phenylalanine (HPA) in the presence of three constant concentrations of I and in the absence of I at pH 7.5 and 30 °C were linear and intersected on the vertical axis, which indicates competitive inhibition. The value of k_{cat} is 100 s^{-1} and K_m is 1.7×10^{-3} M in the absence of inhibitor, which compares well with values found previously (Davies et al., 1968b). The linear plot of $1/V$ vs. the concentration of I is shown in Figure 2 from which a K_i value of 1×10^{-3} M was obtained. A horizontal line through the point of intersection of the lines strikes the vertical axis at the value of $1/V_{max}$ determined from the plots of $1/V$ vs. $1/(S)_0$. Substrate activation has been previously observed for carboxypeptidase A catalyzed hydrolysis of HPA (Auld & Vallee, 1970a); however, no such effects were observed in this study at the concentrations employed.

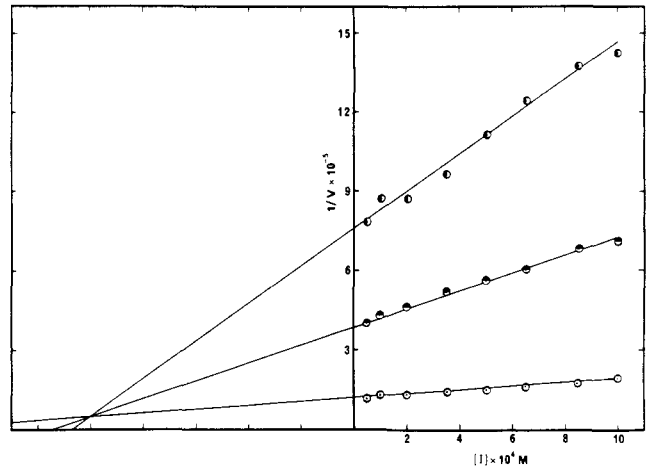


FIGURE 2: Plots of $1/V$ vs. *N*-(phenoxycarbonyl)-L-phenylalanine (I) concentration for reaction of 5×10^{-5} (◐), 1×10^{-4} (◓), and 5×10^{-4} M (◎) hippuryl-L-phenylalanine with carboxypeptidase A at 30 °C. Velocity is expressed as the change in concentration of product per minute measured at 254 nm. The pH was 7.5 (0.05 M Tris and μ = 0.5 M with NaCl).

Table II: Values of k_{cat} and K_m for CPA-Catalyzed Hydrolysis of *N*-(Phenoxycarbonyl)-L-phenylalanine (I) and *O*-(Phenoxycarbonyl)-L-β-phenyllactic Acid (II) at 30 °C

compd	pH	k_{cat} (s^{-1})	$K_m \times 10^3$ (M)	k_{cat}/K_m (M^{-1} s^{-1})
I	6.50	0.018	0.147	120
	6.93	0.042	0.265	158
	7.45	0.25	1.53	166
	7.95	0.28	1.75	162
	8.49	0.28	1.91	146
	8.99	0.18	2.05	86
	9.39	0.14	3.01	47
II	6.50	0.45	0.075	6000
	7.00	0.73	0.076	9600
	7.50	1.02	0.075	13600
	8.00	1.25	0.085	14700
	8.50	1.59	0.077	20650
	9.00	1.50	0.080	18750
	9.50	1.54	0.089	17300
	9.98	1.60	0.149	10740

Although CPA catalysis of the rate of phenol release from *N*-(phenoxycarbonyl)-L-phenylalanine (I) was not detected at enzyme concentrations of 10^{-8}–10^{-9} M (the concentrations in inhibition experiments of Figures 1 and 2), at the higher enzyme concentration of 3×10^{-6} M a catalytic reaction could be observed. Nicely linear plots of V vs. $V/(S)_0$ were obtained at pH values greater than 7.0 from which the values of k_{cat} and K_m in Table II were obtained. Note that the K_m at pH 7.5 is similar to the K_i obtained in the inhibition experiments. The values of k_{cat} are a bell-shaped function of pH with a maximum at pH 8 (see Table II). At pH <7 there was marked downward curvature in the plots of V vs. $V/(S)_0$ at substrate concentrations greater than 10^{-3} M, which indicates substrate inhibition, in contrast with the linearity of such plots at pH >7. The values of k_{cat} and K_m at pH 6.93 and 6.50 were therefore determined by employing data obtained at substrate concentrations less than 10^{-3} M. In the plot of log k_{cat}/K_m vs. pH (not shown), there is a clearly defined pK_2^E of 8.8 ± 0.1.

