Bovine Procarboxypeptidase A: Kinetics of Peptide and Ester Hydrolysis†

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ABSTRACT: Bovine procarboxypeptidase A exhibits intrinsic hydrolytic activity toward haloacyl amino acids (Behnke and Vallee, 1972), as well as toward conventional peptide and ester substrates for carboxypeptidase A (Bazzone, 1974; Uren and Neurath, 1974). The kinetics of hydrolysis of a series of such substrates by native procarboxypeptidase has now been examined in detail in order to ascertain the extent to which the binding and catalytic sites of carboxypeptidase preexist in the zymogen. Distinct differences in the substrate binding sites of the zymogen compared with the enzyme are apparent from their respective kinetic profiles as well as from the effects of modifiers on their activities. Substrate activation with the dipeptides BzGly-L-Phe and CbzGly-L-Phe, well known for carboxypeptidase, is exhibited also by the zymogen, but the corresponding substrate inhibition by CbzGly-L-Phe and BzGly-OPhe is absent. Moreover, the substrate inhibition of carboxypeptidase by CbzGlyGly-L-Phe and BzGly-OPhe is replaced by substrate activation in the zymogen. Benzoylglycine and cyclohexanol, potent modifiers of carboxypeptidase hydrolysis of peptides, barely affect the corresponding zymogen activities. Additionally, β-phenylpropionate, a noncompetitive inhibitor of carboxypeptidase hydrolysis of peptides, competitively inhibits zymogen peptidase activity. Metal removal and substitution of cobalt for zinc in the zymogen results in effects on activity similar to those for carboxypeptidase, indicating that this component of the catalytic site is relatively unchanged in the process of zymogen activation. On the other hand, the pH dependence of peptidase activity and carboxyl group modification both indicate an altered environment and reactivity for the catalytically essential carboxyl group of Glu-270, consistent with the reduced catalytic efficiency of the zymogen. However, since the hydrolytic efficiency of the zymogen relative to the enzyme varies widely with substrate composition, the difference in carboxyl group reactivity cannot account entirely for the observed differences in kinetic behavior of the zymogen and enzyme. Thus, while the essential features of the binding and catalytic sites of carboxypeptidase preexist in the zymogen, activation results in alterations in both of these active site components to generate the ultimate specificity and capacity of the enzyme.

Studies of bovine procarboxypeptidase A involving metal substitutions, organic modifications, as well as substrate and inhibitor binding, have previously shown that significant features of the active center of carboxypeptidase A, the enzyme to which the zymogen gives rise, already exist in the parent protein (Piras and Vallee, 1967; Freisheim and Neurath, 1967). The virtual identity of the absorption and circular dichroic spectra of the cobalt zymogen and those of the cobalt enzyme led us to conclude that procarboxypeptidase should be enzymatically active in accord with views on the entatic state of metalloenzymes (Vallee and Williams, 1968). Procarboxypeptidase indeed catalyzes the hydrolysis both of haloacyl amino acids and of conventional oligopeptide substrates of the native enzyme (Behnke and Vallee, 1972; Bazzone, 1974; Uren and Neurath, 1974) as well as an analogous ester substrate (Neurath and Walsh, 1970).

A model proposed for substrate and product binding to carboxypeptidase incorporates the consequences of chemical modifications and accounts for the complex kinetic behavior of the enzyme as well as the differential effects of modifiers on the esterase and peptidase activities of the native and chemically modified enzymes (Vallee et al., 1968). It postulates multiple, distinct, but overlapping binding sites for esters and peptides and their hydrolysis products. Detailed kinetic analysis of zymogen-catalyzed hydrolysis of such substrates and comparison with the well-known kinetic properties of carboxypeptidase would provide a sensitive means to evaluate the extent to which the binding and catalytic sites of carboxypeptidase already exist in the zymogen and permit assessment of possible changes in these sites upon proteolytic activation.

We have studied the zymogen-catalyzed hydrolysis of peptide and ester substrates of increasing length and with different N-terminal blocking groups and determined the pertinent kinetic parameters and kinetic anomalies when present (Bazzone, 1974). The zymogen-catalyzed hydrolysis of the N-blocked dipeptides CbzGly-L-Phe and BzGly-L-Phe and the ester analogues BzGly-OPhe and AcGly-OPhe have already been examined (Uren and Neurath, 1974). We have ascertained the effects of known modifiers of carboxypeptidase activity on that of procarboxypeptidase and of metal removal and replacement, pH variation, and chemical modification. The results indicate that procarboxypeptidase activation is accompanied both by alterations in substrate binding and a change in the apparent ionization constant of a catalytically essential residue of the enzyme.

Methods and Materials

Carboxypeptidase A (Anson) and trypsin were obtained from Worthington Biochemical Corp. Bovine pancreatic acetone powder was obtained from Roth Products, Inc. Pro-

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‡ Fellow of the National Cancer Institute, 1972–1974, Grant No. 5F02 CA53002-02.

carboxypeptidase A S_5 was prepared and purified according to the method of Uren and Neurath (1972). Apoprocarboxypeptidase was prepared by dialysis of the native protein against four changes of OP,[1] 0.1 M NaCl–50 mM Tris, pH 7.5, followed by four changes of metal-free buffer without OP.

N-blocked di-, tri-, and tetrapeptides and the esters BzGly-OPhe and BzGlyGly-OPhe, synthesized and characterized as described by Auld and Vallee (1970a), were gifts of Dr. D. S. Auld. Chloroacetyl-L-phenylalanine and trifluoroacetyl-L-phenylalanine were gifts of Dr. C. A. Spilburg. Dichloroacetyl-L-phenylalanine was synthesized from $(Cl_2Ac)_2O$ and L-phenylalanine and recrystallized before use (Greenstein and Winitz, 1961).

