Kinetics of Carboxypeptidase A. The pH Dependence of Tripeptide Hydrolysis Catalyzed by Zinc, Cobalt, and Manganese Enzymes*

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ABSTRACT: The zinc, cobalt, and manganese carboxypeptidase A (Cox) catalyzed hydrolysis of tripeptides has been examined over the pH range 5–10. The hydrolysis of these tripeptides can be described adequately by a reaction scheme requiring minimally three forms of free enzyme species, $EH_2 \rightleftarrows EH \rightleftarrows E$, and two of enzyme–substrate complex, one nonproductive, EH_2S, the other productive, EHS. The metal determines both the maximal activity of the enzyme toward peptides (Co > Zn > Mn), and influences the acid dissociation constant which characterizes the pH profile of k_{cat} as well as the acid limb of the k_{cat}/K_m–pH profile (Co > Zn > Mn). However, the effect of these metal substitutions on the values of K_m for these peptides is markedly less than that on k_{cat} and the acid dissociation constant which effects the pH dependence of K_m is independent of the metal. At high concentrations of acyl amino acids and dipeptides a variety of pH–rate profiles are observed. At low substrate concentration where k_{cat}/K_m is being measured, bell-shaped pH–rate profiles are obtained which are virtually identical with those for tri- and tetrapeptides. However, their pH dependence differs from those of the esters examined so far, particularly in the alkaline pH region.

The kinetics of peptide and ester hydrolysis are compared and discussed.

Kinetic anomalies have long been apparent in the carboxypeptidase A catalyzed hydrolysis of dipeptides and their ester analogs. Both substrates and products cause varying degrees of activation and inhibition (McClure *et al.*, 1964; Kaiser *et al.*, 1965; Whitaker, 1966; Davies *et al.*, 1968a,b; Vallee *et al.*, 1968). However, these features are not intrinsic to the enzyme. We have synthesized tri- and tetrapeptide substrates whose hydrolyses are devoid of such anomalies (Auld and Vallee, 1970), permitting the unambiguous determination of their kinetic constants, k_{cat} and K_m, and an analysis of the hydrogen ion dependencies of these parameters. By substituting cobalt and manganese for the native zinc in carboxypeptidase the metal dependencies of the pK_a''s, characterizing the pH profiles of k_{cat} and k_{cat}/K_m for peptides, can also be determined. A preliminary report on this work has appeared (Auld, 1969).

Materials and Methods

Beef Pancreas Carboxypeptidase.[1] Three times recrystallized zinc carboxypeptidase A, [(CPD)Zn], was prepared from beef pancreas acetone powder by the method of Cox *et al.* (1964).[2] The crystals were washed three times with deionized distilled water and dissolved in 1 M NaCl–0.05 M Tris, pH 7.5, to yield stock solutions of 2×10^{-4} M. The peptidase and esterase activities, expressed as turnover numbers, were 8000 ± 500 min^{-1} and 6500 ± 500 equiv of H^+/min per mole of enzyme, at pH 7.5, 25°, when assayed using the standard substrates of 0.02 M carbobenzoxyglycyl-L-phenylalanine (CbzGly-L-Phe) and 0.01 M hippuryl-*dl*-β-phenyllactic acid (HPLA), respectively. Although different preparations of carboxypeptidase A were used, the same preparation was always used for a particular series of experiments. The preparations of cobalt and manganese carboxypeptidase have been described (Davies *et al.*, 1968b). Carboxypeptidase referred to in the text as Anson enzyme was obtained from Worthington Biochemical Corp. and was used without further purification. The experimental procedures for preparing metal-free glassware and buffers have been detailed in a previous communication (Davies *et al.*, 1968b).

Deuterium oxide, 99% pure, was obtained from Bio-Rad. All reagents used were of the highest purity available.

Substrates. Peptides were either synthesized as formerly described (Auld and Vallee, 1970) or purchased from Cyclo Chemical Corp. The peptides were recrystallized until the free amine present was found to be less than 0.1%. Thin-layer chromatography, butanol–glacial acetic acid–water (4:1:1), on silica gel (Eastman Chromogram Sheet 6060 with fluorescent indicator) was used to determine the products of their carboxypeptidase A catalyzed hydrolysis. In all cases only the carboxyl terminal amino acid was cleaved in the reaction. Over the pH range 5–11, the amount of L-amino

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[1] The abbreviations used for blocking groups and amino acids will be Bz, benzoyl; Cbz, carbobenzoxy. [(CPD)Me] refers to metallocarboxypeptidase A where (CPD) represents apoenzyme and the brackets indicate the firm binding of metal to it; Me will be Zn, Co, or Mn. The abbreviations used for buffers will be ammediol, 2-amino-2-methyl-1,3-propanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.

[2] Carboxypeptidase A referred to in the text is carboxypeptidase A (Cox) unless otherwise specified (Petra and Neurath, 1969).

acid formed was always within 2% of theory, as determined by ninhydrin analysis.

Activity Measurements. The concentration of the unblocked amino acid formed during hydrolysis was determined by either of two automated ninhydrin methods. The first method used aliquot withdrawal and has been described previously (Auld and Vallee, 1970). The second employs continuous withdrawal of sample from a water jacketed cell, the quenching being accomplished by mixing immediately with ninhydrin in 0.2 M citrate buffer, pH 5.5, in 50% methyl Cellosolve. For the continuous sampling technique, 4.8 ml of a 0.10 M buffer 1.0 M in NaCl was mixed with 5 ml of a peptide solution, 1.0 M in NaCl. After the solution was equilibrated the reaction was initiated by the addition of 0.2 ml of enzyme. The pH of the assay was constant throughout the 6–8 min of the assay. Using the continuous withdrawal procedure, full hydrolysis of a 1×10^{-4} M substrate gave approximately one optical density unit, allowing facile determination of initial rates (*i.e.*, 10% hydrolysis or less).

