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# Kinetics of Carboxypeptidase A. II. Inhibitors of the Hydrolysis of Oligopeptides*

David S. Auld† and Bert L. Vallee

ABSTRACT: The carboxypeptidase-catalyzed hydrolyses of benzoylglycylglycyl-L-phenylalanine and carbobenzoxyglycylglycyl-L-phenylalanine do not display the substrate activation characteristic of their dipeptide analogs, benzoylglycyl-L-phenylalanine and carbobenzoxyglycyl-L-phenylalanine. Moreover, a known activator of the hydrolysis of the dipeptides, carbobenzoxyglycine, competitively inhibits the hydrolyses of these tripeptides with a $K_i$ of 0.027 M.

As with dipeptides, acetylation of carboxypeptidase A (Anson) reduces activity toward the tri- and tetrapeptides to less than 3% of the control. The relative order of the Michaelis constant, $K_m$, can now be established as Co = Zn = Ni > Mn for the respective metallocarboxypeptidases. $K_m$ is likely to be a measure of the binding affinity of the tripeptide since no relationship is found between the values of $K_m$ and $k_{cat}$ when one particular metallocarboxypeptidase is examined on its action on a series of tripeptides or when a series of metallocarboxypeptidases act on one tripeptide. The order of $k_{cat}$ is the same as for the dipeptides, *i.e.*, Co > Zn = Ni > Mn. These kinetic studies reveal significant differences in the hydrolysis of these peptides and esters. Indole-3-acetate, phenylacetate, and β-phenylpropionate are all noncompetitive inhibitors of tripeptide hydrolyses. This class of compounds has long been known to be competitive inhibitors of the hydrolysis of esters. These studies are consistent with the proposed multiple loci model (Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. C., Auld, D. S., and Sokolvsky, M. (1968), *Biochemistry 7*, 3547).

The kinetics of hydrolyses of CbzGly-L-Phe and BzGly-L-Phe catalyzed by a series of metallocarboxypeptidases are characterized by substrate activation. The range of substrate concentration over which the activation occurs varies slightly for the different metalloenzymes. Similarly, all the various metallocarboxypeptidases exhibit substrate inhibition when employing hippuryl-β-DL-phenyllactic acid, the substrate which commonly serves for esterase activity measurements. Since the Lineweaver–Burk plots are multiphasic, it would seem likely that the extrapolated parameters "$V_{max}$" and "$K_m$" might be composite terms. Thus, it was of interest to note that there was apparent ordering of the constants for the substrates, regardless of the metal present, indicating that the course of the kinetic complications may be due, in part, to multiple interactions of the small synthetic dipeptide and depsipeptide substrates used for the kinetic analysis (Davies *et al.*, 1968a,b). A model proposing binding of substrates at multiple loci is consistent with the data obtained thus far (Vallee, 1967; Vallee *et al.*, 1968, 1969). The model suggests that an increase in the length of the substrate and/or bulk of the blocking group should place more constraints on its manner of binding, and the enzymatic hydrolysis of such substrates should approach Michaelis–Menten kinetics. This hypothesis has been tested by investigating the kinetics of hydrol-

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ysis of the tripeptide homologs, CbzGlyGly-L-Phe and BzGlyGly-L-Phe. Preliminary reports of the present data have been made (Auld, 1968).

## Materials and Methods

*Beef Pancreas Carboxypeptidase.*[1] Five-times-recyrstallized zinc carboxypeptidase A[2] was prepared from beef pancreas acetone powder by the method of Cox *et al.* (1964). The crystals were washed three times with deionized distilled water and dissolved in 1 M NaCl–0.05 Tris (pH 7.5), to yield $2 \times 10^{-4}$ M stock solutions. The apparent proteolytic coefficients, $C$, of the preparations employed were $41 \pm 2$ at pH 7.5, 0°, and the esterase activity expressed as the turnover number or as a zero-order rate constant, $k$ was $6.6 \pm 0.5 \times 10^3$ of equiv $H^+$/min per mole of enzyme, at pH 7.5, 25° when assayed using the standard substrates of 0.02 M carbobenzoxyglycyl-L-phenylalanine and 0.01 M hippuryl-DL-β-phenyllactic acid.