The dicarbonate ester II is also a substrate for CPA. A plot of velocity vs. enzyme concentration for the hydrolysis of 7.87×10^{-5} M II at pH 7.5 with the CPA concentration being varied from 10^{-7} to 9×10^{-7} M was linear but extrapolated

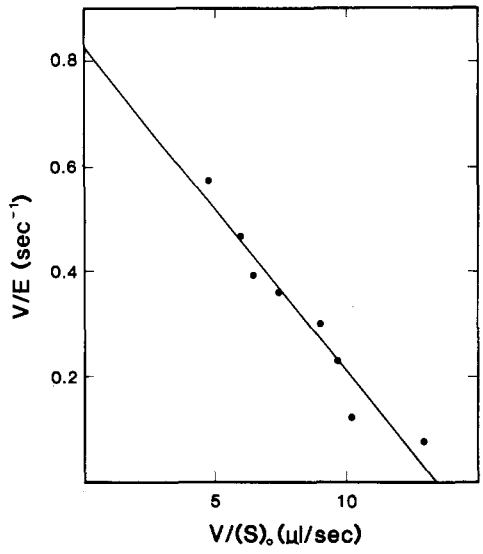


FIGURE 3: Typical V/E vs. $V/(S)_0$ plot for the carboxypeptidase A catalyzed hydrolysis of *O*-(phenoxycarbonyl)-L-β-phenyllactic acid at 30 °C and pH 7.00 (0.05 M Tris–0.5 M NaCl).

Table III: Glutamic Acid-270 Modification

enzyme	k_{cat} (s^{-1}) (pH 7.5), CPL	% control	k_{cat} (s^{-1}) (pH 7.5), II	% control
native	155	100	1.24	100
methoxyamide CPA + inhibitor[a]	109	70.3	0.88	71.0
methoxyamide CPA[b]	11.7	7.5	0.08	6.5

[a] Enzyme preincubated with β-phenylpropionic acid was treated with Woodwards reagent K and the methoxyamine according to the method of Petra (1971). [b] The enzyme was treated with Woodwards reagent K followed by conversion to the methoxyamide according to the method of Petra (1971).

to zero enzyme concentration slightly above the origin. This indicated that nonenzymatic hydrolysis is affecting the velocity by <10%. The velocities were, however, corrected for this spontaneous hydrolysis. Enzymatic rate constants for the hydrolysis of II were calculated from V/E vs. $V/(S)_0$ plots of initial rate data, of which Figure 3 is a typical example. Values of k_{cat} and K_m are given in Table II; k_{cat} is pH independent at pH >8. The limiting k_{cat} value is 1.60 s^{-1}. The K_m is pH independent in the range 6.5–9 but with gentle increases at the extremes of both low and high pH. The pH-independent value of K_m is 7.6×10^{-5} M. The log k_{cat}/K_m vs. pH profile is bell-shaped with values of 6.9 and 9.6 ± 0.2 for pK_1^E and pK_2^E, respectively. Initial velocity plots of absorbance vs. time were linear even at very high concentrations of enzyme (2×10^{-5} M) and $(S)_0 \gg (E)_0$ in reactions of both I and II at pH 7.5.[2]

The carboxyl group of Glu-270 was modified to the methoxyamide by the method of Petra (1971) employing *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodwards reagent K) followed by treatment with methoxyamine. This modified enzyme is inactive toward II at pH 7.50 as shown by the data in Table III. Prior incubation of the enzyme with the inhibitor β-phenylpropionic acid at a concentration of 4 mM protects the enzyme against inactivation by Woodwards reagent K and methoxyamine, thereby showing that the active site is being modified.