Mes, Hepes, Tris, ammediol, and carbonate buffers (0.05 M) were used in the determination of the pH–rate profiles. The effect of the buffer on the reaction was examined by choosing a substrate concentration near K_m and determining the rate of hydrolysis in the presence of 0.01, 0.05, and 0.10 M buffer at its pK_a. Among the buffers used, the carbonate buffer was slightly inhibitory. Above pH 8.5 the determination of k_{cat} in this buffer was approximately 20% lower than in Tris or ammediol buffers, irrespective of the pH studied.

The temperature was controlled to ±0.1°. A Radiometer pH M 26 scale expanded pH meter was used for measuring pH values before and after assays. The meter was standardized at 25° with Harleco pH 4 and 7 reference buffers. Using this procedure the pH 10 reference buffer read within 0.05 unit of theory. In no case was an assay used in which the drift in pH during the reaction exceeded 0.04 pH unit. At alkaline pH values corrections were made for the high sodium content (1 M NaCl) of the assay mixtures.

Stability of the Enzyme. At pH 7.5 and 10.0 and in the absence of substrates the enzyme is stable at concentrations approximating those of the assay for at least three times the time interval used in the assay. At pH values below 6 in the absence of excess metal ion the enzyme continuously lost activity, as judged by assays performed at the incubation pH. In addition, incubation of the enzyme at such a low pH, while assaying at pH 7.5, also resulted in progressive loss of activity. However, at these low pH values excess metal ion stabilized the activity. Thus at pH 4.95 and 25°, when either carboxypeptidase A (Cox) or (Anson), 2×10^{-6} M, was incubated and assayed in the presence of 10^{-4} M Zn^{2+} no more than 12% activity was lost over a period of 1 hr.

Determination of Kinetic Parameters. The values of k_{cat} and K_m defined in eq 1 were calculated from Lineweaver–Burk plots by the method of least squares. The substrate concentration was varied approximately fivefold both above and below K_m. The errors in intercepts and slopes were calculated from the standard error of estimate, standard deviations, and the Student t distribution at the 90% confidence level.

$$V_0/[E_T] = k = \frac{k_{cat}[S]}{K_m + [S]} \quad (1)$$

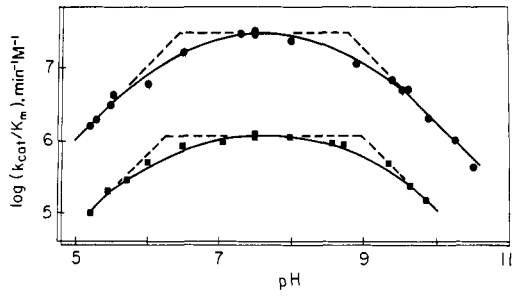

FIGURE 1: The pH dependence of log (k_{cat}/K_m) for CbzGlyGly-L-Phe (●) and BzGlyGly-L-Phe (■) hydrolysis. Conditions of assay: 1.0 M NaCl, 0.05 M Mes, Hepes, Tris, or carbonate buffer, 25°. The dotted lines have slopes of 1, 0, and −1, their intercepts yielding the values of pK_a'.

The best value for K_m is then intercept/slope and its probable error is calculated in the usual manner (Daniels *et al.*, 1956).

The thermodynamic parameters pK_{EH_2} and pK_{EH} were determined from activity measurements at low substrate concentrations and pK_{EH_2S} from activity measurements at high substrate concentrations as a function of pH. Two methods of calculation were employed. In one the parameters were obtained by successive approximation, using eq 3 and 5. An SDS 940 computer aided in the curve-fitting procedure. The parameters of the appropriate equation were allowed to take on several values over a predetermined range, and the criteria for the best fit were threefold. First, the sum of calculated velocities that differed from the observed velocities by more than 15% of the observed velocities was made a minimum. Second, the sum of calculated velocities that differed by less than 10% of the observed velocities was made a maximum. Last, the average per cent deviation, D, of calculated values, C, from experimental values, O, was made a minimum (eq 2) where N is the number of data points.

$$D = \frac{100}{N} \sum_{i=1}^{N} \frac{C_i - O_i}{O_i} \quad (2)$$

The second method obtains the values of the parameters from the minus abscissa intercept of the appropriate equation (8, 9, or 10) using the least-squares procedure described earlier.

Results

pH Dependence of Hydrolysis of Peptides. The Lineweaver–Burk plots for the hydrolysis of tripeptides from initial rate data are linear at least over a tenfold concentration range spanning the K_m for all pH values studied. The variation of log (k_{cat}/K_m) as a function of pH for the carboxypeptidase A catalyzed hydrolysis of CbzGlyGly-L-Phe and BzGlyGly-L-Phe over the pH range 5–10 at 25° is shown in Figure 1. Lines of slope one, zero, and minus one are drawn through the data in the acid, neutral, and alkaline pH regions, respectively. Approximate apparent pK's can be obtained from the intercepts of these lines (Dixon, 1953).