*Apo- and Metallocarboxypeptidases.* Apocarboxypeptidase was prepared by dialysis of $2 \times 10^{-4}$ M carboxypeptidase against four changes of a 100-fold volume excess of $2 \times 10^{-3}$ M 1,10-phenanthroline in 1.0 M NaCl–0.05 M Tris buffer (pH 7.5), followed by dialysis *vs.* four changes of dithizone-extracted buffer (Coombs *et al.*, 1964).

*Metallocarboxypeptidases.* Apoenzyme samples ($10^{-4}$ M) were dialyzed at 4° with a 100-fold volume excess of $10^{-3}$ M metal ion in 1 M NaCl–0.05 M Tris (pH 7.5). The enzyme was stored in this manner until use. Enzyme was diluted to $10^{-5}$ M with $10^{-3}$ M metal ion solutions in order to ensure better than 99% formation of the metalloenzymes (Coleman and Vallee, 1961). Each metalloenzyme prepared in this fashion displayed activities characteristic of the particular metal substituted for zinc (Coleman *et al.*, 1966; Davies *et al.*, 1968b). The amount of zinc present in the apoenzyme and all of the metalloenzymes, as determined by atomic absorption spectrometry (Fuwa *et al.*, 1964) was always in the range of $5 \times 10^{-3}$ to $2.0 \times 10^{-2}$ g-atom of zinc per mole of enzyme. The mercury and cadmium enzymes exhibit residual peptidase activity of 2 and 0.9% of the native enzyme, respectively, when assayed with both CbzGly-L-Phe and CbzGlyGly-L-Phe.

*Acetylcarboxypeptidase.* Acetylcarboxypeptidase was prepared from either carboxypeptidase A (Cox) or A (Anson) by acetylation with a 100-fold molar excess of *N*-acetylimidazole for 1 hr at 25° in pH 7.5, 1.0 M NaCl–0.02 M Veronal buffer. Excess *N*-acetylimidazole and decomposition products were removed by dialysis against two changes of 1 M NaCl–0.02 M Veronal (pH 7.5) buffer.

*Metal Solutions.* Standard solutions of cobalt, nickel, manganese, and zinc ions were prepared from the spectrographically pure metal salts obtained from Johnson Matthey Co., Ltd. Solutions were diluted with metal-free buffer to give the desired pH and molarity and checked for possible zinc contamination before use. The precautions taken to prevent zinc contamination have been described previously (Davies *et al.*, 1968b).

*Protein Concentrations.* Protein concentrations were measured by the absorbance at 280 mμ using the Zeiss PMQ spectrophotometer. A molar absorptivity of $6.42 \times 10^4$ $M^{-1}$ $cm^{-1}$ was used for all metalloenzymes and $5.92 \times 10^4$ $M^{-1}$ $cm^{-1}$ for the corresponding acetylenzymes (Simpson *et al.*, 1963).

*Substrates.* CbzGly-L-Phe was purchased from Miles Corp. Peptide intermediates were purchased from Cyclo Chemical Corp. in the highest available purity. Carbobenzoxyglycylglycyl-L-leucine and carbobenzoxyglycylglycyl-L-valine were purchased from Cyclo Chemical Corp. and recrystallized from ethanol–water before use. Carbobenzoxy-L-phenylalanylglycylglycyl-L-phenylalanine was a gift from Dr. Mordechai Sokolovsky.

*Carbobenzoxyglycylglycyl-L-phenylalanine Methyl Ester.* One equivalent each of L-phenylalanine methyl ester hydrochloride, *N*-carbobenzoxyglycylglycine, and *N,N*-dicyclohexylcarbodiimide, and 1.1 equiv of triethylamine were dissolved in dimethylformamide at 0°. The reaction mixture was stirred for 2 hr at 4°, then left to stand overnight at room temperature. Precipitated dicyclohexylurea was filtered and the mother liquors evaporated to dryness under vacuum keeping the temperature under 35°.