Discussion

Carbamate Ester Inhibition of Carboxypeptidase A. The carbamate ester I possesses the required structural features for strong binding to carboxypeptidase A and must be binding in the site for peptide substrates. Compound I competitively inhibits the CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine ($K_i = 1.0 \times 10^{-3}$ M at pH 7.5) but exhibits noncompetitive inhibition toward hydrolysis of hippuryl-L-β-phenyllactic acid ($K_i = 1.4 \times 10^{-3}$ M at pH 7.5), which has been classified as a specific ester substrate (Bunting et al., 1974). The K_i value of 1.4×10^{-3} M for I shows that it binds to the enzyme much more strongly than the carbamate ester carbobenzoxyglycine (Cbz-Gly) for which K_i values of 2.9×10^{-2} M and 1.6×10^{-2} M have been found for inhibition of the hydrolysis of Cbz-Gly-Gly-L-Phe (Auld & Vallee, 1970a) and *O*-(*trans*-cinnamoyl)-DL-β-phenyllactate (Awazu et al., 1967), respectively. *N*-Carbobenzoxy-L-phenylalanine is a competitive inhibitor toward Cbz-Gly-Gly-L-Phe with $K_i = 6 \times 10^{-4}$ M (Byers & Wolfenden, 1973).

When the active site of an enzyme is large and a relatively small substrate or inhibitor is being studied, caution must be employed in the interpretation of results since binding might then occur in a nonspecific manner. Kinetic anomalies such as substrate activation or substrate inhibition, implying multiple binding modes, have frequently been associated with binding of small molecules to CPA. Acylated tripeptides, on the other hand, behave more simply than shorter homologues (Auld & Vallee, 1970a). The interaction of carbobenzoxyglycine with CPA gives rise to competitive inhibition toward tripeptides (Auld & Vallee, 1970a) and the specific ester substrate CPL (Awazu et al., 1967). In the CPA-catalyzed hydrolysis of dipeptides it behaves as an activator (Davies et al., 1968), and in the hydrolysis of hippurylglycolate, a nonspecific ester substrate, Cbz-Gly acts to suppress substrate activation (Kaiser et al., 1965). It should be noted that Cbz-Gly does not possess an aromatic R group in the amino acid portion of the molecule, a preferred feature for strong binding to CPA (Ludwig & Lipscomb, 1973). Thus, it is not surprising that unusual inhibition and activation effects are observed. In contrast, I does not give rise to such abnormal effects at pH 7.5.

The linear inhibition plots of Figures 1 and 2 indicate that one inhibitor molecule is binding per active site at pH 7.5. Binding of more than one inhibitor molecule or both inhibitor and amide substrate simultaneously [see Figure 12 of Ludwig and Lipscomb (1973)] would give rise to nonlinear $1/V$ vs. (I) plots. Partial competitive inhibition in which an EIS complex is formed will give linear $1/V$ vs. $1/(S)_0$ plots but curvature in the $1/V$ vs. (I) plots (Segel, 1975). Linear mixed-type inhibition can be obtained in cases where an EIS complex is totally unreactive, but then both V_{max} and K_m will be affected; i.e., the plots of $1/V$ vs. $1/(S)_0$ would intersect behind the vertical axis (Segel, 1975). Thus, an EIS complex is not formed in reactions of HPA, and it is therefore reasonable to conclude that I is binding to the active site in a similar or identical manner as specific amide and peptide derivatives of L-phenylalanine. It has been suggested that ester and peptide substrates might bind in separate but adjoining sites on the enzyme (Bunting & Kabir, 1977; Auld & Holmquist, 1974). In those studies carboxylic acid inhibitors were found to be competitive vs. esters and noncompetitive vs. amides. Separate sites for binding of esters and amides are also indicated by the different types of inhibition effects exerted

[2] Only a small absorbance change would be associated with an initial burst in these reactions of I and II. However, biphasic kinetics were also not observed in reactions of the *p*-nitro analogue of I catalyzed by CPA, in which case an initial rapid-burst reaction would produce a large absorbance change and would be readily detectable (unpublished results).

by I in the hydrolysis of HPA and HPL but from the reverse standpoint; i.e., I is competitive vs. amides and noncompetitive vs. esters. Unlike carboxylic acid inhibitors, I also exhibits substrate properties; therefore, it is clear that the inhibitor's effect is due to binding in a catalytically important site.