Stock peptide substrate solutions were prepared either in 1.0 M NaCl–50mM Tris, pH 7.5, or in 1.0 M NaCl with the pH adjusted to ~6.5. All solutions and buffers used for activity measurements were extracted with 0.001% dithizone in carbon tetrachloride. Metal-free glassware was used throughout to prevent contamination by adventitious metal ions.

Absorbance was measured using a Zeiss PMQ-II spectrophotometer. Metal analyses were performed by atomic absorption spectrometry as described previously (Fuwa et al., 1964).

Sedimentation velocities of purified [(PCPD)Zn] were measured in a Beckman Model E Ultracentrifuge; solvent viscosity was measured by using an Ostwald viscometer and solvent density by pycnometry at 20.0 °C. A partial specific volume of 0.732 ml/g was used to calculate $s_{20,w}$ for the zymogen.

Protein concentrations in solution were determined from absorbance at 280 nm using $E_{280}^{0.1\%} = 1.77$ for [(PCPD)Zn], assuming a molecular weight of 6.3×10^4 daltons (Uren and Neurath, 1972), and at 278 nm using $E_{280}^{0.1\%} = 1.88$ and a molecular weight of 3.46×10^4 daltons for carboxypeptidase A (Simpson et al., 1963). Stock zymogen solutions were made 0.1–1.0 mM in DFP to prevent proteolytic activation. After treatment with DFP, the solutions were dialyzed overnight against buffer not containing DFP. Solutions prepared in this manner reproducibly exhibited a turnover number toward CbzGly-L-Phe (20 mM) of 90 ± 15 min^{-1} for a period of at least 2 weeks. For a given dilution of stock zymogen, control assays using 20 mM CbzGly-L-Phe were performed at the end of each set of samples in order to assure that zymogen activation did not occur. Fresh dilutions were made each day just prior to assay.

Carboxypeptidase A crystals were washed three times with metal-free distilled water and dissolved in 2.0 M NaCl–50 mM Tris, pH 7.5, to result in a stock solution of approximately 0.14 mM. An intermediate ten-fold dilution was made from this solution to determine protein concentration. A further dilution was made just prior to assay.

All peptidase assays at pH 7.5 were carried out in 1.0 M NaCl–50 mM Tris, 25 ± 0.1 °C using the automated ninhydrin assay of Auld and Vallee (1970a). Peptidase assays of [(PCPD)Co] were carried out in 0.1 mM Co^{2+} to ensure cobalt zymogen formation.

Esterase assays were carried out in 0.2 M NaCl at pH 7.5 using a Radiometer ABU 12 autoburet employing 2 mM NaOH under a nitrogen atmosphere. Assays were carried out at 25 ± 0.1 °C in a jacketed, thermostated vessel.

At all pH values studied, the zymogen was found to be stable at the concentrations used in assays for at least twice the assay time interval.

Mes, Hepes, and Tris buffers were used to determine the pH rate profiles. The effect of the buffer on the reaction rate was examined by choosing a substrate concentration near K_m and determining the rate of hydrolysis in the presence of 10, 50, and 100 mM buffer at pH 6.4 for Mes, 7.2 for Hepes, and 7.5 for Tris. None of the buffers affected the rate of hydrolysis of BzGlyGly-L-Phe appreciably.

The pH before and after assays for the pH–rate studies was measured with a Kruger and Eckels Model 130 digital pH meter equipped with a Radiometer GK 2321C electrode. The meter was standardized at 25 °C with Harleco pH 7.0 and pH 10.0 reference buffers. Using this procedure, the pH 4.0 reference buffer read within 0.04 pH unit of the stated value. The drift in pH during an assay never exceeded 0.05 unit. At pH values below 6.0, activity measurements were made in the presence of 0.1 mM Zn^{2+} to counteract any pH-dependent loss of metal (Piras and Vallee, 1967).

Carboxyl group modification was carried out by incubating the zymogen (0.55 mg/ml) with CMC, 20 mM, at pH 5.5, 6.0, and 6.5 for 60 min at 0 °C. Aliquots (25 μl) were then quenched by dilution into 100 μl of 50 mM Tris–1.0 M NaCl, pH 7.5, 0 °C. For peptidase assays with 20 mM CbzGly-L-Phe these samples were further diluted tenfold. Modifications were performed in the presence of excess Zn^{2+} (20 μM) to prevent loss of zinc from the zymogen.

The time-dependent inhibition of the zymogen (0.36 μM) by OP was followed by incubation with 2 mM chelating agent in 1.0 M NaCl–50 mM Tris, pH 7.5 at 25 °C (final volume 1.9 ml). Peptidase assays (10 mM BzGly-L-Phe) were performed in 2 mM OP, 1.0 M NaCl–50 mM Tris, pH 7.5, 25 °C, by addition of 0.1 ml of 0.2 M BzGly-L-Phe to the incubation mixture after various incubation times.

Results

Physical and Enzymatic Characteristics of [(PCPD)Zn]. The native zymogen exhibits a single, symmetrical peak in the ultracentrifuge with an $s_{20,w}$ of 5.0 (5.9 mg/ml) corresponding to the two subunit S_5 form (Uren and Neurath, 1972). The zymogen does not bind to an affinity chromatographic column designed to isolate carboxypeptidase A (Peterson and Sokolovsky, 1974); it elutes as a single peak with a specific activity identical with that of the protein solution applied. Thus the exopeptidase activity is intrinsic to the zymogen and not due to possible contaminating activation products.

[(PCPD)Zn] exhibits intrinsic exopeptidase activity toward carboxypeptidase A substrates (Table I). It is inactive toward the chymotrypsin substrate acetyltyrosine ethyl ester, peptides having a C-terminal D-amino acid and the carboxypeptidase B substrate hippuryl-L-arginine.

