Intrinsic Enzymatic Activity of Bovine Procarboxypeptidase A S_5†

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ABSTRACT: Bovine procarboxypeptidase A S_5 possesses inherent catalytic activity toward ester and peptide substrates of carboxypeptidase A. A kinetic analysis of this zymogen activity demonstrates that for ester substrates k_{cat} is $\frac{1}{20}$th that of carboxypeptidase A and K_m is 100–300 times larger. For peptide substrates k_{cat} is $\frac{1}{100}$th to $\frac{4}{100}$th that of the enzyme but K_m is relatively unchanged. The inhibition constants (K_i) for the dipeptide glycyl-L-tyrosine are also similar for the zymogen and the enzyme, suggesting that the substrate binding site is preformed in the zymogen. The catalytically functional elements of carboxypeptidase A (zinc, tyrosine, and glutamic acid) are also involved in the activity of the zymogen. Chelation of zinc with 1,10-phenanthroline inhibits both peptidase and esterase activity. Nitration of tyrosine with tetranitromethane abolishes peptidase activity and diminishes esterase activity. Following activation, peptidase activity remains negligibly low but esterase activity increases to 140% of that of native carboxypeptidase. Carboxyl modification with bromoacetyl-*N*-methyl-L-phenylalanine inhibits peptidase activity of the zymogen and prevents zymogen activation. When the zymogen is labeled with radioactive reagent and subsequently degraded, the radioactive label is found in a dipeptide (Phe-Glu), which is identical with that obtained from similarly labeled carboxypeptidase. The pH dependence of carboxyl modification of the zymogen by Woodward's reagent K corresponds to the ionization of a group having a pK_a of 5.0 as compared to 7.0 for the enzyme. It is proposed that activation of procarboxypeptidase A induces a change in conformation which alters the hydrogen bond structure of Glu_{270} and enhances the reactivity of this nucleophile.

Bovine pancreatic procarboxypeptidase A S_5 is an aggregate of two zymogens: subunit I, the immediate precursor of carboxypeptidase A, and subunit II, the zymogen form of a chymotrypsin-like enzyme. The enzymatic steps leading to the formation of carboxypeptidase A have been described by Brown *et al.* (1963) and more recently reexamined by Uren and Neurath (1972). A number of observations suggest that certain components of the active site of carboxypeptidase exist in the zymogen. Piras and Vallee (1967) have reported that peptide and ester substrates, substrate analogs, and inhibitors retard the incorporation of ^{65}Zn into procarboxypeptidase A, indicating that the substrate binding site is preformed in the zymogen. Freisheim *et al.* (1967) have observed that acetylation of the succinylated zymogen (subunit I), followed by tryptic activation, has the same functional consequences as acetylation of succinyl carboxypeptidase A. It was concluded, therefore, that the tyrosine residues of the active site are reactive in the enzyme precursor. Yamasaki *et al.* (1963) have reported that bovine procarboxypeptidase A displays a low level of activity toward ester and peptide substrates, but they did not exclude the possibility that this activity was due to contamination by active enzyme. More definitive evidence for intrinsic enzymatic activity of the zymogen was provided by Lacko and Neurath (1970) for dogfish procarboxypeptidase A and by Reeck and Neurath (1972) for lungfish procarboxypeptidase B. Behnke and Vallee (1972) have found that cobalt bovine procarboxypeptidase A catalyzes the hydrolysis of haloacylated amino acids at rates comparable to zinc carboxypeptidase A. Enzymatic activity seems to be an intrinsic property of other zymogens as well, notably those of serine proteases (Robinson *et al.*, 1973; Morgan *et al.*, 1972; Gertler *et al.*, 1974).

We have reexamined the nature of the intrinsic enzymatic activity of bovine procarboxypeptidase A S_5 and attempted to relate this activity to the structure and function of carboxypeptidase A. It was the purpose of the present investigation to (1) determine the kinetics of the zymogen-catalyzed hydrolysis of peptide and ester substrates of carboxypeptidase A, (2) investigate the involvement of the functional elements of the enzyme—zinc, tyrosine, and glutamic acid—in the activity of the precursor, and (3) relate the catalytic properties of the zymogen to the three-dimensional structure of crystalline carboxypeptidase A.

Experimental Section

Materials

Enzymes. Bovine procarboxypeptidase A S_5 (PCP A S_5)[1] was prepared as described previously (Uren and Neurath, 1972). Bovine carboxypeptidase A (Anson) (lot COA-7AC) and lysozyme (lot LY 624) were obtained from Worthington Biochemical Corp. Thermolysin (lot TOBBO1) was obtained from Daiwa Kasei K. K., Osaka, Japan.

Reagents. Organic reagents were obtained from the following sources: *N*-α-acetyl-L-cysteine, Calbiochem; L-cysteic acid, Nutritional Biochemical Corp.; *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K), Aldrich; glycyl-L-tyrosine, Fox Chemical Co.; β-phenylpropionic acid, Eastman

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received March 14, 1974.* Taken in part from a dissertation submitted by J. R. U. to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of doctor of philosophy (Uren, 1972). This work has been supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (NP-18N). A preliminary report of this work has been published previously (Uren *et al.*, 1972).

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[1] Abbreviations used are: PCP A S_5, procarboxypeptidase A having a sedimentation coefficient of 5 S; HPLA, hippuryl-D,L-β-phenyllactic acid; Z-Gly-Phe, carbobenzoxyglycyl-L-phenylalanine; Bz-Gly-Phe, hippuryl-L-phenylalanine; AcTyrOEt, acetyl-L-tyrosine ethyl ester; AGPLA, acetylglycyl-L-phenyllactic acid.

Chemical Co.; 1,10-phenanthroline, G. Frederick Smith Chemical Co.; tetranitromethane, Aldrich. *N*-Bromoacetyl-*N*-methyl-L-phenylalanine was prepared by Dr. G. Michael Hass (Hass and Neurath, 1971a) and [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine was prepared as described by Hass and Neurath (1971b). All inorganic reagents were reagent grade.

Column Media. Sephadex G-25, G-100, and SE-Sephadex C-25 were purchased from Pharmacia Fine Chemicals; Dowex 1-X2 was from Bio-Rad Laboratories; and DE-52-cellulose was from Whatman Reeve Angel. *N*-2-(Ethylcellulose)glycyl-D-phenylalanine was synthesized as described by Uren (1971).

Substrates. Hippuryl-β-phenyllactic acid (HPLA), carbobenzoxyglycyl-L-phenylalanine (Z-Gly-Phe), and hippuryl-L-phenylalanine (Bz-Gly-Phe) were obtained from Fox Chemical Co.; acetyl-L-tyrosine ethyl ester (AcTyrOEt) was from Cyclo Chemical Co.; and acetylglycyl-L-phenyllactic acid (AGPLA) was synthesized by Mr. Peter Grogg.