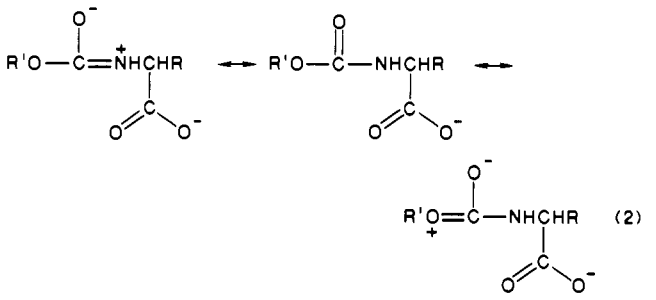
At high enzyme concentration (3×10^{-6} M) CPA catalysis of the release of phenol from the carbamate ester I can be readily detected. The K_m of 10^{-3} M for I compares favorably with the K_m values (pH 7.5) for many of the best amide and peptide substrates. For example, K_m for benzoyl-Gly-Gly-Phe is ca. 10^{-3} M (Auld & Vallee, 1970a) as is that determined for hippuryl-L-phenylalanine in this work. Results have been presented that are compatible with K_m being a measure of the peptide substrates's binding affinity (Auld & Vallee, 1970a,b). Since $K_m = K_i$ in the hydrolysis of I, it is clear that $K_m = K_s$; i.e., K_m represents the dissociation constant of the enzyme–substrate complex. Consequently, if an anhydride intermediate is produced with initial phenol release, as in eq 1, its formation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \text{ (anhydride)} \xrightarrow{k_3} RCOO^- + E + \text{(phenolate)} \quad (1)$$

(k_2) must be rate determining. Phenol must bind weakly to CPA since it is a rather poor inhibitor of reactions catalyzed by the enzyme; 50% inhibition in the hydrolysis of 10^{-3} M HPL is achieved by a 0.05 M concentration (Davies et al., 1968a). The reaction of eq 1 would therefore be essentially irreversible, and K_m would be $K_s k_3/k_2$ if anhydride hydrolysis was rate determining. Only if the carbamate nitrogen was protonated in a tetrahedral intermediate might phenylalanine be the initial leaving group. Such a process does not occur in the chemical intramolecular reactions of phenolic carbamate esters (Hutchins & Fife, 1973a,b; Hegarty et al., 1974); phenol is the leaving group in those reactions. However, the decline in k_{cat} with increasing pH suggests that an acidic group is involved in the CPA-catalyzed hydrolysis of I. The values of K_m and K_s would also be equal if the reaction proceeds without the formation of an intermediate.

Even though the phenoxy group of I would provide a good leaving group, the value of k_{cat} is relatively small (approximately 500-fold less than the k_{cat} values of tripeptide substrates and 400-fold less than those of amide substrates such as hippuryl-L-phenylalanine). Larger substrates are characterized by relatively large k_{cat} values. This may be due to a better steric fit to functional groups in the active site, although the increased possibilities for binding contacts do not result in appreciably lower K_m values (Auld & Vallee, 1970a; Ludwig & Lipscomb, 1973). Thus, binding energy may be expended to produce a more favorable rate. A twisting effect aided by multiple binding contacts would reduce the resonance interaction between the amide nitrogen and the carbonyl and thereby make the carbonyl group more susceptible to nucleophilic attack. Such an effect would be especially difficult with the carbamate I because of the large resonance interaction (eq 2) and the small size of the substrate. Thus, the k_{cat} values of I may in part demonstrate the importance of this resonance effect; i.e., $K_m(K_s)$ is normal but k_{cat} is small.