Kinetics of Hydrolysis of Haloacyl Amino Acids. The substrates chosen for study include haloacylated amino acids (Table I), the simplest and one of the earliest known classes of substrates for carboxypeptidase A (Neurath and Schwert, 1950). [(PCPD)Co] hydrolyzes these substrates with turnover numbers which are high relative to those for native carboxypeptidase A (Behnke and Vallee, 1972).

[1] Abbreviations used are: DFP, diisopropyl phosphofluoridate; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; OP, *o*-phenanthroline; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethyl-piperazine-*N*-2′-ethanesulfonic acid; [(PCPD)Zn] and [(CPD)Zn], native procarboxypeptidase and carboxypeptidase, respectively; [(PCPD)Co] and [(CPD)Co], cobalt-substituted procarboxypeptidase and carboxypeptidase, respectively.

Table I: Inherent Peptidase and Esterase Activity of [(PCPD)Zn].

Substrate	V/e^a (min^{-1}) [PCPD]Zn
Peptide	
F_3Ac-L-Phe	196
ClAc-L-Phe	60
Cl_2Ac-L-Phe	43
BzGly-L-Phe	70
BzGlyGly-L-Phe	10
BzGlyGlyGly-L-Phe	16
CbzGly-L-Phe	24
CbzGlyGly-L-Phe	45
CbzGlyGlyGly-L-Phe	36
Ester	
BzGly-L-OPhe	135
BzGlyGly-L-OPhe	50

[a] Activities measured at 1 mM substrate. See Methods for conditions.

Native zymogen-catalyzed hydrolyses of chloroacetyl-L-phenylalanine, dichloracetyl-L-phenylalanine, and trifluoroacetyl-L-phenylalanine follow normal Michaelis–Menten kinetics over the concentration range from 0.7 to 10 mM. The k_{cat} value of the zymogen for chloroacetyl-L-phenylalanine is nearly 20% of that of the native enzyme, the highest relative k_{cat} value observed for any peptide substrate we have studied (Table II). The K_m values for the zymogen and the enzyme are virtually identical. However, addition of a second chlorine atom at the α-carbon of the N-acyl group results in a decrease in both k_{cat} and K_m for the zymogen while, for the enzyme, both parameters increase. Dichloroacetyl-L-phenylalanine is the only substrate examined with which the zymogen K_m value is significantly lower than that for the enzyme.

For both the zymogen and the enzyme, the k_{cat} values for trifluoroacetyl-L-phenylalanine are higher than for either of the other haloacyl substrates, consistent with the inductive polarization caused by the fluorine atoms. Thus, even though trifluoroacetyl-L-phenylalanine is hydrolyzed by the zymogen at 4% of the rate of the native enzyme, it is an excellent substrate for both, characterized by high turnover rates and normal Michaelis–Menten kinetics.

Kinetics of Hydrolysis of N-Blocked Oligopeptides. The N-blocked dipeptides, CbzGly-L-Phe and BzGly-L-Phe, have long served as standard dipeptide substrates for carboxypeptidase A. Their enzyme-catalyzed hydrolysis is characterized by striking kinetic anomalies, apparent in the nonlinear profiles of their double reciprocal plots (Davies et al., 1968a). For the native enzyme, their Gly_2- and Gly_3-homologues follow normal Michaelis–Menten kinetics allowing unequivocal delineation of kinetic parameters in contrast to the dipeptides (Auld and Vallee, 1970b). The kinetics of the zymogen-catalyzed hydrolysis of all these substrates was therefore examined to compare both the characteristics of the substrate binding site of the zymogen with those previously observed for the enzyme and the catalytic efficiency of the zymogen with that of carboxypeptidase as a function of substrate size and the nature of the blocking group.

Double reciprocal plots for the zymogen-catalyzed hydrolysis of BzGly-L-Phe and BzGlyGly-L-Phe are qualitatively similar to those previously observed for carboxypeptidase; at concentrations of BzGly-L-Phe higher than 1 mM, both zymogen and enzyme are subject to substrate activation, as indicated by the increase in slope of the V^{-1} vs. S^{-1} profiles. With BzGlyGly-L-Phe both the zymogen and the enzyme exhibit normal Michaelis–Menten kinetics over the same concentration range studied for BzGly-L-Phe. For both substrates the k_{cat} and K_m values of the zymogen differ significantly from those of the enzyme (Table III).

Table II: Comparison of Kinetic Parameters[a] for [(CPD)Zn] and [(PCPD)Zn] with Haloacylamino Acids.

Substrate	[(PCPD)Zn] k_{cat}	[(PCPD)Zn] K_m	[(CPD)Zn][b] k_{cat}	[(CPD)Zn][b] K_m
ClAc-L-Phe	154	1.7	860	1.2
Cl_2Ac-L-Phe	66	0.5	5 000	10
F_3Ac-L-Phe	870	3.5	20 000	1.7

[a] Units of k_{cat} and K_m are min^{-1} and M $\times$ 10^3. [b] Data for ClAcPhe and Cl_2AcPhe from Behnke and Vallee (1972); data for F_3AcPhe from this work.

The kinetic profiles for zymogen-catalyzed hydrolysis of the N-carbobenzoxy di- and tripeptides differ qualitatively from those of carboxypeptidase. Above 2 mM CbzGly-L-Phe both the native enzyme and the zymogen exhibit substrate activation (Figure 1). However, above 40 mM substrate the native enzyme also exhibits substrate inhibition which is not apparent for the zymogen up to 0.1 M substrate. Such substrate inhibition is more marked for cobalt carboxypeptidase and occurs above 20 mM (Davies et al., 1968a); the cobalt zymogen on the other hand is not inhibited up to 50 mM.