Methods

Intrinsic Activity of PCP A S_5. The esterase activity toward HPLA and AGPLA was determined by dissolving the substrate (0.1 M) in 0.25 M NaCl–5 mM Tris-HCl (pH 7.5). After addition of the zymogen, the reaction was followed in a pH-Stat at 25° (pH 7.5). The enzymatic hydrolysis of the peptide substrates Z-Gly-Phe and Bz-Gly-Phe was measured spectrophotometrically (Whitaker *et al.*, 1966) at 224 and 254 nm ($\Delta\epsilon$ = 1190 and 360, respectively) in 0.475 M KCl–0.05 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0). Proteolytic activity was measured by incubating the zymogen with 2 mM lysozyme in 0.2 M NaCl–0.05 M Tris-HCl (pH 7.5) at 37° and removing aliquots at 1-hr intervals. The protein was precipitated with 6% sulfosalicylic acid, and amino groups were determined in the supernatant solution by use of a Technicon Auto Analyzer which was calibrated with a phenylalanine standard. The release of leucine was confirmed by use of a Spinco Model 120 amino acid analyzer.

Inactivation of PCP A S_5 by 1,10-Phenanthroline. PCP A S_5 (32.2 μM) was incubated with 1 mM 1,10-phenanthroline in 1 M NaCl–0.05 M Tris-HCl (pH 7.5) at room temperature. Intrinsic esterase activity toward 0.1 M L-HPLA was measured in the presence of 1 mM 1,10-phenanthroline to prevent recombination with metal during the assay. Intrinsic peptidase activity toward 0.01 M Z-Gly-Phe was measured with a Technicon Auto Analyzer by reaction with ninhydrin.

Nitration with Tetranitromethane. The zymogen (95 μM) was incubated with 830 μM tetranitromethane in 1 M NaCl–0.05 M Tris-HCl (pH 8.0) at room temperature. Intrinsic esterase activity was measured toward 0.1 M L-HPLA and intrinsic peptidase activity toward 1 mM Bz-Gly-Phe. At 15-min intervals, 0.1-ml aliquots were withdrawn and added to 2 ml of 6 N HCl containing an excess of phenol to absorb any nitrite produced (Sokolovsky and Riordan, 1970). These samples were hydrolyzed at 110° and subjected to amino acid analysis.

Reaction with Woodward's Reagent K. The zymogen was incubated with Woodward's reagent K in 1 M NaCl–5 mM imidazole. The pH was adjusted to the desired value and kept constant by the addition of 0.1 M NaOH in a pH-Stat at 25°. Aliquots (0.05 ml) were withdrawn at various times and rapidly frozen in tubes at −60°. After thawing in the presence of 1 ml of 0.1 M L-HPLA, the aliquots were assayed for esterase activity in a pH-Stat as described above. Since Woodward's reagent K interfered with the peptidase assay, peptidase activity was not determined.

Reaction with Bromoacetyl-N-methyl-L-phenylalanine. PCP A S_5 (1 mg/ml) was incubated with various concentrations of this inhibitor in 1 M NaCl–0.05 M Tris-HCl (pH 7.5) at 25°. Intrinsic esterase activity was measured toward 0.1 M L-HPLA and intrinsic peptidase activity toward 1 mM Bz-Gly-Phe.

Inactivation of PCP A S_5 by [^{14}C]Bromoacetyl-N-methyl-L-phenylalanine. The zymogen (0.8 mg/ml) was dissolved in 2 ml of 1 M NaCl–0.05 M Tris-HCl (pH 7.5) and incubated with 5 mM [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine. Aliquots (0.2 ml) were withdrawn at 0-, 1-, 2-, and 3-hr intervals and desalted on a 1 × 40 cm column of Sephadex G-25 by elution with 0.1 M KH_2PO_4 (pH 6.5). Intrinsic peptidase activity toward 1 mM Bz-Gly-Phe was measured as described above. Initial activity was determined by extrapolating the first-order loss in intrinsic activity after 1, 2, and 3 hr to zero time. Activatability was determined by activating the desalted samples for 0, 1, 2, and 3 hr with 50 μl of trypsin (1 mg/ml) at 37° and measuring activity toward 10 mM D,L-HPLA. Specific radioactivity of the reagent was determined as described by Hass and Neurath (1971a). The radioactivity of aliquots of the desalted protein and the protein concentration were determined as described previously (Uren and Neurath, 1972).

Digestion with Thermolysin. PCP A S_5 (0.9 μmol) was incubated for 3 hr with 10 mmol of the radioactive inhibitor in 1 M NaCl–0.05 M Tris-HCl (pH 7.5). The reaction mixture was dialyzed for 1 day against 2 changes of 2 l. of 1 M NaCl–0.05 M Tris-HCl (pH 7.5) and then for 3 days against a continuous flow of 5% acetic acid, and lyophilized. The lyophilized material was suspended in 9 ml of 2 mM calcium acetate–0.01 M Tris-HCl (pH 7.5). This material was digested at 37° with thermolysin (1 mg/ml) which was added in three 1-ml aliquots. Digestion was carried out for 2 hr in a pH-Stat maintained at pH 7.5 by the addition of 0.1 M NaOH. After centrifugation, 60–85% of the radioactivity was found in the supernatant solution. The soluble peptides were lyophilized.

Chromatography of the Thermolysin Digest. The lyophilized, soluble peptides from the digestion with thermolysin were chromatographed on a SE-Sephadex C-25 column under the conditions described by Hass and Neurath (1971b) except that the second linear gradient of pyridine–acetic acid (0.5–2.0 M) was omitted because the radioactive material was eluted by application of the first gradient. The amino acid composition of the eluted peptides was determined in a Beckman Model 120B amino acid analyzer. As a blank, an identical strip of paper containing no ninhydrin-reacting material was eluted, hydrolyzed, and analyzed.

Stereoscopic Plot of the Environment of Glu_{270}. The stereoscopic plot of the environment of Glu_{270} in carboxypeptidase A was drawn with a Calcomp plotter from calculations of a CDC 6400 computer using the Oak Ridge Thermal Ellipsoid Plot (ORTEP) program written by Carroll Johnson. The atomic coordinates of the atoms of carboxypeptidase A were supplied by Dr. William N. Lipscomb (Quiocho and Lipscomb, 1971) prior to publication.

Results

Purified PCP A S_5 catalyzes the hydrolysis of peptide and ester substrates of carboxypeptidase A (*vide infra*). The following chromatographic analyses demonstrate that this activity is an intrinsic property of the zymogen and not due to adventitious contamination by carboxypeptidase A.