A number of peptides varying in length, C-terminal residues, and N-terminal blocking groups have pH–log rate constant profiles identical with those shown in Figure 1 when investi-

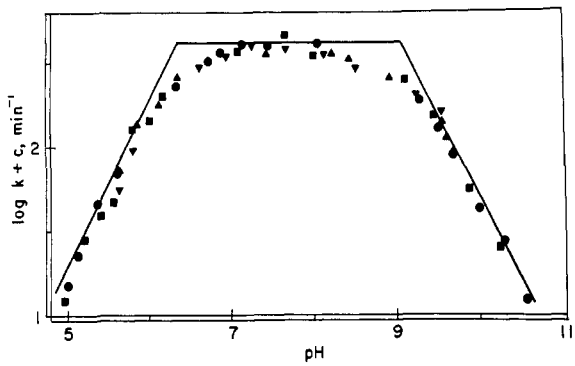

FIGURE 2: The pH dependence of the hydrolysis of 5×10^{-4} M chloroacetyl-L-phenylalanine (▼, $c = 0.25$), 2×10^{-4} M BzGly-L-Phe (▲, $c = -0.5$), BzGlyGly-L-Leu (●, $c = 1.0$), and CbzGlyGlyGly-L-Phe (■, $c = 0$). Conditions of assay were the same as in Figure 1. The solid lines have slopes of 1, 0, and -1, their intercepts yielding the values of pK_a'.

gated at substrate concentrations well below their value of K_m as determined at pH 7.5 (Figure 2). The profiles of the pH–log rate constant shown are normalized to the same rate constant in the pH region 7.5–8.5. In all instances ionization of two protein groups with pK_a''s of about 6.2 ± 0.1 and 9.0 ± 0.1 units characterize these pH profiles.

The curve for CbzGlyGly-L-Phe hydrolysis is resolved into its components of log k_{cat} and pK_m in Figure 3. Between pH 5 and 7, log k_{cat} increases, as a group in the enzyme–substrate complex ionizes with a pK_a' of 6. Thereafter, the rate remains constant. In contrast, pK_m is essentially constant from pH 5 to 8, but decreases thereafter on ionization of an enzyme group with a pK_a' of 9. The pH profiles for log k_{cat} and pK_m for BzGlyGly-L-Phe hydrolysis are essentially identical with those for CbzGlyGly-L-Phe. The kinetic parameters determined for CbzGlyGly-L-Leu at pH values 5.1, 5.5, 6.0, 7.5, 9.4, and 9.8 are also in accord with these general findings.

The simplest reaction scheme which satisfies the observed pH dependence of the hydrolysis of these tripeptides is shown in Figure 4. Three forms of free enzyme EH_2, EH, and E and two forms of enzyme substrate complexes, one inactive, EH_2S, the other active, EHS, are required minimally for the derivation of theoretical equations which fit the data. In this scheme, K_{EH_2} and K_{EH} represent ionization of groups in the

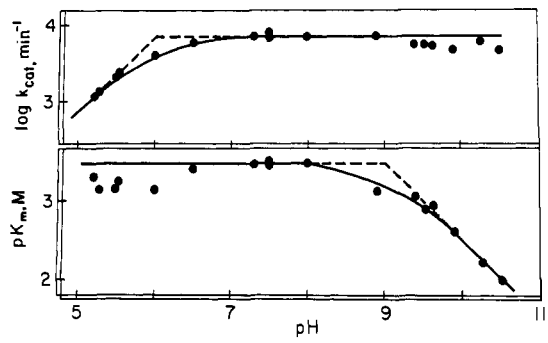

FIGURE 3: The pH dependence of log k_{cat} and pK_m for CbzGlyGly-L-Phe hydrolysis. Conditions of assays were the same as in Figure 1.

REACTION SCHEME

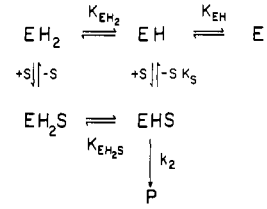

FIGURE 4

free enzyme and K_{EH_2S} denotes ionization of an enzyme–substrate complex. With increasing pH, activity will first increase due to the formation of EH which can form the productive enzyme–substrate complex, EHS, and then decrease due to the formation of E, which apparently can no longer bind substrate. Instability of the enzyme at pH values greater than 11 has prevented kinetic studies which might determine if the substrate still binds to the E form with a much weaker affinity constant.

The hydrogen ion dependence of the kinetic parameters k_{cat} and K_m which pertain for these hydrolyses are given in eq 3 and 4 and that for k_{cat}/K_m in eq 5

$$k_{cat} = \frac{k_2}{1 + a_H/K_{EH_2S}} \tag{3}$$

$$K_m = K_S \frac{(1 + a_H/K_{EH_2} + K_{EH}/a_H)}{1 + a_H/K_{EH_2S}} \tag{4}$$

$$\frac{k_{cat}}{K_m} = \frac{k_2/K_S}{1 + a_H/K_{EH_2} + K_{EH}/a_H} \tag{5}$$

where k_2, K_S, K_{EH_2}, K_{EH}, and K_{EH_2S} are constants defined in the reaction scheme and a_H is the hydrogen ion activity (Figure 4).

Good estimates of pK_{EH_2S}, pK_{EH_2}, and pK_{EH} can be obtained from the log plots of k_{cat}, k_{cat}/K_m, and K_m (Figures 1 and 3). The kinetic parameter of eq 1 which controls the activity of the enzyme below pH 7 is k_{cat} while K_m is the only parameter affecting activity above pH 7. The reduction of random errors in the determination of the pK_a''s which characterize the pH profiles of k_{cat} and k_{cat}/K_m can therefore be accomplished most easily by activity measurements under well-defined conditions.