Ethyl acetate and 1 N HCl were added to the flask. Any precipitate was filtered and the filter cake was washed with ethyl acetate. The ethyl acetate phase of the filtrate was separated from the aqueous phase and extracted successively with 0.5 N HCl, water, and 5% $NaHCO_3$, washing well with water after the last extraction. The ethyl acetate phase was dried with $Na_2SO_4$ and petroleum ether (bp 30–60°) added until crystallization was initiated. The melting point of the crude product was 94–98° uncorrected.

*Carbobenzoxyglycylglycyl-L-phenylalanine.* One equivalent of carbobenzoxyglycylglycyl-L-phenylalanine methyl ester and 1.05 equiv NaOH were added to acetone and the solution was stirred at room temperature for 1 hr. Large excesses of NaOH, prolonged hydrolysis times, and high temperatures should be avoided to reduce side reactions (Maclaren, 1958). The acetone solution was poured into an equal volume of water and any precipitated solids were removed by filtration. HCl (6 N) was added to the filtrate until the pH was approximately 1. An oily, white solid formed which hardened after a few minutes. The sample was recrystallized twice from ethanol–water, mp 109–111°. *Anal.* Calcd[3] for $C_{21}H_{23}N_3O_6$ (413.41): C, 61.0; H, 5.6; N, 10.2. Found: C, 61.2; H, 5.6; N, 10.4.

*Carbobenzoxyglycylglycylglycyl-L-phenylalanine* was synthesized by the same method as above using carbobenzoxyglycylglycylglycine and L-phenylalanine methyl ester hydrochloride as starting materials, mp 138–140°. *Anal.* Calcd for $C_{23}H_{26}N_4O_7$ (470.46): N, 11.8. Found: N, 11.2.

*Benzoylglycylglycyl-L-phenylalanine.* Glycylglycyl-L-phenylalanine (1.0 g) was added to 10 ml of water and 1 ml of 10 N NaOH was added to form a clear solution followed by 0.4

[1] The abbreviations used for blocking groups and amino acids throughout the text will be Bz, benzoyl; Cbz, carbobenzoxy; Ci, cinnamoyl. The abbreviations for the metalloenzymes are used in the formulation and figures only and when required for differentiation [(CPD)Zn], zinc carboxypeptidase A; with (CPD) representing apoenzyme and the brackets indicating the firm binding of zinc or other metals to it, *e.g.*, [(CPD)Me] refers to the metallocarboxypeptidases where Me may be Cd, Co, Hg, Mn, or Ni; [(AcCPD)Zn], zinc carboxypeptidase acetylated with *N*-acetylimidazole.

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

[3] Elemental analysis performed by Dr. S. M. Nage of M.I.T.

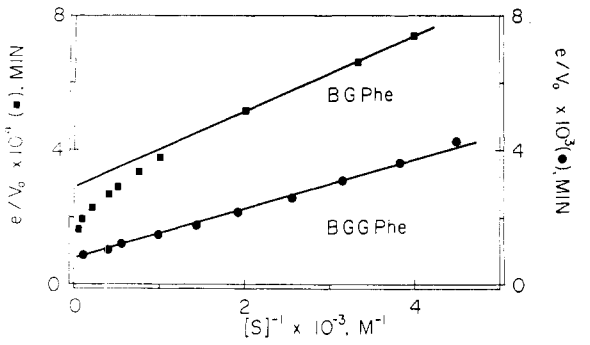


FIGURE 1: Lineweaver–Burk plots for carboxypeptidase-catalyzed hydrolysis of a di- and tripeptide pair. Conditions of assay were 1.0 M NaCl–0.05 M Tris (pH 7.5) and 25°.

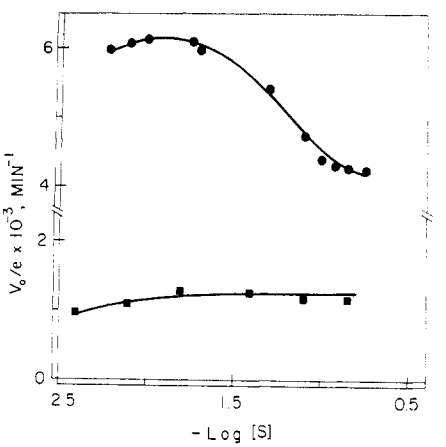


FIGURE 2: Velocity *vs.* substrate profiles for high concentrations of BzGlyGly-L-Phe (■) and CbzGlyGly-L-Phe (●). Ionic strength was held constant at 1.0 with NaCl in a 0.05 M Tris buffer, pH 7.5 and 25°.