(1) When the zymogen was subjected to chromatography on DE-52 cellulose (Figure 1), fractions of the major peak of PCP A S_5 showed constant specific esterase acitivity toward HPLA. The specific peptidase activity toward Z-Gly-Phe decreased

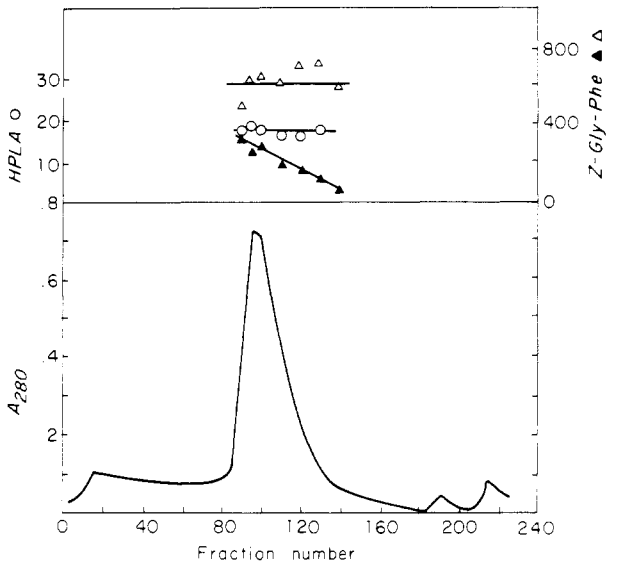

FIGURE 1: Chromatography of bovine PCP A S_5 on DE-52 cellulose. The column (2.5 × 85 cm) was eluted by a 2 l. × 2 l. gradient of 0.1–0.2 M KH_2PO_4 (pH 6.5) at a flow rate of 1.5 ml/min at 4°. Fraction volume was 16.4 ml/tube. Intrinsic activities of the major component are plotted in the top part of Figure 1. The symbols refer to the following substrates: (○) HPLA; (▲) Z-Gly-Phe before and (△) after dialysis to remove phosphate ions. Units of activity are μmoles min^{-1} mg^{-1} for HPLA and nmol min^{-1} mg^{-1} for Z-Gly-Phe.

with increasing tube number unless phosphate—a known inhibitor of carboxypeptidase (Lumry *et al.*, 1951)—was first removed from the eluting buffer by dialysis. Under the same conditions of chromatography, carboxypeptidase A eluted with the breakthrough fraction.

(2) Gel filtration of purified PCP A S_5 on Sephadex G-100 also yielded a main component of constant specific esterase (HPLA) and peptidase (Z-Gly-Phe) activity. Since the molecular weight of the active enzyme (36,000) is much lower than that of the zymogen (63,000), the presence of significant quantities of carboxypeptidase A would have caused the specific activities to increase with increasing tube number.

(3) Purified PCP A S_5 was not adsorbed on an affinity column of *N*-(2-ethylcellulose)glycyl-D-phenylalanine which adsorbs carboxypeptidase A (Uren, 1971). The specific esterase activity (HPLA) of the zymogen was unaffected by filtration through this column.

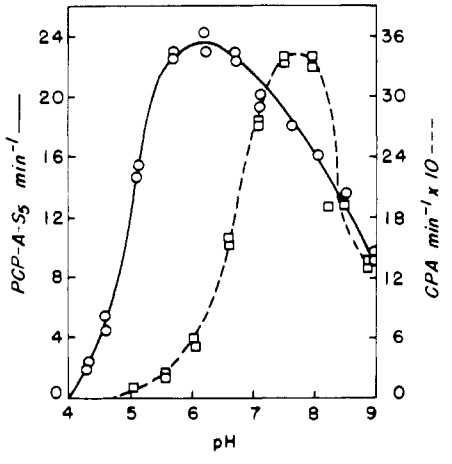

FIGURE 2: pH dependence of the rate of hydrolysis of 1 mM Z-Gly-Phe by PCP A S_5 (○) and by carboxypeptidase A (□) at 25°. The substrate was dissolved in 0.05 M Tris-acetic acid buffers, containing 0.45 M KCl. A spectrophotometric assay was used.

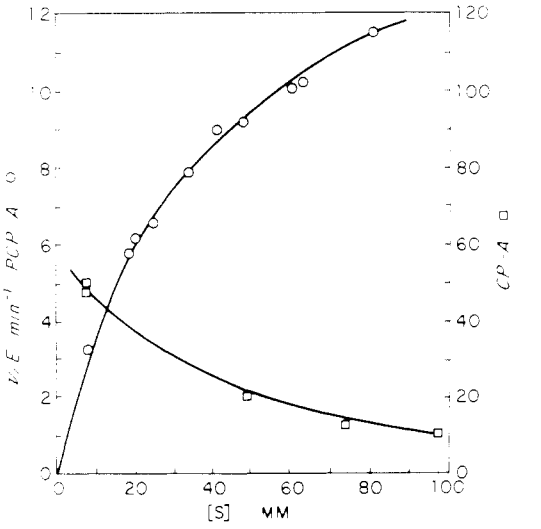

FIGURE 3: Substrate concentration dependence of the hydrolysis of L-HPLA by PCP A S_5 (○) and by carboxypeptidase A (□) at 25°. The substrate was dissolved in 5 mM Tris (pH 7.5) containing 0.25 M NaCl. Assays were performed in a pH-Stat. Ordinate units are 1/100th of those given.

(4) Three different preparations of purified PCP A S_5 gave specific activities of 31.8 ± 1.5 μmol min^{-1} mg^{-1} toward HPLA and 258 ± 10 μmol min^{-1} mg^{-1} toward Z-Gly-Phe. This constancy indicates that the observed activity is an inherent property of the zymogen.

Effects of Solvent Composition on Activity. At pH 7.5 the specific esterase activity (HPLA) of PCP A S_5 varies little with increasing ionic strength above 0.25 M NaCl. The activity is also independent of pH between pH 6.5 and 9. These properties are similar to those of carboxypeptidase A (Riordan and Vallee, 1963; McClure and Neurath, 1966). The pH dependence of the peptidase activity of PCP A S_5 is shown in Figure 2 and compared to that of carboxypeptidase A measured under identical conditions. It is evident that under the specific conditions of assay, the pH optimum of the zymogen activity (pH 6.0) is 1.5 pH units lower than that of the enzyme (pH 7.5).

Kinetic Parameters. The kinetics of the zymogen-catalyzed hydrolysis of two ester and two peptide substrates were determined and compared to the enzyme-catalyzed hydrolysis. As shown in Figure 3, the zymogen-catalyzed hydrolysis of L-HPLA followed Michaelis-Menten kinetics in the substrate concentration range where carboxypeptidase A shows excess

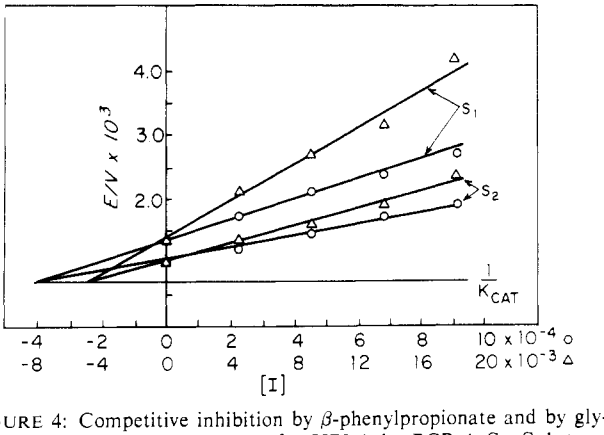

FIGURE 4: Competitive inhibition by β-phenylpropionate and by glycyl-L-tyrosine of the hydrolysis of L-HPLA by PCP A S_5. Substrate concentrations were 1.82 × 10^{-2} M (S_1) and 3.64 × 10^{-2} M (S_2), respectively. Assay conditions are described in the legend to Figure 3.