Determination of pK_{EH_2} and pK_{EH} for Different Metallocarboxypeptidases. Since pK_{EH_2} and pK_{EH} are separated by nearly 3 pH units, very precise measurements of their values are possible from velocity measurements as a function of pH, obtained at substrate concentrations well below their K_m and where the initial rate data are given by eq 6.

$$V_0/[E_T] = k = \frac{k_{cat}[S]}{K_m} \tag{6}$$

These studies were performed using CbzGlyGly-L-Leu and CbzGlyGly-L-Val as substrates. For CbzGlyGly-L-Val the pH–velocity profiles were essentially identical when the substrate concentrations used for activity measurements were

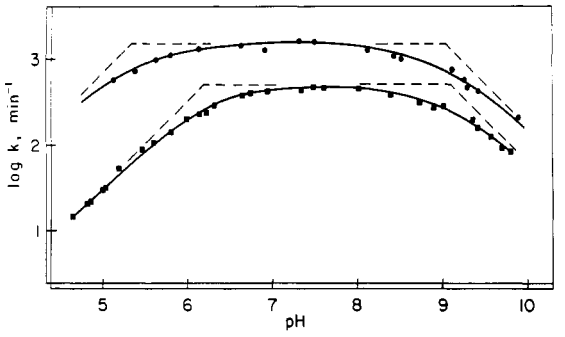

FIGURE 5: Hydrolysis of 1.92×10^{-4} M CbzGlyGly-L-Leu by [(CPD)-Co] (●) and [(CPD)Zn] (■). See Results section for the concentrations of metal ions in the assays. Condition of the assay: 1.0 M NaCl, 0.05 M Mes, Hepes, Tris, or ammediol buffers, at 25°. The solid lines are theoretical using eq 5 and the best values of pK_{EH_2}, pK_{EH}, and k_2/K_s obtained by the curve-fitting procedure. The values found were 5.33, 9.04, and $7.72 \times 10^{+6}$ min^{-1} M^{-1} for [(CPD)Co] and 6.19, 9.11, and $2.60 \times 10^{+6}$ min^{-1} M^{-1} for [(CPD)Zn]. The average per cent deviation of the experimental points from the calculated curve was 7.0% for [(CPD)Co] and 4.3% for [(CPD)Zn].

30- and 100-fold below its K_m at pH 7.5 and 25°. The activities with CbzGlyGly-L-Leu were obtained at a substrate concentration approximately 15-fold below its minimum K_m (Figure 5).

Below pH 5.8 velocities decrease more rapidly than would be predicted from eq 5. However, a small excess of Zn^{2+} increases activity to a constant value. Loss of zinc is the likely cause of this additional pH-dependent decrease in activity, since over the pH range 6–4 a parallel loss of peptidase activity and zinc ions has been shown previously when carboxypeptidase A (Allan) is dialyzed at these H^+ ion concentrations (Vallee *et al.*, 1960). Therefore, below pH 5 the reaction scheme shown should be modified by an additional equilibrium (eq 7).

$$[(EH_2)Zn] + nH^+ \rightleftharpoons [(EH_2)H_n] + Zn^{2+} \qquad (7)$$

Thus, *e.g.*, the enzymatic rates for the hydrolysis of 1.0×10^{-4} M CbzGlyGly-L-Leu, at pH 4.82, are increased by nearly 300% for a tenfold molar excess of Zn^{2+}, but additional increases to 100- and 1000-fold excesses do not further increase the velocity appreciably (Table I). This enhanced activity in the presence of excess Zn^{2+} rapidly decreases as the pH increases. At pH 6.15, velocities for the hydrolysis of CbzGlyGly-L-Leu, determined in the presence of 0-, 2000- and 20,000-fold molar excesses of Zn^{2+}, are identical within 5%.

A 2000-fold or greater molar excess of Zn^{2+} at pH 7.5 or above inhibits the enzyme. Enzyme solutions incubated with a 2000-fold excess of Zn^{2+} at pH 10.0 for 1 hr result in normal activity when diluted tenfold into assay mixtures containing 0.02 M CbzGly-L-Phe at pH 7.5, indicating that the inhibition is rapidly reversible, a phenomenon which was not pursued further. Metal ions were not added to assays of the native enzyme above pH 6.2.

Over the pH range 7–10, velocities of cobalt carboxypeptidase were independent of the free Co^{2+} concentration in the range from 10^{-4} to 10^{-2} M metal ion. However, over the pH range 5–6.5 only 10^{-3}–10^{-2} M concentrations of free Co^{2+} gave velocities which were independent of the metal ion concentration (Figure 5).

TABLE I: Effect of Excess Zinc Ions on the Hydrolysis of CbzGlyGly-L-Leu.[a]

pH	$[Zn^{2+}]$ added, M	k, min^{-1}	$[Zn^{2+}]$ added/$[E_T]$
4.82	0	6.52	0
4.82	10^{-6}	18.8	10
4.82	10^{-5}	19.7	100
4.82	10^{-4}	19.9	1,000
6.15	0	240	0
6.15	10^{-6}	222	200
6.15	10^{-5}	228	2,000
7.63	0	500	0
7.63	10^{-6}	499	200
7.63	10^{-4}	58	20,000
9.53	0	150	0
9.50	10^{-6}	61	30
9.49	10^{-4}	3.2	3,000

[a] Assays were performed at a CbzGlyGly-L-Leu concentration of 1.92×10^{-4} M in 1.0 M NaCl, 0.05 M Mes, Tris, or ammediol buffers at 25°. Zinc ions were added as $ZnSO_4$. Equivalent concentrations of Na_2SO_4 had no effect on activity.

For manganese carboxypeptidase catalyzed hydrolysis of CbzGlyGly-L-Val the concentrations of free Mn^{2+} were held constant at 10^{-4} M (Figure 6). Further increases to 10^{-3} or 10^{-2} M Mn^{2+} decreased the velocities by 10–20%, independent of pH. The best values of pK_{EH_2} and pK_{EH} obtained from these pH profiles by curve-fitting procedures are given in the captions of Figures 5 and 6.