Similarly, over the substrate concentration range from 0.2 to 20 mM, native carboxypeptidase hydrolyzes CbzGlyGly-L-Phe with normal Michaelis–Menten kinetics, while inhibition appears at higher substrate concentration (Auld and Vallee, 1970b). On the other hand, the zymogen exhibits normal Michaelis–Menten kinetics for the hydrolysis of this substrate over the range from 0.6 to 3 mM with substrate activation becoming apparent thereafter (Figure 2). Inhibition of the zymogen was not observed up to 90 mM substrate.

The extrapolated K_m values of zymogen- and enzyme-catalyzed hydrolysis of CbzGly-L-Phe are approximately the same, while those of k_{cat} for the zymogen are only about 1% of those of the enzyme in both regions of the biphasic profile. K_m and k_{cat} for both species with CbzGlyGly-L-Phe differ markedly.

Zymogen-catalyzed hydrolysis of the tetrapeptide BzGlyGly-L-Phe follows normal Michaelis–Menten kinetics over the substrate concentration range from 0.25 to 10 mM. Similarly, the corresponding N-carbobenzoxy tetrapeptide CbzGlyGlyGly-L-Phe obeys Michaelis–Menten kinetics in the range from 0.3 to 5 mM. Again both k_{cat} and K_m for the zymogen differ from the corresponding enzyme values.

Kinetic Parameters for Esters. The kinetics of zymogen-catalyzed hydrolysis of BzGly-OPhe are normal over the concentration range from 0.7 to 5 mM, with substrate activation becoming apparent thereafter up to 40 mM (Figure 3). In marked contrast, carboxypeptidase exhibits extreme substrate inhibition above 5 mM, (McClure et al., 1964; Davies et al., 1968a). The corresponding kinetic parameters for the linear regions at low substrate concentration differ markedly: the k_{cat} value for the zymogen, 600 min^{-1}, is only about 2% of that of the enzyme, while the K_m value (3.6 mM) is some 40 times greater (Table IV).

Zymogen-catalyzed hydrolysis of BzGlyGly-OPhe follows Michaelis–Menten kinetics over the range from 0.9 to

Table III: Comparison of Kinetic Parameters[a] for [(PCPD)Zn] and [(CPD)Zn] with Peptide Substrates.

Substrate	[(PCPD)Zn]				[(CPD)Zn][b]			
	Low [S][c]		High [S]		Low [S][c]		High [S]	
	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m
CbzGly-L-Phe	61	1.1	150	5.0	5460	1.95	10 080	5.9
BzGly-L-Phe	213	1.8	890	13.0	5520	0.81	8 700	2.7
CbzGlyGly-L-Phe	99	1.1	450	16.0			8 000	0.25
BzGlyGly-L-Phe			45	4.0			1 200	0.81
CbzGlyGlyGly-L-Phe			45	2.2			3 300	0.91
BzGlyGlyGly-L-Phe			140	8.3			2 600	3.7

[a] Units of K_m and k_{cat} are M × 10^3 and min^{-1}, respectively. [b] Values for [(CPD)Zn] taken from the literature: CbzGly-L-Phe and BzGly-L-Phe. (Davies et al., 1968a); BzGlyGly-L-Phe, CbzGlyGly-L-Phe, and CbzGlyGlyGly-L-Phe (Auld and Vallee, 1970a); BzGlyGlyGly-L-Phe (Auld and Holmquist, 1974). [c] Where values are not given for low [S], kinetics were linear over entire range. See text for concentration ranges.

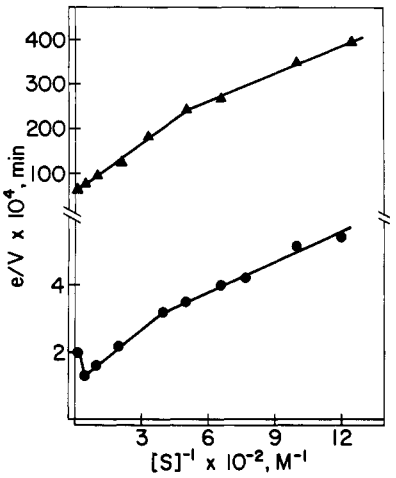

FIGURE 1: Double reciprocal profiles for the hydrolysis of CbzGly-L-Phe by [(PCPD)Zn] (▲) and [(CPD)Zn] (●) in 1 M NaCl, 50 mM Tris, pH 7.5, 25 °C.

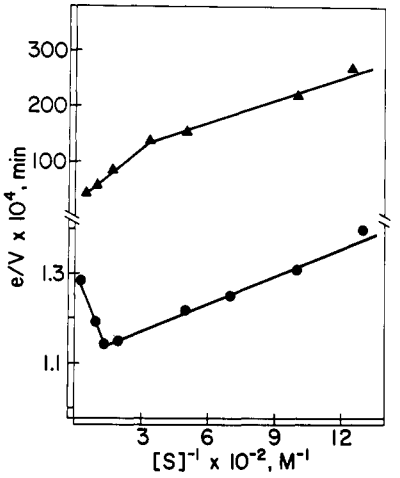

FIGURE 2: Double reciprocal profiles for the hydrolysis of CbzGlyGly-L-Phe by [(PCPD)Zn] (▲) and [(CPD)Zn] (●). Assay conditions as in Figure 1.

13 mM. The k_{cat} value for the zymogen, 260 min^{-1}, is about 1% that for carboxypeptidase, while the K_m value (5 mM) is about 15-fold greater (Auld and Holmquist, 1974; Table IV).

Effects of Modifiers on Peptidase and Esterase Activity of Procarboxypeptidase. Various agents such as hydrophobic alcohols as well as products of dipeptide hydrolysis activate carboxypeptidase-catalyzed dipeptide hydrolysis but inhibit that of depsipeptides (Davies et al., 1968b). In contrast, with the zymogen, increasing concentrations of either cyclohexanol or benzoylglycine perturb activity toward CbzGly-L-Phe minimally. Benzoylglycine inhibits the esterase activity of the zymogen toward BzGly-OPhe (V/V_0 = 0.50 at 50mM benzoylglycine); this inhibitory effect is virtually identical with that on esterase activity of carboxypeptidase under the same conditions (Davies et al., 1968b). Cyclohexanol also inhibits hydrolysis of BzGly-OPhe but, at comparable concentrations, its effect on the zymogen is only about half of that on carboxypeptidase.