TABLE I: Comparison of Kinetic Constants of Procarboxypeptidase A S_5 and Carboxypeptidase A.[a]

Substrate	Procarboxypeptidase A S_5		Carboxypeptidase A	
	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
Peptides				
Z-Gly-Phe	1.0 ± 0.2[b]	31 ± 2[b]	2.0 ± 0.1[b,c]	3,000 ± 180[b,c]
	0.67 ± 0.01[d]	31 ± 2[d]		
Bz-Gly-Phe	2.7 ± 0.7[d]	182 ± 41[d]	1.9[b,e]	7,080[b,e]
Esters				
L-HPLA	33.4 ± 1.7[f]	1,570 ± 40[f]	0.12 ± 0.1[b]	28,000 ± 1,000[b]
AGPLA	33 ± 4[f]	1,900 ± 100[f]	0.3[f,g]	36,000[f,g]

[a] T = 25°. For other experimental conditions, see the text. [b] Measurements in 50 mM Tris-HCl–0.475 M KCl (pH 7.5). [c] Uren (1972). [d] Measurements in 50 mM 2-(*N*-morpholino)ethanesulfonate (pH 6.0). The buffers used for measurements of zymogen and enzyme activities were adjusted to the respective pH optima for peptide hydrolysis. [e] Whitaker *et al.* (1966). [f] Measurements in 5 mM Tris–0.25 M NaCl (pH 7.5). [g] R.-M. McDonald, unpublished observations.

substrate inhibition. These findings are analogous to those previously reported by Lacko and Neurath (1970) for dogfish procarboxypeptidase A and carboxypeptidase A, respectively. The zymogen-catalyzed hydrolysis of the other ester substrate, AGPLA, also followed Michaelis–Menten kinetics over the entire substrate concentration range (10–100 mM). The kinetic constants, K_m and k_{cat}, for the hydrolysis of all four substrates by PCP A S_5 were calculated from linearized plots of the data and are given in Table I together with the K_m and k_{cat} values for the hydrolysis of these substrates by carboxypeptidase A.

Inhibition. The hydrolysis of L-HPLA by the zymogen was competitively inhibited by β-phenylpropionate and by glycyl-L-tyrosine (Figure 4). In 5 mM Tris–0.25 M NaCl (pH 7.5) at 25°, the inhibition constants (K_i) were 5 mM for β-phenylpropionate and 0.4 mM for glycyl-L-tyrosine (Table II). Analogous data for the inhibition of the enzyme-catalyzed hydrolysis of L-HPLA are also included in Table II.

Proteolysis. Besides hydrolyzing small ester and peptide substrates, PCP A S_5 also possesses activity toward proteins. On a molar basis, the zymogen released carboxyl-terminal leucine from egg white lysozyme at approximately 1/7th the rate of carboxypeptidase A.

Modification of Functional Groups. Zinc. The involvement of metal (zinc) in the activity of the zymogen was demonstrated by the parallel first-order rate of inhibition of esterase and peptidase activities when PCP A S_5 was incubated at pH 7.5 with 1 mM 1,10-phenanthroline (Figure 5). The slow inhibition observed herein differs from the very rapid loss of peptidase activity (Felber *et al.*, 1962) when carboxypeptidase A is incubated under similar conditions with this chelating agent. The half-life of inhibition was 80 min for the zymogen as compared to less than 1 min for the enzyme (Felber *et al.*, 1962).

Nitration with Tetranitromethane. When PCP A S_5 was incubated with an 8.7-fold molar excess of reagent under the conditions described by Riordan *et al.* (1967) for the inhibition of carboxypeptidase A, a rapid loss of intrinsic peptidase and a slower loss of intrinsic esterase activity were observed (Figure 6). According to these data, an average of one residue of tyrosine per molecule was nitrated within the first 10 min, accompanied by an 80% loss of intrinsic peptidase activity and a 24% loss of intrinsic esterase activity. After 1 hr of incubation, 91% of peptidase activity and 45% of esterase activity were lost, and two additional tyrosine residues were nitrated. The unequal loss of esterase and peptidase activities is similar to the differential response of these activities when the enzyme is nitrated.

The nitrated zymogen containing on the average 3.5 nitro groups per molecule was subjected to gel filtration on Sephadex G-100 and then activated with trypsin. A 10.7 μM solution of the zymogen in 0.1 M KH_2PO_4 (pH 7.0) was incubated with trypsin (1 μM) at 37° for 200 min. During activation, the esterase activity (10 mM D,L-HPLA) increased to a level 140% that of native carboxypeptidase A. The peptidase activity (1 mM Bz-Gly-Phe) prior to activation of the nitrated zymogen was

TABLE II: Comparison of Inhibition Constants of Procarboxypeptidase A S_5 and Carboxypeptidase A.[a]

Inhibitor	K_i (mM)	
	Procarboxypeptidase A S_5	Carboxypeptidase A
β-Phenylpropionate	5.0	0.15[b]
Glycyl-L-tyrosine	0.4	0.2[c]
D-HPLA	45	4

[a] The substrate was L-HPLA (18.2 and 36.4 mM) in 5 mM Tris–0.25 M NaCl (pH 7.5). Measurements at 25°. Inhibition was competitive in all cases except the inhibition of carboxypeptidase A by D-HPLA which was noncompetitive (Lange *et al.*, 1972). [b] Using hippuryl-L-mandelate as substrate (Kaiser and Carson, 1965). [c] Using Z-Gly-Phe as substrate (Yanari and Mitz, 1957).

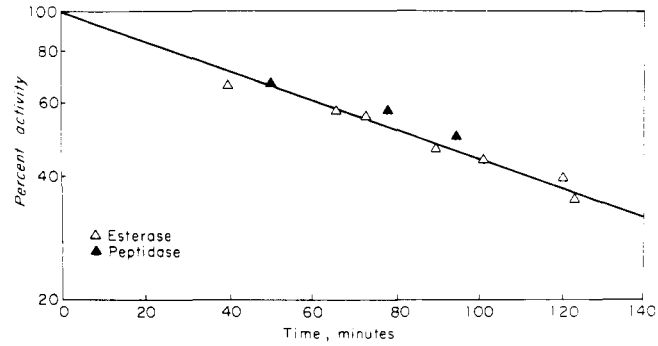

FIGURE 5: Inactivation of intrinsic activity of PCP A S_5 by 1,10-phenanthroline. The zymogen (32.2 μM) was incubated with 1 mM 1,10-phenanthroline in 0.05 M Tris–1 M NaCl (pH 7.5) at room temperature. Activities were measured as described in Methods.

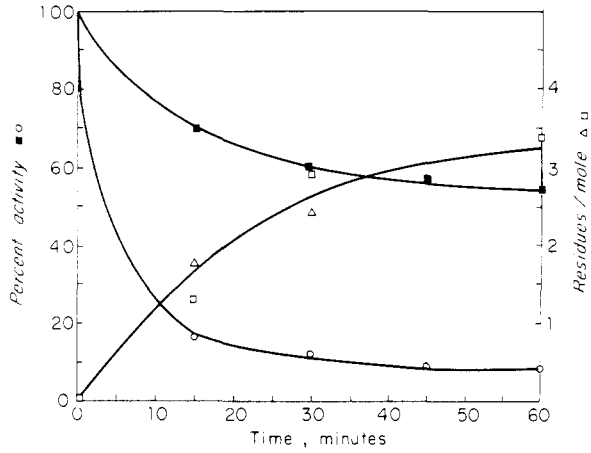

FIGURE 6: Nitration of PCP A S_5 with tetranitromethane. The symbols are as follows: (■) esterase activity; (○) peptidase activity; (△) nitrotyrosine recovered; (□) tyrosine residues lost.

only 0.5% that of the enzyme and remained at that low level during activation. These results are analogous to those described by Riordan *et al.* (1967) for the effects of nitration on the esterase and peptidase activities of carboxypeptidase A.