The k_{cat} values for I decline as pH is decreased below 7. This is also the case in the plot of log k_{cat}/K_m vs. pH (pK_a^E values of 6.7 and 8.8). Thus, as with other substrates for CPA there is an important apparent pK_a near 7. The occurrence of substrate inhibition at pH less than 7 (such inhibition is not observed at pH >7) indicates that a conformational change is occurring in association with the apparent pK_a^E, which



(2)

R = benzyl; R' = phenyl

permits binding of I in an additional site. Nonproductive binding in an additional site would lower k_{cat} and K_m identically (Fersht, 1977). Note in Table II that at pH 6.93 both k_{cat} and K_m are substantially less than at higher pH but that k_{cat}/K_m remains nearly unchanged. Nonproductive binding might not occur with large substrates, but the conformation change at pH <7 could still be important catalytically if it also occurs in the ES complex and could influence the apparent pK_a^{ES} of 6–7. Thus, a pH-dependent conformational change of the type that is very likely occurring in the reaction of I could be of general significance in CPA-catalyzed reactions.

CPA-Catalyzed Hydrolysis of O-(Phenoxycarbonyl)-L-β-phenyllactate. The carbonate diester II is also a substrate for carboxypeptidase A. The value of K_m (pH 6.5–9) is 7.6×10^{-5} M, which is slightly less than that of the specific ester substrate *O*-(*trans*-cinnamoyl)-L-β-phenyllactic acid (1.9×10^{-4} M) (Hall et al., 1969). The values of pK_1^E and pK_2^E of 6.9 and 9.6 obtained from the plot of log (k_{cat}/K_m) vs. pH are also comparable to those found for CPL (6.5 and 9.4). Thus, II is very likely binding in the active site in the same manner as specific esters. Phenol release in the hydrolytic reaction is quantitative, and all of the phenol is released in a single rapid reaction. However, k_{cat} values for II are 100-fold less than those obtained for hydrolysis of CPL in the pH range 6.5–9 and 400-fold less than in the hydrolysis of HPL at pH 7.5. The value of k_{cat}/K_m at pH 7.5 for II is 1.4×10^4 M^{-1} s^{-1} as compared with 1.7×10^2 M^{-1} s^{-1} in the case of I. Therefore, the carbonate ester is a better substrate than the analogous carbamate. The ratio k_{cat}/K_m is the second-order rate constant for reaction of the free enzyme with substrate and is unaffected by any nonproductive binding of the substrate. This constant is therefore the most reliable parameter to employ in assessing relative substrate ability (Fersht, 1977). The difference in substrate ability for I and II resides largely in the less favorable K_m value of I. The difference in the k_{cat} values for I and II at pH 7.5 is only a factor of 4, which is similar to the 5-fold difference in k_{cat} for HPA and HPL. The difference in k_{cat}/K_m for I and II (82-fold) is likewise very similar to that for HPA and HPL (100-fold).

The values of k_{cat} for CPA-catalyzed hydrolysis of II are pH independent in the pH range 8–10 and decline with decreasing pH below pH 8. The hydrolysis of CPL is characterized by a k_{cat} vs. pH profile that is sigmoidal below pH 9 but with a rapidly rising arm at pH >9 (Hall et al., 1969; King & Fife, 1983). It will be noted that such an apparent OH^--catalyzed reaction does not occur in hydrolysis of II at pH values below 10. It has been considered that breakdown of an anhydride intermediate is the rate-determining step in the CPA-catalyzed hydrolysis of β-phenyllactate esters (Makinen et al., 1979; Kaiser & Kaiser, 1972), and the increasing rate constants with increasing pH at pH >9 are in accord with Zn(II)-promoted attack of OH^- on an anhydride. In contrast, the pH independence of the k_{cat} values of II at high pH cannot reflect OH^- attack on an anhydride inter-

mediate or the substrate unless the pK_a of metal ion liganded water is abnormally low [see Discussion in King and Fife (1983)].