Inhibition by β-Phenylpropionate. β-Phenylpropionate competitively inhibits the zymogen-catalyzed hydrolysis of BzGlyGly-L-Phe with a K_i of 0.4 mM, in marked contrast to its noncompetitive inhibition of carboxypeptidase-catalyzed hydrolysis under these conditions (K_i of 0.1–0.2 mM; Auld and Vallee, 1970a). The inhibition of BzGlyGly-OPhe hydrolysis is competitive in both cases with K_i's of 0.5 and 0.12 mM (Auld and Holmquist, 1974) for the zymogen and enzyme, respectively.

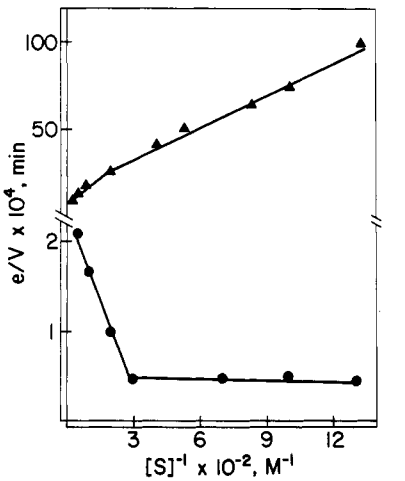

FIGURE 3: Double reciprocal profiles for the hydrolysis of BzGly-OPhe by [(PCPD)Zn (▲) and [(CPD)Zn] (●) in 0.2 M NaCl, pH 7.5, 25 °C.

Removal and Replacement of Metal from the Zymogen. The essentiality of the zinc atom for catalytic activity of the zymogen was also investigated. Incubation with 2 mM OP at 25 °C, pH 7.5, results in time-dependent inhibition, complete within 2 h. Under similar conditions, carboxypeptidase is inhibited completely in about 10 min (Felber et al., 1962). Removal of the chelating agent by dialysis simulta-

Table IV: Kinetic Parameter Ratios For [(PCPD)Zn] and [(CPD)Zn] with Peptides and Esters.

Substrate	$k_{cat}\frac{[(PCPD)Zn]}{[(CPD)Zn]}$	$K_m\frac{[(PCPD)Zn]}{[(CPD)Zn]}$	$\frac{(k_{cat}/K_m)\,[(CPD)Zn]}{(k_{cat}/K_m)\,[(PCPD)Zn]}$
Peptide			
ClAc-L-Phe	0.18	1.5	8.3
Cl_2Ac-L-Phe	0.01	0.05	5
F_3Ac-L-Phe	0.04	2.0	50
BzGly-L-Phe	0.04	2.2	55
BzGlyGly-L-Phe	0.04	4.8	120
BzGlyGlyGly-L-Phe	0.05	2.1	42
CbzGly-L-Phe	0.01	0.6	60
CbzGlyGly-L-Phe	0.01	5.0	500
CbzGlyGlyGly-L-Phe	0.01	2.1	210
Ester			
BzGly-L-OPhe	0.02	47.0	2300
BzGlyGly-L-OPhe	0.01	17.0	1900

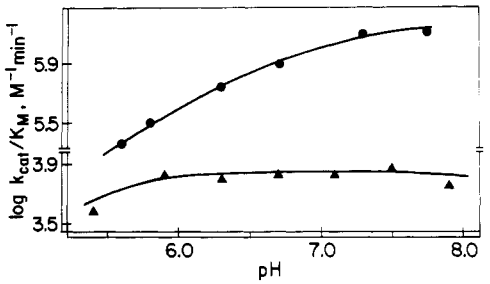

FIGURE 4: The pH–rate profiles for the hydrolysis of BzGlyGly-L-Phe by [(PCPD)Zn] (▲) and [(CPD)Zn] (●). Assays were performed in 50 mM Mes, Hepes, or Tris buffer in 1 M NaCl, at 25 °C.

neously removes the metal and <0.01 g-atom of Zn/mol remains thereafter. The resultant apoenzyme is inactive but can be reactivated by zinc.

Substitution of Co^{2+} for Zn^{2+} in the zymogen increases k_{cat} but does not appreciably affect K_m for dipeptides. With CbzGly-L-Phe the profile of the double reciprocal plot for [(PCPD)Co] is biphasic, and substrate activation becomes apparent above 2 mM. The kinetic parameters extrapolated from the linear region at low substrate concentration are 134 min^{-1} for k_{cat} and 1.1 mM for K_m, while the corresponding values from the region at high substrate concentration are 200 min^{-1} and 3.3 mM. Similarly, with BzGly-L-Phe, the profile is biphasic with substrate activation apparent above 2 mM. The kinetic parameters extrapolated at low substrate concentration are 323 min^{-1} for k_{cat} and 2.0 mM for K_m, while, at high substrate concentration, the corresponding values are 1428 min^{-1} and 15 mM.

Carboxyl Group Modification. Inactivation of carboxypeptidase with the water-soluble carbodiimide, CMC, correlates with the modification of a single carboxyl residue with an apparent pK_a for the inactivation of 7.0 (Riordan and Hayashida, 1970); the rate of inactivation increases as pH decreases. Similarly, exposure of the zymogen to 20 mM CMC at 0 °C for 1 h results in progressive loss of peptidase activity as the pH decreases (Table V) but at a given pH the extent of inactivation is far less than for carboxypeptidase under the same conditions.

pH Dependence of Peptide Hydrolysis. Over the pH range from 5.4 to 7.9, double reciprocal plots for zymogen-catalyzed hydrolysis of BzGlyGly-L-Phe are linear over at least a tenfold concentration range bracketing K_m. At pH values below pH 6.0, Zn^{2+} must be added in excess to assay mixtures so that full activity is maintained. Variations in log k_{cat}/K_m as a function of pH for the zymogen and enzyme are compared in Figure 4. The pH profile for the zymogen is flat from pH 5.9 to 7.5 while, in contrast, that of the enzyme falls off rapidly over this pH range (Auld and Vallee, 1970b).