Carboxyl Group Modification. WOODWARD's REAGENT K. The involvement of a carboxyl group in the intrinsic activity of the zymogen was investigated by reaction with Woodward's reagent K (Petra, 1971; Petra and Neurath, 1971). When PCP A S_5 (19 μM) was incubated with 5, 10, and 20 mM of reagent for 0–5 min at 25° in 1 M NaCl–5 mM imidazole-HCl, at pH 6.4 and pH 4.7, a pseudo-first-order loss of activity was observed for relatively short periods, corresponding to 40–60% inactivation. Further additions of the reagent caused further loss of activity, implying a rapid turnover of the reagent. These inactivation rates did not change with increasing inhibitor concentrations, suggesting that the zymogen was saturated with inhibitor. A plot of the pseudo-first-order rate constants of inhibition against pH (Figure 7) followed the calculated titration curve of a group having a pK_a of 5.0 and a maximum inactivation rate of 0.6 min^{-1}. This pK_a is 2 pH units lower than that which controls the inhibition of carboxypeptidase A by this reagent (Petra, 1971). Inhibition at pH 6 was prevented by β-phenylpropionate (36 mM). Measurements of the effect of Woodward's reagent K on peptidase activity were precluded by interference of the reagent with the assay.

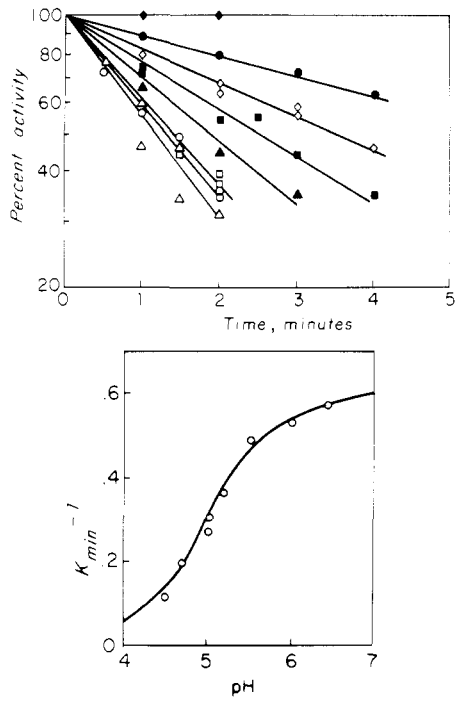

FIGURE 7: (Top) Effect of pH on the rate of inactivation of PCP A S_5 by Woodward's reagent K. The zymogen (19 μM) was incubated with the reagent (20 mM) in 0.05 M imidazole-1 M NaCl at the desired pH at 25°. Aliquots were withdrawn and assayed as described in Methods. The symbols are as follows: (♦) pH 6.0 in the presence of 36.3 mM β-phenylpropionate; (●) pH 4.5, k = 0.115; (◇) pH 4.7, k = 0.198; (■) pH 5.0, k = 0.277; (▲) pH 5.2, k = 0.364; (□) pH 5.5, k = 0.495; (○) pH 6.0, k = 0.532; (△) pH 6.4, k = 0.576. k represents pseudo-first-order rate constants of inactivation. (Bottom) Rate of inactivation as a function of pH. The curve was calculated for a pK_a of 5.0 and a k_{max} of 0.6 sec^{-1}.

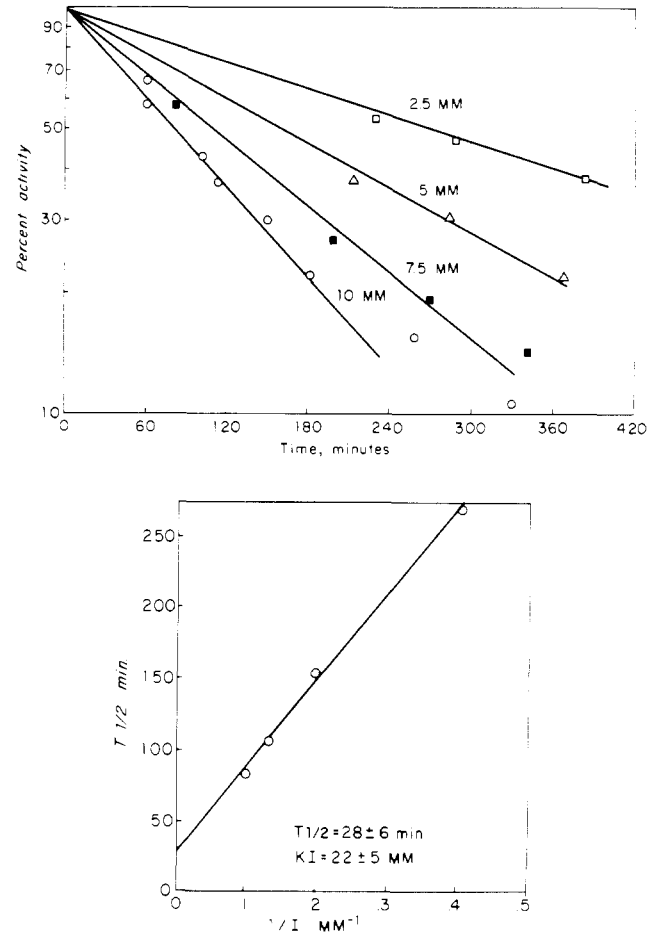

FIGURE 8: (Top) Effect of reagent concentration on the inactivation of PCP A S_5 by bromoacetyl-*N*-methyl-L-phenylalanine. The enzyme (1 mg/ml) was incubated with various concentrations of reagent in 0.05 M Tris–1 M NaCl (pH 7.5) at 25°. Esterase and peptidase activities were measured as described in Methods. (Bottom) Half-time of inactivation plotted against reciprocal of the reagent concentration according to the equation (Hass and Neurath, 1971a): $t = (K_i T/[I]) + T$, where t is the half-time of inactivation at a given inhibitor concentration, $[I]$, T the half-time at saturating inhibitor concentration, and K_i the enzyme-inhibitor dissociation constant.