ml of benzoyl chloride added in small portions at 0° with vigorous stirring over approximately 10 min. HCl (6 N) was added to acidify the solution and the white precipitate was filtered and washed with water. The crude yield was 95%. The sample was recrystallized twice from ethanol–water, mp 218–219°. *Anal.* Calcd for $C_{20}H_{21}N_3O_5$ (383.45): C, 62.7; H, 5.5; N, 11.0. Found: C, 63.0; H, 5.7; N, 10.6.

*Benzoylglycylglycyl-*L*-leucine.* Prepared from glycylglycyl-L-leucine using the same procedure as for BzGlyGly-L-Phe.

*Cinnamoylglycylglycyl-*L*-phenylalanine* was prepared from glycylglycyl-L-phenylalanine and cinnamoyl chloride by the method used by McClure and Neurath (1966) for cinnamoyl phenylalanine. Ethanol–water was used for recrystallization, mp 209–210°. *Anal.* Calcd for $C_{22}H_{23}N_3O_5$ (409.4): C, 64.5; H, 5.7; N, 10.3. Found: C, 64.5; H, 6.5; N, 10.3.

Free amine present in the peptides was found to be less than 0.1%. Thin-layer chromatography on silica gel (Eastman Chromogram sheet 6060 with fluorescent indicator) was used to determine the products of the carboxypeptidase-catalyzed hydrolysis. Solvents used were 1-butanol–glacial acetic acid–water (4:1:1) and 1-butanol–acetone–acetic acid–5% $NH_4OH$–water (4.5:1.5:1:1:2). Ninhydrin spray was used to detect the free amine and fluorescence quenching to detect the blocked amino acid product. Extent of carboxypeptidase-catalyzed hydrolysis of the peptide was also used as a criterion of purity. The amount of L-amino acid formed was always within 2% of theory as determined by ninhydrin analysis. Only the first acid was cleaved by enzymatic hydrolysis.

*Activity Measurement.* Stock 0.2 M solutions of peptides were prepared in 0.8 M NaCl–0.05 M Tris (pH 7.5) and extracted with a carbon tetrachloride solution of dithizone to remove metals before use. All further dilutions were performed with 1 M NaCl–0.05 M Tris (pH 7.5) buffer previously extracted with dithizone.

Stock solutions of metalloenzyme ($10^{-4}$ M) containing metal ions[4] ($10^{-3}$ M) were diluted daily to $10^{-5}$ M with buffer, containing metal ion ($10^{-3}$ M) and kept at 4°. Further dilutions to $10^{-6}$ or $10^{-7}$ M were made with buffer containing metal ion ($10^{-4}$ M) and kept on ice until used. These solutions, though stable for at least 2 hr, were used within 1 hr.

All rate measurements were determined in 1.0 M NaCl–0.05 M Tris (pH 7.5) buffer at 25 ± 0.1°. Metalloenzymes other than the native enzyme were assayed in the presence of metal ion ($10^{-4}$ M). Assays were performed over the concentration range of $5 \times 10^{-5}$ to $2 \times 10^{-1}$ M substrate by a modification of the ninhydrin method (Snoke and Neurath, 1949) using a Technicon Auto Analyzer. Depending on the concentration range being inspected assay solutions from 0.9 to 6.8 ml were prepared and 0.1 or 0.2 ml of enzyme was added to initiate the reaction. Sample aliquots from 0.1 to 1 ml were withdrawn at fixed time intervals and the reaction was quenched by addition to 1 ml of 1 M citrate buffer (pH 5.0) containing EDTA ($10^{-4}$ M) in 2.5 ml of polystyrene cups. Standard curves were prepared employing the amino acid being formed during the hydrolysis. Under these conditions full hydrolysis of peptides forming phenylalanine gave an optical density of 0.40 unit at $1 \times 10^{-4}$ M substrate.