Table V: pH Dependence of Inactivation of [(CPD)Zn] and [(PCPD)Zn] by CMC.

pH	$V/V_c \times 100$ [(CPD)Zn] [a]	$V/V_c \times 100$ [(PCPD)Zn]
6.5	40	95
6.0	5	88
5.5	0	80

[a] Data from Riordan and Hayashida (1970).

Discussion

Zinc and cobalt substituted procarboxypeptidases exhibit intrinsic catalytic activity both toward haloacylamino acids (Behnke and Vallee, 1972), and the more conventional peptide and ester substrates of carboxypeptidase (Table I). Compared with carboxypeptidase, the zymogen is much less active, though the degree to which this is true varies with the substrate. Detailed kinetics of zymogen-catalyzed hydrolysis of all substrates in Table I were examined not only to determine whether the reduced catalytic rates of the zymogen are due to weakened binding, less efficient catalysis, or both, but also to investigate the extent to which the zymogen exhibits kinetic anomalies known for carboxypeptidase A.

The size and minimal number of determinants required for the binding of the haloacylamino acids suggest that they might be better substrates for the zymogen than the larger N-blocked peptide substrates of carboxypeptidase. However, detailed comparison of kinetic parameters for zymogen- and enzyme-catalyzed hydrolysis of conventional carboxypeptidase substrates and haloacylamino acids shows that factors other than size and structural complexity must contribute significantly to the relative catalytic efficiency of the zymogen (Table IV). Thus, for example, despite their marked differences in size and composition, BzGly-L-Phe, BzGlyGlyGly-L-Phe, and F_3Ac-L-Phe exhibit virtually identical k_{cat} and K_m ratios.

Kinetic anomalies, apparent from the nonlinearity of the double reciprocal profiles, characterize the carboxypeptidase-catalyzed hydrolysis of the dipeptides BzGly-L-Phe and CbzGly-L-Phe (Figure 1) and of the ester BzGly-OPhe (Figure 3) (Davies et al., 1968a). Multiple productive and

nonproductive substrate binding modes resulting in either substrate activation or inhibition have been postulated to account for this complex behavior (Vallee et al., 1968). The zymogen also exhibits complex kinetic behavior, consistent with multiple substrate binding modes, but which differ in kind and degree from that previously observed for carboxypeptidase. Thus, while both exhibit substrate activation with CbzGly-L-Phe and BzGly-L-Phe, the anomalies with the zymogen are more marked. This is apparent from the differences in k_{cat} and K_m extrapolated from the low and high substrate concentration regions. In addition, the marked substrate inhibition of carboxypeptidase by CbzGly-L-Phe above 40 mM is completely absent in the zymogen, even at a concentration 20-fold above the K_m value extrapolated from the high substrate region. Moreover, for CbzGlyGly-L-Phe, marked substrate inhibition of the enzyme replaces the substrate activation of the zymogen (Figure 2). Such differences indicate that the binding sites of the two proteins for these peptides are not identical. Binding site differences are also evident both from the reversal of the kinetic anomaly of the zymogen for the ester BzGly-L-OPhe and the marked differences of k_{cat} and K_m of the zymogen and enzyme for both peptides and esters (Table IV).

Comparison of kinetic parameters for acylamino acids, peptides, and depsipeptides, extrapolated from the region of low substrate concentration for those substrates which exhibit nonlinear kinetics, reveals that, relative to carboxypeptidase, the maximal rates for zymogen-catalyzed hydrolysis are reduced (Table IV, column 1). In contrast, the K_m values for the zymogen are generally higher (Table IV, column 2). Differences in substrate activation and inhibition of the two proteins are likely reflected in the K_m values so that their comparison may not be physically meaningful. Comparison of the ratio (k_{cat}/K_m) for the enzyme to that for the zymogen with the various substrates may minimize these effects by correcting for nonproductive binding modes. Such a comparison (Table IV, column 3) reveals that the relative catalytic efficiency of the zymogen varies considerably over the range of peptide and ester substrates studied, as a function both of length and of blocking group. Moreover, for the esters BzGly-OPhe and BzGlyGly-OPhe, (k_{cat}/K_m) values of enzyme relative to zymogen are 50- and 15-fold higher, respectively, than those of their corresponding peptide analogues, primarily due to the much higher relative K_m values of the ester–zymogen pairs. The latter suggest that, in the process of activation, the binding site for esters in the zymogen undergoes more extensive alterations than that for peptides.

Given a particular C-terminus of peptide substrates, variations in the amino acid composition of the remainder of the substrate are known to affect both k_{cat} and K_m for carboxypeptidase, extending up to at least five amino acid residues and a blocking group (Abramowitz et al., 1967). The data in Table III demonstrate similar sensitivity of the kinetic parameters of the zymogen and are indicative of analogous extension of the contact areas. However, changes in substrate composition affect the kinetic parameters of the zymogen differently from those of the enzyme (Tables III and IV), suggesting that substrate recognition subsites differ either in kind or in relative spatial orientation in the two proteins.