BROMOACETYL-*N*-METHYL-L-PHENYLALANINE. Since Woodward's reagent K is rapidly hydrolyzed and has several paths of decomposition, the kinetics of carboxyl modification of the zymogen were determined with the site-specific reagent bromoacetyl-*N*-methyl-L-phenylalanine (Hass and Neurath, 1971a,b). The effect of inhibitor concentration on the rates of intrinsic peptidase activity (pH 7.5) at 25° is shown in the top part of Figure 8. Both esterase (HPLA) and peptidase (Bz-Gly-Phe) activities were inhibited at the same pseudo-first-order rate. A reciprocal plot (Figure 8, bottom) of the inhibitor

concentration against the half-time of inactivation yielded a half-time (at saturating inhibitor concentration) of 28 ± 6 min compared to 3.6 min determined under the same conditions for the inactivation of the enzyme (Hass and Neurath, 1971a). The enzyme-inhibitor dissociation constants, K_i, are 22 ± 5 mM for the zymogen and 4.8 mM for the enzyme.

The stoichiometry of inactivation was determined by incubation of the zymogen with [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine of known specific radioactivity (see Methods). The correlation between incorporation of labeled inhibitor, loss of intrinsic peptidase activity, and loss of ability to be activated by trypsin is shown in Figure 9. Reaction with the reagent caused a parallel loss of intrinsic activity and activatability by trypsin, suggesting that the labeled residue was functional in both the zymogen and the active enzyme. Linear extrapolation of the data to zero activity indicated that 0.84 active site/molecule had reacted with the site-specific reagent. The fraction which did not react with the reagent was devoid of both intrinsic activity and the ability to be activated by trypsin.

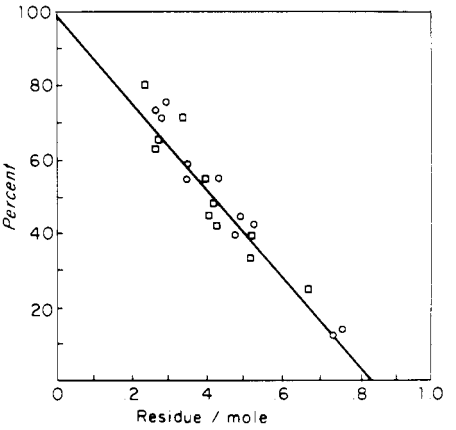

FIGURE 9: Stoichiometry of inactivation of PCP A S_5 by [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine. Procedures are described in Methods. (□) represents per cent intrinsic activity and (○) per cent activatability by trypsin.

Following acid hydrolysis of the ^{14}C-labeled zymogen, radioactive material was recovered in a ninhydrin-negative fraction which eluted in the amino acid analyzer after cysteic acid and before aspartic acid. This elution position corresponds to glycolic acid, an expected hydrolysis product of the reaction of bromoacetyl-*N*-methyl-L-phenylalanine with a carboxyl group (Hass and Neurath, 1971b).

Peptide Isolation. In order to identify the modified carboxyl group, the ^{14}C-labeled zymogen was denatured by acid and digested with thermolysin (see Methods). The enzymatic digest was chromatographed on a column of SE-Sephadex C-25 under the conditions described by Hass and Neurath (1971b). The elution position of the radioactive material was identical with that of pool SE-2 described by these authors for carboxypeptidase A labeled with [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine. The radioactive fractions were pooled, lyophilized, and chromatographed on a Dowex 1-X2 column (Hass and Neurath, 1971b). On this column, too, the radioactive material eluted at the same position as that of the ^{14}C-labeled enzyme. The radioactive fractions were pooled, lyophilized, and subjected to preparative paper electrophoresis at pH 6.5. The R_F of the labeled peptide relative to glutamic acid was 0.45 as compared to 0.43 for the labeled peptide isolated from carboxypeptidase A (G. M. Hass, personal communication). The radioactive peptide was eluted, hydrolyzed in 6 N HCl, and subjected to amino acid analysis. As a blank, an identical strip of paper containing no ninhydrin-reacting material was similarly treated and analyzed. Based upon specific activity, the peptide (30 nmol) contained 0.9 residue/molecule of glutamic acid, 0.8 residue of phenylalanine, and 0.1 residue of aspartic acid (contaminant). Because the peptide was obtained by digestion with thermolysin, which cleaves peptide bonds adjacent to hydrophobic residues, it can be concluded that its dipeptide sequence was Phe-Glu rather than Glu-Phe. The same dipeptide was isolated by Hass and Neurath (1971b) from carboxypeptidase A labeled with [^{14}C]bromoacetyl-*N*-methyl-L-phenylalanine and was identifed as residues 269 and 270 in the amino acid sequence of the enzyme (Bradshaw *et al.*, 1969).

Discussion

Previous reports had indicated that procarboxypeptidases A and B possess intrinsic activity toward substrates of the corresponding enzymes (Yamasaki *et al.*, 1963; Lacko and Neurath, 1970; Reeck and Neurath, 1972). The possibility was not rigorously excluded, however, that these activities were due to spontaneous activation or were associated with extraneous contaminants. The present experiments leave no doubt that the catalytic activity of PCP A S_5 is an inherent property of the zymogen. The following observations support this conclusion. (1) The intrinsic activity remains constant under various chromatographic conditions. (2) The kinetic behavior of the zymogen toward esters is different from that of carboxypeptidase A. (3) The pH optimum and the pH dependence of peptidase activity differ from those of the enzyme. (4) Two typical inhibitors of carboxypeptidase A (β-phenylpropionate and D-HPLA) are less tightly bound to the zymogen than to the enzyme. (5) The site-specific reagent bromoacetyl-*N*-methyl-L-phenylalanine reacts with 0.84 active site/zymogen molecule. If the intrinsic activity were associated with an extraneous enzyme similar to carboxypeptidase A, such a contaminant would have to respond identically to ion exchange and affinity chromatography, to gel filtration, and to the affinity reagent bromoacetyl-*N*-methyl-L-phenylalanine. Such coincidence in properties seems implausible.

The lower enzymatic activity of the zymogen as compared to the enzyme could be due to a less efficient catalytic mechanism, a less efficient binding of substrates, or both. Comparison of the kinetic constants (K_m and k_{cat}) for the hydrolysis of peptide substrates (Table I) indicates that the turnover (k_{cat}) of the zymogen is approximately 1/20th (or less) that of the enzyme; yet the values for K_m are nearly the same. Although in general K_m contains algebraic terms involving both binding and turnover, Coleman and Vallee (1962) have shown that the binding constant (K_s) for peptide binding to apocarboxypeptidase A corresponds to the Michaelis constant (K_m) for the hydrolysis of this peptide by the holoenzyme. These authors concluded therefore that K_m was a measure of binding. Since in the present comparison between zymogen and enzyme k_{cat} is greatly different but K_m remains relatively constant, it may be concluded that here, too, K_m is a binding constant. Support for this interpretation was also derived from inhibition studies (Table II) which demonstrated that zymogen and enzyme have similar K_i values for the inhibition of ester hydrolysis by glycyl-L-tyrosine.[2] The fact that the zymogen and the enzyme have

[2] Since no data are available for the inhibition of ester hydrolysis by glycyl-L-tyrosine, the value for the inhibition of peptide hydrolysis (Z-Gly-Phe) was used for comparison. This substitution seems valid since, by and large, the inhibition constants are similar for peptide and ester hydrolysis (Petra, 1970). Moreover, the K_i value of 0.2 mM is comparable to the K_m value of 0.7 mM when glycyl-L-tyrosine serves as substrate (Izumiya and Uchio, 1959), indicating that the enzyme-inhibitor dissociation constant is of the stated order of magnitude.