Modification of the carboxyl group of Glu-270 to the methoxyamide by the method of Petra (1971) greatly inhibits activity toward *O*-(phenoxycarbonyl)-L-β-phenyllactate just as it does toward CPL (King & Fife, 1983). Thus it is clear that an intact carboxyl group (or carboxylate anion) is necessary for enzyme catalysis. A simple interpretation is that Glu-270 is directly involved in the catalytic process either as a nucleophile or as a general base, but it is, of course, also possible that the carboxyl group of Glu-270 is structurally necessary to maintain the active site in the proper conformation.

A nucleophilic mechanism might be anticipated in the hydrolysis of II, considering the possibility of phenol expulsion. It is chemically reasonable that phenol would be the initial leaving group in such a reaction because of the large difference in the pK_a values of phenol (10) and the β-phenyllactate alcohol group (14.7) estimated by the method of Fox and Jencks (1974). If nucleophilic attack by Glu-270 were occurring in that manner, then as in the case of I, formation of the anhydride should be rate determining because of the reduced tendency of phenol to bind in the active site. Rate-determining anhydride breakdown would not be expected unless reversibility occurs, as in eq 3, with $k_{-2} > k_3$. L-β-Phenyllactate, initially

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\overset{\bullet}{R}OH}{ES' \text{ (anhydride)}} \xrightarrow{k_3} RCOO^- + E \quad (3)$$

liberated in the hydrolysis of CPL, will, on the other hand, bind strongly in the active site ($K_i = 5.8 \times 10^{-5}$ M at pH 7.5) (Hall et al., 1969) presumably in position to reverse the reaction (Hall & Kaiser, 1967; Kaiser & Kaiser, 1972). In view of the much lower pK_a of phenol than β-phenyllactate (4.7 pK_a units), expulsion of the latter from a tetrahedral intermediate in preference to the former would require a strained C–O bond or proton transfer to the leaving group. Since k_{cat} is pH independent at pH >8, such a proton transfer might only occur from water and would take place most readily if water were the nucleophile adding to the carbonyl group.

The relatively small values of k_{cat} and k_{cat}/K_m for I and II are significant in view of the much greater reactivity of those compounds in nonenzymatic hydrolysis reactions than carboxylic ester and amide substrates for CPA. For example, the second-order rate constant k_{OH} for OH^--catalyzed hydrolysis of CPL in only 0.017 M^{-1} s^{-1} at 30 °C (King & Fife, 1983). Therefore, the high nonenzymatic hydrolytic reactivity of I and II due to the phenolic leaving group is not manifested in the k_{cat} and k_{cat}/K_m values. As discussed, the small k_{cat} values could reflect an imprecise alignment in the active site. However, the K_m values are normal and give no evidence of unusual binding. The rate of anhydride formation should be markedly enhanced by a significant reduction of the leaving group pK_a. Consequently, the small k_{cat} values in combination with the absence of burst kinetics at high enzyme concentrations[2] and the relationship $K_m = K_i$ (with I) indicate that a nucleophilic reaction in which phenol is the leaving group cannot be a favorable process. The effect of the phenoxy group with both I and II may be due mainly to its relatively small size and perhaps in part to its inductive electron-withdrawing effect. Such an effect should decrease the facility of breakdown of a tetrahedral intermediate to give β-phenyllactic acid or phenylalanine.

Rate-Determining Step. From X-ray crystallographic studies of CPA with bound peptides and a ketonic inhibitor analogous to esters (O replaced by $-CH_2$), Rees and Lipscomb (1981) and Lipscomb (1980) have suggested that the catalytic site for both esters and peptides is the same and is that in which the aromatic residue can fit in the hydrophobic pocket and the terminal carboxylate group interacts with Arg-145. The different inhibition patterns of esters and peptides (Auld & Holmquist, 1974), which had been explained on the basis of different sites for the two types of substrates, were then attributed to different rate-determining steps. Hydrolysis of esters was considered to involve rate-determining breakdown in the catalytic site S_1', whereas the rate-determining step with peptides was suggested to be movement from the subsite S_2, near Phe-279, Tyr-198, and Arg-71, into S_1' [see also Cleland (1977)]. The greater difficulty of binding peptides than esters in S_1' was thought to reside in the twisting effect necessary to achieve binding to the metal ion and the hydrophobic pocket and to allow hydrogen bonding with Tyr-248. As noted, resonance in a carbamate ester (eq 2) would be especially pronounced, and therefore twisting the molecule would be difficult. However, a structural change from phenylalanine to β-phenyllactic acid produces the same relative change in k_{cat} and k_{cat}/K_m for the phenoxy and hippuryl derivatives. Likewise, a structural change from hippuryl to phenoxy produces the same change in k_{cat}/K_m in each series (4×10^2). This would not be expected if association with the catalytic site was rate determining for amides but not esters, considering the differences in size and reactivity of these compounds. A chemical step is very likely rate limiting with both types of substrates.