The multiple site model for substrate binding to carboxypeptidase accounts for the effects of various hydrophobic alcohols and products of hydrolysis on peptidase and esterase activities (Vallee et al., 1968). Thus binding of cyclohexanol or benzoylglycine to carboxypeptidase prevents peptide substrate inhibition by blocking nonproductive peptide binding without interfering with productive binding, and leading to net activation. At the same time these agents can prevent productive binding of the ester resulting in inhibition since the ester binding site, while separate from, nevertheless overlaps that for the peptide. The minimal effects of these agents on the peptidase activity of the zymogen are thus consistent with significant alteration in the inhibitory binding mode for dipeptides. Importantly, the corresponding effects of these agents on esterase activity of the zymogen indicate that their minimal effect on peptidase activity is not due to lack of binding of the modifiers. The different effects of benzoylglycine on peptide and ester hydrolysis, observed for both the zymogen and enzyme, are consistent with separate but overlapping modes of ester and peptide binding (Vallee et al., 1968) in both proteins; moreover, the proposed differences in mechanism of carboxypeptidase-catalyzed hydrolysis of esters and peptides likely extend to the zymogen (Auld and Holmquist, 1974).

Substrate activation and inhibition of carboxypeptidase which are observed with N-blocked dipeptides and the ester BzGly-OPhe preclude a physical interpretation of K_m and k_{cat} since, in these instances, the constants likely represent composite terms. This problem has been overcome through use of the tripeptide homologues of BzGly-L-Phe and CbzGly-L-Phe, i.e., BzGlyGly-L-Phe and CbzGlyGly-L-Phe (Auld and Vallee, 1970a). Neither of these tripeptide substrates exhibits substrate activation with carboxypeptidase and, hence, both the extrapolated kinetic parameters and the effects of inhibitors and pH on activity may be interpreted unambiguously since multiple substrate binding modes have not been detected. Since both procarboxypeptidase and carboxypeptidase display normal Michaelis–Menten kinetics for the hydrolysis of the tripeptide BzGlyGly-L-Phe over a wide concentration range, this substrate was chosen to compare the effects of the inhibitor β-phenylpropionate as well as pH on zymogen and enzyme activity.

β-Phenylpropionate competitively inhibits the zymogen-catalyzed hydrolysis of BzGlyGly-L-Phe but the inhibition of the enzyme is noncompetitive. This might be due either to different binding modes of substrate, of β-phenylpropionate or of both. The similarity of K_i values for the zymogen and enzyme with this inhibitor together with the nearly identical effects of β-phenylpropionate on the spectral properties of cobalt procarboxypeptidase and cobalt carboxypeptidase and on their hydrolysis of chloroacetyl-L-phenylalanine (Behnke and Vallee, 1972) strongly suggest similar modes of inhibitor binding to both proteins. The difference in type of zymogen and enzyme inhibition therefore suggests different modes of binding to the zymogen and enzyme for BzGlyGly-L-Phe.

Over and above the differences in the substrate binding sites of the zymogen and the enzyme, the pH–rate profiles show significant differences in the pK_a of a catalytically essential residue. In carboxypeptidase a residue ionizing in the pH region from 5 to 7 is involved in activity, apparently the carboxyl group of glutamic acid 270 (Riordan and Hayashida, 1970; Petra, 1971; Petra and Neurath, 1971; Hass and Neurath, 1971a,b; Nau and Riordan, 1974), consistent with the kinetic studies of the native enzyme (Auld and Vallee, 1970b). On the other hand, the pH–activity profile of the zymogen, which falls off only below pH 5.9 (Figure 4), suggests a lower pK_a for the carboxyl group. The greatly reduced extent of inactivation of the zymogen by the carbo-

diimide reagent (Table V) is consistent with this view. These conclusions are entirely analogous to those reached recently based on chemical modification of Glu-270 in [(PCPD)Zn] with both Woodward's reagent K and *N*-bromoacetyl-*N*-L-phenylalanine (Uren and Neurath, 1974).

If the reduced catalytic efficiency of the zymogen were due solely to the change in the environment of a single catalytically essential residue, its catalytic efficiency relative to that of the enzyme might be expected to be constant, regardless of the nature of the substrate. This is clearly not the case (Table IV, column 3). Thus, while activation of the zymogen might be due, in part, to changes in the reactivity of Glu-270, alterations in the substrate binding site may also contribute significantly to the catalytic capacity of carboxypeptidase. In this respect binding to the zymogen of the affinity reagent *N*-bromoacetyl-*N*-methyl-L-phenylalanine is four- to fivefold weaker than to the enzyme, consistent with an altered binding site (Uren and Neurath, 1974). This, coupled with the sevenfold increase in $t_{1/2}$ for inactivation at a saturating *N*-bromoacetyl-*N*-methyl-L-phenylalanine concentration suggests that the reagent binds less favorably for interaction with Glu-270. Hence, conformational changes upon substrate binding, critical for efficient catalysis in the zymogen, may be hindered, precluding proper substrate recognition.

While the differences in substrate binding to zymogen and enzyme observed here are predominantly in nonproductive modes, they are nevertheless consistent with the conformational changes previously detected spectrally upon zymogen activation (Behnke and Vallee, 1971). Such changes appear to be related to changes in the substrate binding characteristics and in the reactivity of at least one of the catalytically essential residues of carboxypeptidase. The extent to which these changes are interrelated and of importance to the increase in catalytic efficiency upon zymogen activation cannot be judged with certainty from data presently available.

The metal atom is essential for the catalytic activity of the zymogen, as is apparent from the functional consequences of its removal and replacement. Moreover, the changes in k_{cat} due to cobalt substitution closely parallel those observed for carboxypeptidase (Davies et al., 1968a; Auld and Vallee, 1970a; Auld and Holmquist, 1974). The metal binding sites of both proteins are also closely similar, as is evident from the spectral properties of the two cobalt-substituted proteins (Behnke and Vallee, 1972). Thus, the reduced catalytic efficiency of the zymogen does not appear to result from alterations in this component of the catalytic site.