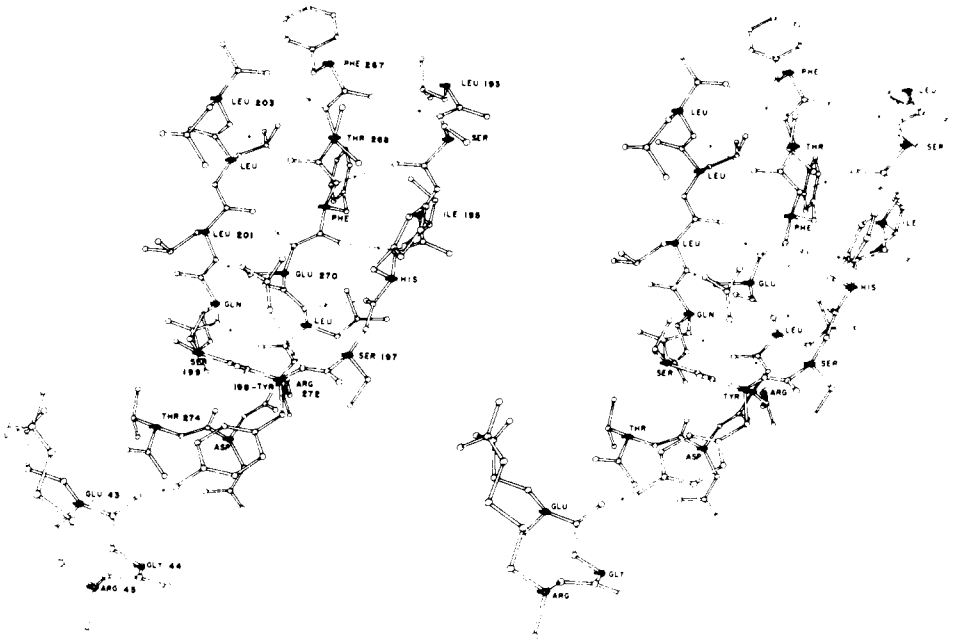

FIGURE 10: ORTEP stereo projection of the atomic environment of Glu-270. Two segments of the molecule are illustrated: residues 193–203 and residues 267–274. In the lower left a segment (residues 43–45) of a neighboring molecule in the crystal lattice is illustrated. The atomic coordinates were provided by Dr. W. N. Lipscomb (Quiocho and Lipscomb, 1971). Calculations are described in Methods.

similar K_m values for peptide hydrolysis and similar K_i values for peptide inhibition suggests that the substrate binding site is largely preformed in the zymogen. In contrast, the catalytic (bond-breaking) mechanism is relatively ineffective prior to activation. The kinetic constants of ester hydrolysis cannot be similarly interpreted since the zymogen and the enzyme differ in *both* k_{cat} and K_m; k_{cat} of the zymogen is about $\frac{1}{20}$th that of the enzyme and K_m is 300–500 times greater. This different response to ester substrates is consistent with the view that peptide and ester substrates are bound differently and are hydrolyzed by different mechanisms by carboxypeptidase A (Vallee and Riordan, 1968).

In order to account for the ineffective bond-breaking mechanism (k_{cat}) of the zymogen, we have examined the reactivity of the three catalytic elements of carboxypeptidase A (zinc, tyrosine, and the carboxyl group of Glu_{270}) in the enzymatic function of PCP A S_5. Addition of the chelating agent 1,10-phenanthroline to the enzyme causes inactivation and loss of zinc (Vallee *et al.*, 1960). Incubation of the zymogen with this chelating agent likewise inhibits both peptidase and esterase activities. Although the zinc content of the zymogen was not measured in our experiments, Piras and Vallee (1967) have shown that this chelating agent causes metal removal with a loss of ability of the zymogen to be subsequently activated by trypsin. Hence zinc is a catalytic element of the zymogen as well as of the enzyme.

Nitration or acetylation of tyrosine residues of the active site abolishes peptidase activity of carboxypeptidase A and enhances apparent esterase activity (Vallee and Riordan, 1968). In the zymogen, too, peptidase activity is lost after nitration and apparent esterase activity is enhanced after activation by trypsin. The reactivity of tyrosine residues toward acylating agents was previously demonstrated by Freisheim *et al.* (1967) who reported that acetylation of succinylated subunit I of procarboxypeptidase, followed by tryptic activation, resulted in the same changes in enzymatic function as occur upon acetylation of succinylcarboxypeptidase. Hence the tyrosyl residue of the active site seems as accessible in the zymogen as in the enzyme.

The involvement of the carboxyl group of Glu_{270} in the activity of carboxypeptidase A was first proposed on the basis of model building and X-ray structure analysis and was subsequently proven chemically by reaction with Woodward's reagent K (Petra, 1971; Petra and Neurath, 1971) and with bromoacetyl-*N*-methyl-L-phenylalanine (Hass and Neurath, 1971a,b). *Both* reagents modify Glu_{270} and inactivate the enzyme. Both reagents also inactivate the zymogen. The site of reaction of bromoacetyl-*N*-methyl-L-phenylalanine with the zymogen was shown to be the same dipeptide, Glu-Phe, which Hass and Neurath (1971b) isolated from the labeled enzyme. Although the site of reaction of Woodward's reagent K with the zymogen has not been identified, it may be assumed to be Glu_{270} also.

Petra (1971) has suggested that the pH dependence of reaction of Woodward's reagent K with carboxypeptidase A is due to the ionization of the carboxyl group of Glu_{270} having a pK_a of 7.0. Hass and Neurath (1971a) have also observed that the pK_a of 7.0 was a property of Glu_{270} itself and not of an ionizable group in its environment. The reaction of the zymogen with Woodward's reagent K is also pH dependent but in comparison to the enzyme, the curve relating pH to inactivation is shifted to a lower pH range, corresponding to a pK_a of 5.0. This lowered pK_a is also consistent with the ascending portion of the pH dependence of peptide hydrolysis by the zymogen (Figure 2). Furthermore, in the zymogen the reactivity of Glu_{270} toward the affinity reagent (bromoacetyl-*N*-methyl-L-phenylalanine) at pH 7.5 is only $\frac{1}{7}$th that in the enzyme.

In the model of crystalline carboxypeptidase A (Lipscomb *et al.*, 1968) the environment of Glu_{270} was examined in detail to detect possible changes in its vicinity that could account for the difference of 2 pK_a units when the enzyme (pK_a = 7.0) is compared to the zymogen (pK_a = 5.0). An increase in environmental polarity or the formation of a hydrogen bond in the zymogen or both could account for the observed changes in pK_a. A stereoscopic plot of the coordinates of the residues in the vicinity of Glu_{270} in the crystal structure of carboxypeptidase A is shown in Figure 10. It is apparent that the segment of the peptide chain containing Glu_{270} is extensively hydrogen-bonded to chain segments on either side. Hence it would be difficult to

extricate Glu_{270} from its hydrophobic environment which presumably confers on it an unusually high pK_a. A more likely proposal would involve the formation of a hydrogen bond between Glu_{270} and another residue within its environment. Such a hydrogen bond would be expected to decrease both the pK_a and the nucleophilicity of Glu_{270}. The disruption of this hydrogen bond during conversion of the zymogen to the enzyme could account for the observed changes in reactivity of this residue.