Since below pH 7.5 the contribution of [E] to the pH profile is negligible, eq 5 can be simplified for this pH region by elimination of the hydrogen ion term containing K_{EH}. Substitution of the resulting equation into eq 6 and rearrangement lead to eq 8. Similarly above pH 8.0, $[EH_2]$ essentially

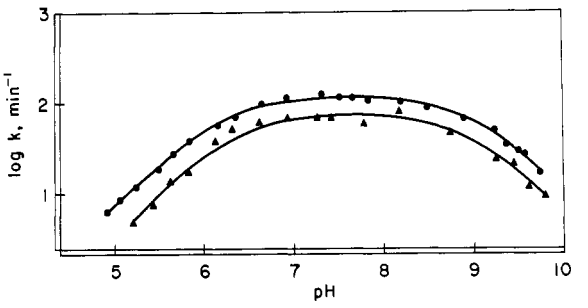

FIGURE 6: Hydrolysis of 5.75×10^{-4} M CbzGlyGly-L-Val by [(CPD)-Zn] (●) and [(CPD)Mn] (■). See Results section for the concentrations of metal ions in the assay. The solid lines are theoretical using eq 5 and the best values of pK_{EH_2}, pK_{EH}, and k_2/K_s obtained by the curve-fitting procedure. The values found were 6.19, 8.99, and $2.14 \times 10^{+5}$ min^{-1} M^{-1} for [(CPD)Zn] and 6.36, 8.94, and $1.36 \times 10^{+5}$ min^{-1} M^{-1} for [(CPD)Mn]. The average per cent deviation of the experimental points from the calculated curve was 4.8% for [(CPD)Zn] and 11.1% for [(CPD)Mn].

TABLE II: Best Values Obtained for pK_{EH_2}, pK_{EH}, and k_2/K_S for Metallocarboxypeptidases.[a]

[(CPD)Me]	Peptide	pK_{EH_2}	$k_2/K_S \times 10^{-5}$, min^{-1} M^{-1} A	$k_2/K_S \times 10^{-5}$, min^{-1} M^{-1} B	pK_{EH}
Co	CbzGlyGly-L-Leu	5.33 ± 0.08	76.5 ± 5.0	71.5 ± 8.4	9.08 ± 0.08
Zn	CbzGlyGly-L-Leu	6.18 ± 0.07	25.8 ± 3.4	24.6 ± 2.3	9.14 ± 0.05
Zn	CbzGlyGly-L-Val	6.14 ± 0.05	1.97 ± 0.23	2.05 ± 0.16	9.04 ± 0.05
Mn	CbzGlyGly-L-Val	6.36 ± 0.10	1.53 ± 0.31	1.34 ± 0.31	8.93 ± 0.12

[a] Assays were performed at 25° in 1.0 M NaCl, 0.05 M Mes, Tris, or ammediol buffers. The concentrations of CbzGlyGly-L-Leu and CbzGlyGly-L-Val were 1.92×10^{-4} and 5.75×10^{-4} M, respectively. Equations 8 and 9 were used for the determination of K_{EH_2} and K_{EH}. Values of k_2/K_S in columns A and B were determined from eq 8 and 9, respectively. Experimental errors are at the 90% confidence level.

$$\frac{1}{k} = \frac{K_m}{k_{cat}[S]} = \frac{K_S}{k_2[S]} + \frac{K_S a_H}{k_2[S]K_{EH_2}} \quad (8)$$

does not affect the shape of the pH–velocity profile and upon simplifying and rearranging, eq 6 can be transformed into eq 9 for the pH region above 8.0. When the reciprocal of the

$$\frac{1}{k} = \frac{K_m}{k_{cat}[S]} = \frac{K_S}{k_2[S]} + \frac{K_S K_{EH}}{k_2[S]a_H} \quad (9)$$

experimental rate constant is plotted *vs.* the hydrogen ion concentration eq 8 or its reciprocal, eq 9, the value of K_S/k_2 can be obtained from the ordinate intercept and the acid dissociation constants K_{EH_2} and K_{EH} from the abscissa intercepts (Table II).

When plotted in this manner the data give the expected linear relationships and the calculated parameters agree closely with those obtained from the curve-fitting procedure (Figure 7).

Determination of pK_{EH_2S}. Over the pH range from 5 to 8, for both BzGlyGly-L-Phe and CbzGlyGly-L-Phe (Figure 3), K_m is essentially insensitive to change in pH. Activity–pH profiles, determined at enzyme saturating concentrations of substrate, should yield precise measurements of K_{EH_2S}, since under these conditions eq 1 reduces to eq 3. Excess Zn^{2+} has little effect on the rates of hydrolysis of 0.02 M BzGlyGly-L-Phe by native carboxypeptidase over the pH range 4.8–6. At pH 4.94 a 10,000-fold excess of Zn^{2+} in the assay increased the velocity by 35%. No effect of such an excess of metal ion is found at other pH values. Cobalt carboxypeptidase was assayed in the presence of at least 10^{-4} M Co^{2+}. Concentrations of 10^{-2} M Co^{2+} did not affect the rate appreciably. The best values for pK_{EH_2S} and k_2 obtained by the curve-fitting procedure for these metalloenzymes are given in the caption of Figure 8. Equation 3 can also be rearranged in order to achieve a linear plot.

$$\frac{1}{k_{cat}} = \frac{a_H}{k_2 K_{EH_2S}} + \frac{1}{k_2} \quad (10)$$

When the values of $1/k_{cat}$ are plotted *vs.* the hydrogen ion activities, the values of K_{EH_2S} and k_2 are obtained from the abscissa and ordinate intercepts, respectively. The data treated in this manner yield values of pK_{EH_2S} and k_2, in good agreement with those obtained by the curve-fitting procedure (Table III and Figure 8).