Even prior to activation the zymogen seems to exhibit the essential features of the active site of carboxypeptidase, albeit in altered form. Moreover, like crystalline carboxypeptidase, the zymogen exhibits reduced catalytic rates relative to carboxypeptidase in solution. Hence it might be tempting to interpret the catalytic properties of the zymogen in terms of the mechanism of action of the enzyme deduced from its three-dimensional crystal structure. However, comparison of the detailed kinetic behavior of carboxypeptidase in the crystalline state (Spilburg et al., 1974) with that observed here for the zymogen reveals that both the pertinent kinetic profiles and the extrapolated kinetic parameters differ markedly throughout. Although it is not possible to compare directly individual kinetic parameters of the crystalline, cross-linked enzyme with those of the zymogen for all of the substrates owing to varying degrees of kinetic anomalies in the two cases, kinetics of hydrolysis of the ester, BzGlyGly-OPhe, are normal for the zymogen and the enzyme in the crystalline state and, hence, a reasonable comparison can be made for this case. For crystalline [(CPD)Zn], k_{cat} is 30 min^{-1} and K_m is 0.3 mM (Spilburg et al., 1974). For [(PCPD)Zn], on the other hand, k_{cat} is 260 min^{-1} and K_m is 5.0 mM, both markedly different from the corresponding parameters of the enzyme crystals. Thus, the factors underlying the reduced catalytic activity of the zymogen and crystalline carboxypeptidase apparently are not the same, and mechanistic deductions for the zymogen from crystalline carboxypeptidase would not seem valid.

A number of recent studies have shown that zymogens need not be catalytically inert. In fact, they exhibit many of the features of the active site essential for the catalytic properties of the resultant enzymes. As illustrated in the present study, comparisons of the catalytic behavior of a zymogen and of its product enzyme serve as indices of the functional consequences incident to alterations in primary, secondary, and tertiary structure. Indeed, detailed investigation of the catalytic behavior of other zymogens relative to that of their product enzymes should provide valuable new information for the elucidation both of the mechanism of enzyme action and zymogen activation.

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Subunit Dissociation of Mitochondrial Malate Dehydrogenase†

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ABSTRACT: Fluorescence polarization studies of porcine mitochondrial malate dehydrogenase labeled with fluorescein isothiocyanate or fluorescamine indicated a concentration-dependent dissociation of the dimeric molecule with a K_D of 2×10^{-7} N at pH 8.0. These results were confirmed by the concentration dependence of the stability of the enzyme at elevated temperatures and the creation of hybrid molecules with fluorescein and Rhodamine B labeled subunits, in which energy transfer was observed. The binding of NADH resulted in a small shift of the subunit dissociation curve toward monomer, demonstrating that monomer has twice the affinity for reduced coenzyme. NAD^+ binding prevented dissociation of the dimer, even at concentrations below 10^{-8} N. These results indicate that binding of reduced or oxidized coenzymes results in different conformation changes, which are transferred to the subunit interface.

Malate dehydrogenase (EC 1.1.1.37) from porcine heart mitochondria is a dimeric enzyme (Devenyi et al., 1966) with a molecular weight of 70 000 (Thorne and Kaplan, 1963), able to bind two molecules of NADH per dimer (Pfleiderer and Auricchio, 1964). Considering the anomalous kinetics reported for the enzyme (Harada and Wolfe, 1968) and our experience of instability of dilute solutions of the enzyme, the possibility of a monomer–dimer equilibrium was studied. The report of a monomer–dimer equilibrium for beef heart cytoplasmic malate dehydrogenase (Cassman and King, 1972) provided further interest in evaluating this possibility. Fluorescence techniques provided convenient and rapid methods for demonstrating and studying this equilibrium.

The polarization of fluorescence of a protein molecule covalently labeled with a fluorescent dye provides a sensitive method for the determination of rotational relaxation times (Weber, 1953), which would be expected to change with the degree of association of enzyme subunits. It has also been reported that protein association can be studied by singlet energy transfer between species labeled with different chromophores (Gennis et al., 1972). These techniques can be used to demonstrate the equilibrium between monomer and dimer and to determine the effects of coenzyme binding.

In the present study, fluorescence polarization of mitochondrial malate dehydrogenase covalently labeled with fluorescent dyes was determined as a function of enzyme concentration in the presence and absence of coenzymes. In addition, hybrid dimeric enzyme was created using donor–acceptor dye pairs and was used to evaluate the effects of NADH binding on the subunit dissociation.

Materials and Methods

Coenzymes and substrates were purchased from Sigma Chemical Company and used without further purification. Pig heart mitochondrial malate dehydrogenase was purchased from Miles-Seravac (Batch 95 AB) and exhibited maximum fluorescence at 307 nm when excited at 280 nm, indicating the absence of tryptophan-containing proteins. Enzyme activity was determined by the method of Gregory et al. (1971). The concentration of active enzyme was determined by titration with NADH in the presence of hydroxymalonate at pH 6.4 by the method of Holbrook and Wolfe (1972).

The reagents used for fluorescence labeling of the enzyme were fluorescein isothiocyanate on Celite purchased from Calbiochem, Rhodamine B isothiocyanate purchased from Sigma Chemical Company, and fluorescamine purchased from Roche diagnostics. Fluorescein-labeled enzyme was prepared by adding 1 mg of fluorescein isothiocyanate on Celite to 4 ml of 30 μN enzyme in 0.05 M (pH 8.0) Tris-acetate buffer and stirring for 0.5 h at 4 °C. The amount of fluorescein labeling was determined by the absorbance at 490 nm, using an extinction coefficient of 3.4×10^4 $l.^{-1}$ M cm^{-1} (Churchich, 1967). The preparations consistently

† From the Edsel B. Ford Institute, Henry Ford Hospital, Detroit, Michigan 48202. *Received July 15, 1975.* Supported by U.S. Public Health Service Grant HL13035. A preliminary report of this work was presented at the 1973 FASEB Meeting, Abstract 1635.