Initial velocities (*i.e.*, less than 10% hydrolysis) could be determined directly from the recordings. A least-squares program was used to calculate the kinetic parameters, $k_{cat}$, $K_m$, or $K_I$ from the Lineweaver–Burk or Dixon plots. The errors in the intercept and slope were calculated from the standard error of estimate, standard deviations, and the Student t distribution at the 70% confidence level.

## Results

*Hydrolysis of Tripeptides by Metallocarboxypeptidases.* Lineweaver–Burk plots for the carboxypeptidase-catalyzed hydrolysis of CbzGlyGly-L-Phe and BzGlyGly-L-Phe are linear within the concentration range $2 \times 10^{-4}$ to $1 \times 10^{-2}$ M for all of the metalloenzymes investigated. In contrast, the double-reciprocal plot for the hydrolysis of the dipeptide BzGly-L-Phe deviates markedly from linearity in this region, indicative of substrate activation (Figure 1). BzGlyGly-L-Phe hydrolysis does not exhibit substrate inhibition up to 0.17 M and inhibition by CbzGlyGly-L-Phe manifests only above 0.02 M (Figure 2). Above 0.01 M substrate the velocity *vs.* substrate profiles for both of these substrates resemble that of their smaller analogs. The time course of hydrolysis of the tripeptides for all the metalloenzymes studies is also consistent with simpler kinetics. This includes the nickel derivative

[4] No excess zinc ion added to the native enzyme.

TABLE I: Kinetic Constants for Peptide Hydrolysis by Metallocarboxypeptidase.[a]

| Peptide | [(CDP)Zn] | | [(CPD)Ni] | | [(CPD)Co] | | [(CPD)Mn] | |
|---|---|---|---|---|---|---|---|---|
| | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ |
| BzGlyGly-L-Phe | 8.0 | 1.2 | 7.4 | 1.1 | 6.0 | 5.9 | 2.9 | 0.23 |
| CbzGlyGly-L-Phe | 2.5 | 8.0 | 2.5 | 8.6 | 2.0 | 17.0 | 1.3 | 3.6 |
| CbzGlyGly-L-Leu | 30 | 6.5 | | | 24 | 16.7 | 13 | 2.3 |
| CbzGlyGly-L-Val | 170 | 3.1 | | | | | | |
| CbzGlyGlyGly-L-Phe | 9 | 3.3 | | | | | | |
| CiGlyGly-L-Phe | 8 | 3.5 | | | | | | |
| BzGlyGly-L-Leu | 70 | 0.79 | | | | | | |

[a] Assays performed in 0.05 M Tris–1 M NaCl (pH 7.5, 25°), containing $1 \times 10^{-4}$ M metal ion.[4] Units for $K_m$ and $k_{cat}$ are M $\times 10^{-4}$ and $min^{-1} \times 10^{+3}$, respectively.

which displayed neither first- nor zero-order kinetics when assayed *vs.* CbzGly-L-Phe at 25°.[5] Zero-order kinetics are obtained for 0.01 M CbzGlyGly-L-Phe until hydrolysis is about 60% complete, consistent with a $K_m$ lower than $10^{-3}$ M ($K_m = 2.5 \times 10^{-4}$ M). For BzGlyGly-L-Phe, at a concentration of $2 \times 10^{-4}$ M, first-order kinetics are obtained up to at least 80% of hydrolysis, consistent with a $K_m$ of $1 \times 10^{-3}$.