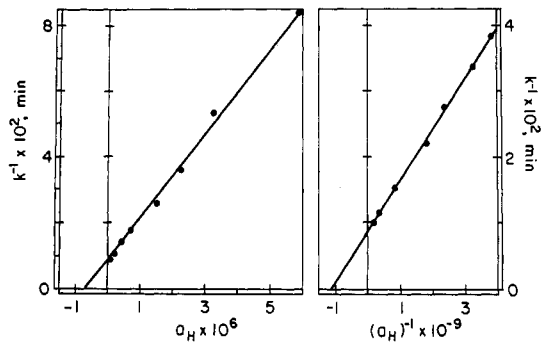

FIGURE 7: Determination of K_{EH_2} and K_{EH} for the [(CPD)Zn]-catalyzed hydrolysis of CbzGlyGly-L-Val hydrolysis using eq 8 and 9. The lines were determined by the method of least squares. The values of pK_{EH_2}, 6.14, and pK_{EH}, 9.04, obtained by this means are in close agreement to those obtained by the curve-fitting procedure (Figure 6).

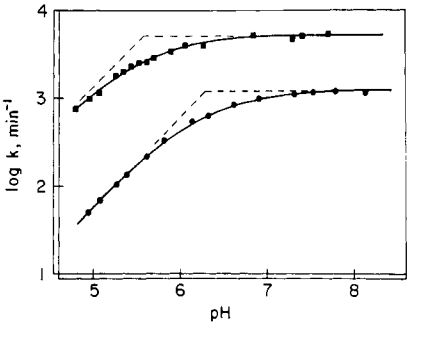

FIGURE 8: Hydrolysis of 0.02 M BzGlyGly-L-Phe by [(CPD)Co] (■) and [(CPD)Zn] (●). See the Results section for the concentrations of metal ions in assay. The solid line is generated using eq 3 and best values of pK_{EH_2S} and k_2 are obtained by curve-fitting procedure. The values found were 5.56 and 4920 min^{-1} for [(CPD)Co] and 6.27 and 1180 min^{-1} for [(CPD)Zn]. The average per cent deviation of the experimental points from the calculated curve was 4.5% for [(CPD)Co] and 2.8% for [(CPD)Zn].

TABLE III: Best Values Obtained for k_2 and pK_{EH_2S} for the Zinc and Cobalt Carboxypeptidase A Catalyzed Hydrolysis of BzGlyGly-L-Phe.[a]

[(CPD)Me]	pK_{EH_2S}	k_2, min^{-1}
Co	5.57 ± 0.03	5000 ± 472
Zn	6.33 ± 0.05	1320 ± 210

[a] Activity measurements were made at 0.02 M BzGlyGly-L-Phe at 25° in 0.05 M Mes–1.0 M NaCl from pH 4.8 to 7.3 and in 0.05 M Tris–1.0 M NaCl from pH 7.3 to 8.5. Equation 10 was employed for the determination of K_{EH_2S} and k_2. Experimental errors are at 90% confidence level.

TABLE IV: Effect of D_2O on the Rate of BzGlyGly-L-Phe Hydrolysis.[a]

pH	$V_0/[E_T]$, min^{-1}	pD	$V_0/[E_T]$, min^{-1}
7.80	1168	7.73	1060
8.15	1110	7.95	1180
8.61	1050	8.59	1050

[a] Conditions of assay 1.0 M NaCl–0.05 M Tris–0.02 M BzGlyGly-L-Phe, 25°; pD = pH meter reading + $(4.29 \times 10^2/T°) - 1.04$ (Fife and Bruice, 1961).

Rate of BzGlyGly-L-Phe Hydrolysis in D_2O. The average rate constant for the hydrolysis of 0.02 M BzGlyGly-L-Phe in H_2O and pH values 7.8, 8.2, and 8.6 is 1113 ± 44 min^{-1}. This value is essentially identical with that obtained in D_2O (*i.e.*, 1097 ± 56 min^{-1}) at pD values 7.7, 8.0, and 8.6 (Table IV).

Discussion

Past kinetic studies of the pH dependence of the hydrolysis of dipeptides, catalyzed by carboxypeptidase A (Anson), have yielded variable and even conflicting results. The values of both k_{cat} and K_m for the hydrolysis of CbzGly-L-Phe have been reported to be minimal at pH 7.5, while a plot of k_{cat}/K_m is bell shaped with a maximum at pH 7.5 (Neurath and Schwert, 1950). However, in other instances where the hydrolysis of both CbzGly-L-Phe and CbzGly-L-Trp was studied virtually no change in k_{cat} and K_m was found on varying pH from 7.3 to 8.6 (Lumry *et al.*, 1951). In addition, when CbzGly-L-Phe is hydrolyzed by a suspension of enzyme crystals "k_{cat}" and "K_m" vary only slightly over the pH range 6–9 (Quiocho *et al.*, 1967).

At present it is difficult to assess the extent to which factors such as substrate activation have complicated the interpretation of such steady state kinetic studies.[3] The ambiguities discernible from kinetic data obtained with dipeptide substrates may derive in part from the substrate concentration ranges employed to obtain the values of k_{cat} and K_m.

Clearly, mechanistic studies employing substrates lacking obvious kinetic anomalies might resolve such problems. In searching for such substrates, we have found that hydrolysis of a number of tri- or tetrapeptides blocked at the N terminus does not reveal substrate activation (Auld, 1968; Auld and Vallee, 1970). Hence, for these peptides, the variations of kinetic parameters as a function of pH and temperature can be assessed and analyzed unambiguously. Thus, over the range of pH 5–10, the pH dependence of hydrolysis of these peptides can be described adequately by a reaction scheme such as shown in Figure 4. In this instance, three enzyme species free of substrate, EH_2, EH, and E, and two enzyme–substrate complexes, one nonproductive, EH_2S, the other productive, EHS, are required minimally. In this scheme, the proton acts as a noncompetitive inhibitor on the acid side of the pH profile, producing EH_2 which still binds substrate to give a nonproductive enzyme–substrate complex, EH_2S. In the alkaline pH region, loss of a proton from EH produces E, whose ability to bind substrate is greatly reduced. Therefore, the bell-shaped pH profiles of k_{cat}/K_m (Figure 1) for the hydrolyses of these tripeptides are composed of two sigmoid curves, one on the acid side, reflecting the pH dependence of k_{cat}, the other on the alkaline side, reflecting that of K_m (Figure 3).