Table IV: Values of k_{cat}/K_m at pH 7.5 for Ester and Amide Substrates for CPA

compd	k_{cat}/K_m (M^{-1} s^{-1})	ratio (ester/amide)
(1) II	1.4×10^4	82
I	1.7×10^2	
(2) HPL	5.7×10^6 [a]	97[a]
	6.6×10^6 [b]	60[b]
	5.3×10^6 [c]	
HPA	5.9×10^4 [a]	
	1.1×10^5 [b]	
(3) Bz-Gly_2-L-OPhe	1.5×10^6 [d]	75
Bz-Gly_2-L-Phe	2×10^4 [d]	
(4) Dns-Gly_3-L-OPhe	7.3×10^6 [d]	84
Dns-Gly_3-L-Phe	8.7×10^4 [d]	
(5) Bz-Gly_3-L-OPhe	1.5×10^6 [d]	125
Bz-Gly_3-L-Phe	1.2×10^4 [d]	

[a] This work, 30 °C. [b] Davies et al. (1968b), 25 °C. [c] Bunting et al. (1974), 25 °C, 0.2 M NaCl. [d] Auld & Holmquist (1974), 25 °C, 1.0 M NaCl.

A similar analysis of the data of Auld and Holmquist (1974) on the CPA-catalyzed hydrolysis of oligopeptides and depsipeptides again reveals that the ratios of k_{cat}/K_m for ester–amide pairs having phenyllactate or phenylalanine as the terminal group are not greatly dependent on structure, the ratios varying from 75 to 125 (Table IV). Thus, through a wide variation in structure, the ratio of k_{cat}/K_m for an ester to k_{cat}/K_m for the analogous amide is nearly independent of structure with large specific substrates and the small nonspecific substrates I and II. The ratio is apparently larger with a cinnamoyl acyl group (4720) (McClure & Neurath, 1966; Kaiser & Kaiser, 1972) because the amide is an especially poor substrate, but the ratio is 185 with the α-(acetylamino)cinnamoyl derivatives and 111 with the α-naphthoylamino-substituted compounds (Suh et al., 1985).

Esters and amides must bind in different subsites. Since the pH dependence of k_{cat} is different for I and II in the high

pH range, it is indeed probable that the catalytic reactions of I and II are also occurring in subsites that are different. That does not, however, preclude the possibility of common features in the mechanisms if substrate-dependent conformational changes of the enzyme are occurring.

A plausible scheme would involve initial formation of ES followed by a conformational change in which the catalytic site is formed. This would then be followed by breakdown of the substrate, which may or may not involve Glu-270. The fact that I is a linear noncompetitive inhibitor toward esters (HPL) very likely means that a conformational change takes place due to its binding (or that one necessary for the reaction of esters is prevented) so that the EIS complex does not break down. Two intermediates have been detected in the hydrolysis of both peptide and ester substrates prior to the rate-determining step (Galdes et al., 1983; Geoghegan et al., 1983). The above scheme provides for at least two intermediates before the product-forming step ($ES_1 \rightleftharpoons ES_2$).

Registry No. I, 56379-89-6; II, 104070-51-1; HPL, 3675-74-9; HPA, 744-59-2; CPA, 11075-17-5; L-Glu, 56-86-0; PhO_2CCl, 1885-14-9; L-$PhCH_2CH(OH)CO_2H$, 20312-36-1.

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