Closer examination of Figure 10 shows that the nitrogen of the peptide bond between Ser_{197} and Tyr_{198} is in the proper orientation to donate its hydrogen to the OE_2 oxygen of Glu_{270}. Disallowing for uncertainties of published coordinates, this interaction would occur over a distance of 3.10 Å and require an angle of 145° between OE_2 of Glu_{270} and N–C_α of Ser_{197}. Donohue (1968) has demonstrated that in organic crystals, a hydrogen bond angle of the above type can be anywhere between 95 and 175°; the distance of 3.10 Å is within acceptable limits. In carboxypeptidase A this peptide bond between residues 197 and 198 is closer to the cis than to the trans configuration (Hartsuck and Lipscomb, 1971). It is not in a true cis configuration because the two α carbons of residues 197 and 198 are not in the same plane. Consequently, this peptide bond must be in an energetically unfavorable conformation unless it is stabilized by side-chain interactions. The proposed hydrogen bond between N of Tyr_{198} and OE_2 of Glu_{270} would be one such interaction; another would be a hydrogen bond between OH of Tyr_{198} and oxygen of Glu_{43} in the neighboring molecule in the crystal lattice (Figure 10). The latter hydrogen-bond distance of 2.22 Å is shorter than expected for hydrogen bonds of this type (2.5–2.9 Å according to Donohue, 1952) and may be due to small errors in the coordinates. The angle of the phenolic hydroxyl to the carboxyl oxygen and carboxyl carbon is an acceptable 137°. Consequently, these two hydrogen bonds could be of sufficient energy to cause the observed peptide-bond distortion.

If in fact part of the stabilization energy comes from an interaction between molecules in the crystal lattice, the question arises whether the distorted peptide bond and the concomitant hydrogen bond to Glu_{270} can exist *in solution*. Comparison of the catalytic properties of the enzyme in the crystalline state and in solution indicates that they are significantly different, probably due to different conformations of the molecules. The specific activity of the crystalline enzyme toward Z-Gly-Phe is about $1/150$ of that in solution (allowing for the effect of cross-linking on activity) and the inhibition constant for β-phenylpropionate $1/100$th (Quiocho and Richards, 1966).[3] The specific activity of the zymogen toward Z-Gly-Phe and its inhibition by β-phenylpropionate are comparable to those of the crystalline enzyme, suggesting that in both cases the postulated hydrogen bond between Glu_{270} and the peptide nitrogen of Ser_{197}–Tyr_{198} is intact and the ionization of the carboxyl group of Glu_{270} is impeded. Johansen and Vallee (1971, 1973) have examined the spectra of arsanilazotyrosine-248 carboxypeptidase A in the crystalline state and in solution and have concluded that the conformation of the crystals are different from that of their solutions. These conclusions have been disputed by Quiocho *et al.* (1972) and Lipscomb (1973).

In summary, we have shown that the peptide binding site seems to preexist in the zymogen and that the three known catalytic elements of carboxypeptidase A—zinc, tyrosine, and glutamic acid—are involved in zymogen activity as well. The reactivity of one of these elements, Glu_{270}, increases when the zymogen is converted to the enzyme. We suggest that the proteolytic removal of the activation peptide could disrupt its interaction with Tyr_{198} and, in turn, normalize the distorted peptide bond with a concomitant rupture of its hydrogen bond to Glu_{270}. This hypothesis would explain the differences in reactivity of Glu_{270} in the enzyme and the zymogen, the differences in pH optimum, as well as the similarities of the properties of the zymogen in solution and the enzyme in the crystalline form. We believe that this proposal is the simplest one to explain the changes in enzymatic function when PCP A S_5 is converted to carboxypeptidase A.

[3] In a more recent paper it is stated that the peptidase activity (Z-Gly-Phe) of the specific crystals of carboxypeptidase A_α used for X-ray structure analysis was one-third of that in solution (Quiocho *et al.*, 1972).

Acknowledgments

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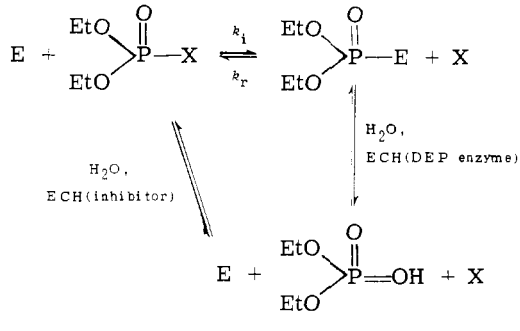
Equilibrium Constants for the Phosphorylation of Acetylcholinesterase by Some Diethyl Phosphorothiolates and Phosphates†

James A. Maglothin and Irwin B. Wilson*

ABSTRACT: Two classes of organophosphorus inhibitors of acetylcholinesterase, phosphorothiolates and phosphates, are compared in terms of their reversible binding with the enzyme, phosphorylating activities, and free energies of hydrolysis. *O,O*-Diethyl *S*-[2-trimethylammonium)ethyl] phosphorothiolate iodide, *O,O*-diethyl *S*-[2-(dimethylammonium)ethyl] phosphorothiolate hydrogen oxalate, *O,O*-diethylphosphorylcholine iodide, and *O,O*-diethyl *O*-[2-(dimethylammonium)ethyl] phosphate hydrogen oxalate display comparable reversible binding affinities for eel acetylcholinesterase, having dissociation constants ranging from 1 to 4×10^{-3} M. The dissociation constant for *O,O*-diethyl *O*-(3,3-dimethylbutyl) phosphate is somewhat greater, 1.1×10^{-2} M. The magnitudes of these dissociation constants are consistent with current knowledge of the factors contributing to molecular complementarity in this system. The phosphorothiolates are potent phosphorylating agents, but no phosphorylating ability is detected for any of the phosphates after removal of active impurities by brief exposure to alkaline solution. The different phosphorylating activities of the phosphorothiolates as compared to the phosphates are consistent with the large differences in the pK_a's of their leaving groups. Rate constants are also obtained for the reverse reaction, the reactivation of the inhibited enzyme by the leaving group. The forward and reverse rate constants allow calculation of the pertinent equilibrium constants for phosphorylation and also the free energies of hydrolysis of the inhibitors. The diethyl phosphorothiolates have free energies of hydrolysis of −23 kcal/mol at pH 7.0 and 25° whereas the values for the diethyl phosphates are ≥−7. The phosphorothiolates are less stable than some similar compounds having leaving groups with even lower pK_a's which suggests that it is appropriate to speak of these compounds as "high-energy compounds" or compounds with a high group transfer potential.

Certain types of organophosphorus compounds are highly potent inhibitors of acetylcholinesterase and other serine esterases. The reaction here illustrated for a diethyl phosphate on the horizontal line is intrinsically reversible although the reaction may in some cases be carried out in an essentially unidirectional manner, either in the forward direction as inhibition, or in the reverse direction as reactivation. In the forward direc-



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