*Acetylcarboxypeptidase-Catalyzed Hydrolysis of Oligopeptides.* Carboxypeptidase is acetylated readily with *N*-acetylimidazole (Simpson *et al.*, 1963), the peptidase activity decreasing to as low as 2 to 3% of the native enzyme when assayed *vs.* CbzGly-L-Phe under standard conditions. Since tripeptides might bind differently than their smaller analogs, peptidase activity of acetylcarboxypeptidase was assayed with a series of longer peptide substrates. At a substrate concentration of $1 \times 10^{-3}$ M the per cent residual peptidase activity for acetylcarboxypeptidase A (Anson) is 3.1% for CbzGly-L-Phe, 2.5% for CbzGlyGly-L-Phe, 2.5% for CbzGlyGlyGly-L-Phe, and 3.4% for CbzPheGlyGly-L-Phe. Hence, increasing the length of the peptide to a tri- or tetrapeptide does not change the residual peptidase activity significantly. The hydrolysis of CbzGlyGly-L-Phe by acetylcarboxypeptidase was studied over the substrate concentration range from $2 \times 10^{-4}$ to $2 \times 10^{-3}$ M. The $k_{cat}$ is 490 $min^{-1}$ and $K_m$ is $2.5 \times 10^{-4}$ M. The value of $K_m$ agrees with that obtained for the hydrolysis of CbzGlyGly-L-Phe by the native enzyme (Table I).

Cox carboxypeptidase was acetylated with a 50-, 100-, 200-, and 500-fold molar excess of *N*-acetylimidazole. In all instances the esterase activity of hippuryl-β-DL-phenyllactic acid at 0.01 M is increased by 600%. Peptidase activity for CbzGly-L-Phe (0.02 M) and CbzGlyGly-L-Phe (0.01 M) is reduced to 7–8% of the control when a 100-fold molar excess of *N*-acetylimidazole is employed and is not decreased further using a 200- or 500-fold molar excess of the agent. The kinetic parameters determined over a range of CbzGlyGly-L-Phe concentration from $1.6 \times 10^{-4}$ to $1 \times 10^{-3}$ M are 790 $min^{-1}$ and $4.5 \times 10^{-4}$ M for the enzyme modified with a 100-fold molar excess. Over the higher substrate concentration range from $10^{-3}$ to $10^{-1}$ M the kinetic parameters are 720 $min^{-1}$ and $1.8 \times 10^{-3}$ M. In addition, the substrate velocity curve for the acetylated enzyme displays no substrate inhibition up to 0.18 M CbzGlyGly-L-Phe.

*Effect of Activators of Dipeptides on the Hydrolysis of Oligopeptides.* At initial substrate concentrations of $2 \times 10^{-4}$ M BzGlyGly-L-Phe and CbzGlyGly-L-Phe, neither 0.10 M benzoylglycylglycine nor 0.10 M carbobenzoxyglycylglycine, products of their respective hydrolyses, appreciably affect the rates of tripeptide hydrolysis. Similarly, cyclohexanol, a good activator of dipeptides, has no affect on the hydrolysis of these or longer peptides such as CbzGlyGlyGly-L-Phe or CbzPheGlyGly-L-Phe.

Carbobenzoxyglycine, a product of the hydrolysis of CbzGly-L-Phe, strongly activates dipeptide substrates at substrate concentrations below $1 \times 10^{-2}$ M (Davies *et al.*, 1968a,b). In marked contrast CbzGly inhibits the hydrolysis of BzGlyGly-L-Phe and CbzGlyGly-L-Phe by the native enzyme with a $K_I$ of approximately $2.7 \times 10^{-2}$ M (Table II). Both Dixon (Figure 3) and Lineweaver–Burk plots are indicative of competitive inhibition.

*Inhibitors of the Hydrolysis of BzGlyGlyPhe and CbzGlyGly-L-Phe.* β-Phenylpropionate is a competitive inhibitor of the carboxypeptidase-catalyzed hydrolysis of hippuryl-L-mandelate (Kaiser and Carson, 1965). It also inhibits the hydrolysis of hippuryl-β-DL-phenyllactic acid (Coleman and Vallee, 1964). The inhibition constant calculated[5] from a $V_i/V_c$ plot at $2 \times 10^{-4}$ M hippuryl-β-DL-phenyllactic acid, $1.3 \times 10^{-4}$, is in good agreement with a $K_i$ of $1.5 \times 10^{-4}$ (Kaiser and Carson, 1965). Figure 4 shows Dixon plots for the β-phenylpropionate-inhibited hydrolysis of BzGlyGlyPhe and CbzGlyGly-L-Phe, when substrate and inhibitor concentration ranges were employed as shown in Table II. Extrapolation to the abscissa shows a common point of intersection, indicative of noncompetitive inhibition. $K_I$ values calculated from these plots for CbzGlyGly-L-Phe and BzGlyGlyPhe hydrolysis are in close agreement with those found for esters. At much higher concentrations of β-phenylpropionate, *i.e.*, $> 10^{-3}$ M, the Dixon plots deviate positively from linearity, indicative of additional inhibitory modes of binding.