In the pH region where K_m is constant, k_{cat} depends strongly on pH providing additional evidence that K_m is proportional to the dissociation constant of the enzyme–substrate complex, K_S, as already indicated by previous kinetic studies with tripeptides. No relationship between values of k_{cat} and K_m was found when one particular metallocarboxypeptidase acted on a series of tripeptides or when a series of metallocarboxypeptidases act on a single tripeptide (Auld and Vallee, 1970).

The hydrolysis of dipeptides at high substrate concentrations results in a variety of pH profiles for k_{cat} (Neurath and Schwert, 1950; Lumry *et al.*, 1951; Hommes, 1962). However, at substrate concentrations where activity is a measure of k_{cat}-K_m, the pH–rate profiles for chloroacetyl-L-Phe, BzGly-L-Phe, BzGlyGly-L-Phe, BzGlyGly-L-Leu, CbzGlyGly-L-Leu, CbzGlyGly-L-Val, and CbzGlyGlyGly-L-Phe are bell shaped and virtually identical (Figure 2). Thus, changing the N-terminal blocking group, the C-terminal residue or peptide chain length does not alter the inflection points at pH 6.2 ± 0.1 and pH 9.0 ± 0.1 representing pK_{EH_2} and pK_{EH}, respectively. Hence, these substrates would seem to interact with the same ionizing groups of the enzyme which are reflected in the pH–rate profiles (Peller and Alberty, 1959).

The inflection points of the pH–rate profiles may provide a clue to the identity of functional residues at the active site of the enzyme. Thus, the pK of 9.0 could be consistent, *e.g.*, with the ionization of an enzyme–metal aquocomplex, *i.e.*, $Me(L_n)(H_2O) \rightleftharpoons Me(L_n)(OH^-) + H^+$, lysyl or tyrosyl residues.

If the ionization $EH \rightarrow E + H^+$ were to reflect the ionization of $Me(L_n)(H_2O) \rightleftharpoons Me(L_n)(OH^-) + H^+$, judging from complex metal ions the acidity of such a coordinated water molecule might be a function of the metal to which it is coordi-

[3] The activation of CbzGly-L-Phe hydrolysis by cyclohexanol has been observed to occur both at 25° and 0° and throughout the pH range from 6 to 9 (unpublished data, R. C. Davies).

nated.[4] However, the values of pK_{EH} for zinc, cobalt, and manganese carboxypeptidase, which are essentially identical, are not consistent with such a postulate (Table II). If an enzyme–metal aquocomplex were indeed to account for ionization of EH, the metalloenzyme would not seem to behave in a manner predicted by models, a possibility which cannot be ruled out (Vallee and Williams, 1968).

All attempts to implicate the participation of lysyl residues or the α-amino group of carboxypeptidase in activity have been unsuccessful (Vallee and Riordan, 1968).

A pK_a of 9.0 could represent participation of a protonated tyrosine. If EH were to represent a protonated tyrosine, then on the basis of the present data its ionization would reduce peptide binding, though this would not exclude other functional roles. The involvement of tyrosyl residues must obviously be considered since a large number of chemical modification have indicated their participation in function (Vallee, 1964; Vallee and Riordan, 1968) and both chemical and X-ray data have implicated conformational changes involving tyrosine (Riordan *et al.*, 1967; Lipscomb *et al.*, 1968). The pH-dependent kinetics here described may likely offer an additional means for the delineation of the role of tyrosines in hydrolysis.

The acidic pK_a' could be in accord with the ionization of a carboxyl or an imidazolyl group, or again, with a $Me(L_n)(H_2O)$ aquocomplex. In contrast to the studies at alkaline pH (*vide supra*), the values of pK_{EH_2} and pK_{EH_2S} at acid pH values *do* vary with metal substitution (Tables II and III) and the primary kinetic effect of the metal in the hydrolysis of tripeptides is on k_{cat} (Auld and Vallee, 1970). Since now the important catalytic species would be the conjugate base, *e.g.*, $Me(L_n)(OH^-)$, the metal might be thought to increase the effective concentration of hydroxide ion at neutral pH. Under such circumstances, the metal might function in a manner similar to that proposed for the carbonic anhydrase catalyzed hydration of carbon dioxide (Davis, 1961; Coleman, 1967; Riepe and Wang, 1968). However, of themselves the present data are not decisive in regard to such a mechanism.

Both chemical modification and X-ray studies have implicated a carboxyl group in the mechanism of action of carboxypeptidase (Vallee and Riordan, 1968; Lipscomb *et al.*, 1968; Petra and Neurath, 1970). As judged by studies of model systems, a pK_a of 6.2–6.3 would be high for a carboxyl group unless it were placed in a hydrophobic environment. Such a group with a pK_a' of 6.1 is known to affect the activity of lysozyme, although in that case, it is postulated to function as a general acid (Blake *et al.*, 1967; Raftery *et al.*, 1969).