Phenylacetate and indole-3-acetate also inhibit the hydrolysis of CbzGlyGly-L-Phe noncompetitively (Figure 5). Table II shows the inhibitor concentration ranges employed. Since the noncompetitive inhibition obtained here might be characteristic only of the longer peptides examined, the

[5] Unpublished observations of R. C. Davies.

TABLE II: Inhibition Constants for Inhibitors of Peptide Hydrolysis by Carboxypeptidase.

| Peptide | Inhibitor | Inhibitor Concentration Range $\times 10^{-4}$ M | $K_i$[a] | Type Inhibition |
|---|---|---|---|---|
| CbzGlyGly-L-Phe | Carbobenzoxyglycine | 300–1300 | $2.9 \pm 0.5 \times 10^{-2}$ | Competitive |
| CbzGlyGly-L-Phe | Indole-3-acetic acid | 0.5–4 | $1.7 \pm 0.1 \times 10^{-4}$ | Noncompetitive |
| CbzGlyGly-L-Phe | Phenylacetic acid | 2–16 | $7.3 \pm 0.7 \times 10^{-4}$ | Noncompetitive |
| CbzGlyGly-L-Phe | β-Phenylpropionic acid | 0.5–3 | $1.2 \pm 0.1 \times 10^{-4}$ | Noncompetitive |
| BzGlyGly-L-Phe | Carbobenzoxyglycine | 200–1200 | $2.5 \pm 0.5 \times 10^{-2}$ | Competitive |
| BzGlyGly-L-Phe | β-Phenylpropionic acid | 0.5–4 | $1.1 \pm 0.1 \times 10^{-4}$ | Noncompetitive |
| BzGlyGly-L-Phe | Glycyl-L-tyrosine | 0.7–4 | $1.0 \pm 0.2 \times 10^{-4}$ | Competitive |
| BzGly-L-Phe | β-Phenylpropionic acid | 0.5–5 | $1.9 \pm 0.5 \times 10^{-4}$ | Noncompetitive |

[a] Inhibition constants determined from Dixon plots for noncompetitive inhibition and from Lineweaver–Burk plots for competitive inhibition.

inhibition by β-phenylpropionate of the hydrolysis of BzGly-L-Phe was investigated over the substrate concentration range from $1 \times 10^{-4}$ to $1 \times 10^{-3}$ M, where linear double-reciprocal plots can be obtained (Davies *et al.*, 1968b). Inhibition studies at 0, 1, and $3 \times 10^{-4}$ M β-phenylpropionate indicate that the acid is also a noncompetitive inhibitor of this substrate with a $K_i = 1.9 \times 10^{-4}$ M. In addition $V_i/V_e$ plots at $3 \times 10^{-2}$ and $3 \times 10^{-4}$ M BzGly-L-Phe at eight different concentrations of β-phenylpropionate yield inhibition constants of $6 \times 10^{-4}$ and $2 \times 10^{-4}$ M, respectively.

Glycyl-L-tyrosine, a very poor substrate for carboxypeptidase, competitively inhibits the hydrolysis of the tripeptides (Figure 6).

## Discussion

A number of studies have emphasized the complex kinetic behavior of the native carboxypeptidase-catalyzed hydrolysis of acylamino acids, dipeptides, and their ester analogs and such kinetic studies have been reviewed (Neurath and Schwert, 1950; Smith, 1952; Carson and Kaiser, 1966; Vallee *et al.*, 1968; Lipscomb *et al.*, 1968; Vallee and Riordan, 1968).

The kinetics of the carboxypeptidase A catalyzed hydrolysis of a number of N-terminal blocked depeptide substrates and ester analogs show varying degrees of substrate activation and inhibition (McClure *et al.*, 1964; Whitaker, 1966; Davies *et al.*, 1968b; Vallee *et al.*, 1968). With these substrates the determination of meaningful kinetic constants for both the native and modified carboxypeptidase has proven difficult.