Based on kinetic studies, histidine was implicated early in the mechanism of carboxypeptidase (Neurath and Schwert, 1950) and later coupling with 5-diazo-1*H*-tetrazole also suggested a role (Sokolovsky and Vallee, 1967). While the X-ray data do not provide evidence for the participation of a histidyl residue either in catalysis or substrate binding (Lipscomb *et al.*, 1968), two histidines are now known to be metal ligands (Bradshaw *et al.*, 1969; Lipscomb *et al.*, 1969). Alteration of one of these two histidyls might conceivably be reflected either in the kinetics, in the functional consequences of chemical modifications, or both. Thus, competition between protons and the metal for one of these histidine ligands might inactivate the enzyme, and with decreasing pH, additional protonation of the other ligands could then contribute to the release of the metal from the enzyme (Vallee *et al.*, 1960).

Thus one may conclude substitutions of cobalt or manganese for zinc demonstrate that the metal not only determines the maximal activity of the enzyme toward peptides, but that in addition, it can influence the acid dissociation constants K_{EH_2S} or K_{EH_2} which characterize the pH profile of k_{cat} and the acid limb of k_{cat}/K_m (Tables II and III). However, these metal substitutions neither affect the values of K_m for these peptides nor the acid dissociation constant, pK_{EH}, which characterizes the pH profiles for K_m (Table II). Hence, on this basis and consistent with previous observations, the metal may function more importantly in the catalytic than in the binding step of the hydrolysis of peptides (Coleman and Vallee, 1962a,b).

Chemical modifications of the enzyme, pH variation, and inhibitors similarly affect the hydrolysis of the peptides studied here though these differ in length, N-terminal blocking groups, and C-terminal residues. However, the differing responses of esterase and peptidase activities to chemical modification of the enzyme have long raised questions concerning the manner of their respective hydrolyses (Vallee *et al.*, 1963; Riordan and Vallee, 1968; Vallee *et al.*, 1970), a problem also revealed subsequently by more detailed kinetic studies. Thus, phenylacetate and β-phenylpropionate are known competitive inhibitors of esters (Kaiser and Carson, 1965), but they noncompetitively inhibit the hydrolysis of tripeptides (Auld and Vallee, 1970). Studies in D_2O have further uncovered differences. The deuterium isotope effect is 1.90 for k_{cat} in the hydrolysis of the ester substrate *O*-(*trans*-cinnomoyl)-L-β-phenyllactate while it is 1.33 for BzGly-L-Phe (Kaiser and Kaiser, 1969). Yet, these substrates are not strictly comparable since the ester substrate is characterized by normal kinetics while BzGly-L-Phe exhibits substrate activation (Davies *et al.*, 1968b; Auld and Vallee, 1970) which may complicate the interpretation of a deuterium isotope effect in that instance. In this regard it should be noted that the tripeptide homolog BzGlyGly-L-Phe shows *no* D_2O effect on k_{cat} over the pH range 7.7–8.6 (Table IV).

The pH dependencies of ester and peptide hydrolysis reveal additional differences. Acetylmandelate displays a narrow, bell shaped pH profile of k_{cat}/K_m, characterized by pK_a' values of 6.9 and 7.5 (Carson and Kaiser, 1966). The large difference in the pK_a' value characterizing the alkaline limb of the k_{cat}/K_m curve for this ester, $pK_a' = 7.5$, and of the peptides studied here, $pK_a' = 9.0$, is of particular interest. If the same enzyme groups are involved in both cases, then identical pH profiles would be expected (Peller and Alberty, 1959). Furthermore, the pH dependence of k_{cat} for acetylmandelate also depends on the ionization of two groups with pK_a' values of 7.2 and 7.9. The marked difference in the shape of this ester profile compared with the sigmoidal curve obtained here for k_{cat} using peptides may also reflect involvement of different groups in their hydrolysis.

The pH dependence of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate has been reported recently (Hall *et al.*, 1969). In this instance the value of k_{cat}/K_m at any pH depends on the ionization of groups with pK_a' values of 6.5 and 9.4. The acid

[4] The pK values for the equilibria $M(H_2O)_x \rightleftharpoons M(H_2O)_{x-1}(OH^-) + H^+$ are 10.59, 9.85, and 8.96 where M is Mn^{2+}, Co^{2+}, and Zn^{2+}, respectively, at 25° and zero ionic strength (Perrin, 1962a,b; Bolzan and Arvia, 1962). In addition, values of 5.7 and 2.0 have been cited for coordinated water molecules in $[Co(NH_3)_5H_2O]^{3+}$ and $[Pt(NH_3)_4(H_2O)_2]^{4+}$, respectively (Cotton and Wilkinson, 1966).

limb of the pH profile for k_{cat} is characterized by a pK_a' of 6.2. These values are much closer to those found here for peptide hydrolysis than those cited above on acetylmandelate. However, the entire pH profile for k_{cat} for *O*-(*trans*-cinnamoyl)-L-β-phenyllactate was thought to be a composite of two simpler profiles, one bell shaped, the other of undetermined shape but rising steeply above pH 8. While such a pH profile for k_{cat} differs from that found here for peptides, it closely resembled that found for the hydrolyses of 0.01 M hippuryl-*dl*-β-phenyllactate which also exhibits a steeply rising component in the region above pH 9 (Riordan and Vallee, 1963).

Thus, the pH-dependent hydrolyses of these peptides and esters differ primarily in the pH region above neutrality. For the peptides k_{cat} is not influenced by the hydrogen ion concentration in the pH region 7.0–10. However, for the esters acetylmandelate and *O*-(*trans*-cinnamoyl)-L-β-phenyllactate, these hydrogen ion concentrations influence k_{cat} markedly (Hall *et al.*, 1969; Carson and Kaiser, 1960).

Such differences may reflect different mechanisms for peptide and ester hydrolysis or pH-dependent substrate inhibition modes that may affect the profiles of the esters studied. Hence, the pH dependence of ester hydrolysis will have to be examined further before more definite conclusions on the possible generality and reasons for the apparent differences between ester and peptide hydrolysis can be discerned.

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