Identification of the source of such kinetic ambiguities was first sought by replacing zinc at the active site of the native enzyme by other metals. However, the anomalies persist when cobalt, manganese, or nickel are substituted (Davies *et al.*, 1968b). Such kinetic anomalies could also arise from other features intrinsic to the enzyme, the properties of the substrate employed or both.

Chemical modification of the reactive tyrosyl groups yield enzymes which have markedly reduced peptidase while retaining esterase activity toward hippuryl-β-DL-phenyllactic acid. Such modifications do not eliminate the marked

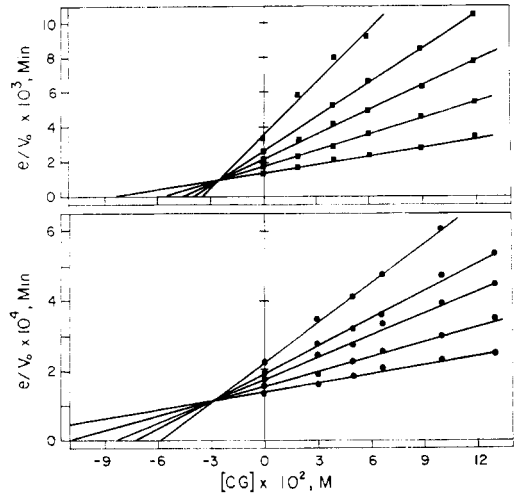


FIGURE 3: Dixon plots for inhibition by CbzGly (CG) of the carboxypeptidase-catalyzed hydrolysis of ByGlzGly-L-Phe (■) and CbzGly-Gly-L-Phe (●). Concentrations of substrate were 2.67, 4.0, 5.34, 8.0, and 13.3 $\times 10^{-4}$ M for BzGlyGly-L-Phe and 2.07, 4.06, 5.41, 8.10, and 13.5 $\times 10^{-4}$ M for CbzGlyGly-L-Phe. Condition of assay: 1.0 M NaCl–0.05 M Tris (pH 7.5) and 25°.

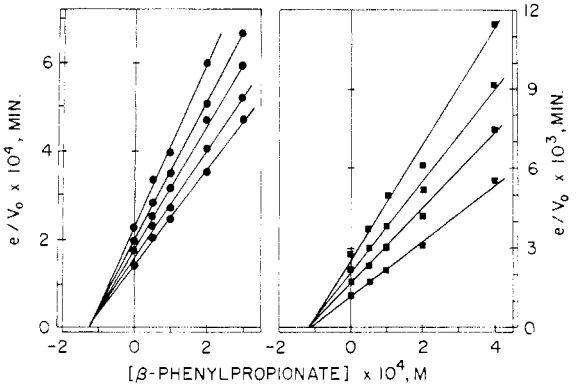


FIGURE 4: Dixon plots for the noncompetitive inhibition of BzGlyGly-L-Phe (■) and CbzGlyGly-L-Phe (●) hydrolysis by β-phenylpropionate. Concentrations of substrate were 3.6, 5.2, 8.0, and 20. $\times 10^{-4}$ M for BzGlyGly-L-Phe and 2.7, 4.0, 5.4, 8.15, and 13.5 $\times 10^{-4}$ M for CbzGlyGly-L-Phe. Assay conditions were 1.0 M NaCl–0.05 M Tris (pH 7.5) and 25°.

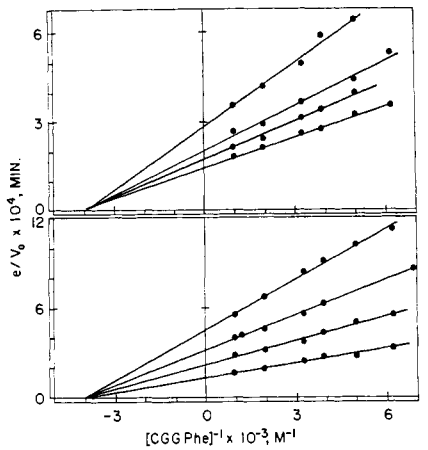


FIGURE 5: Lineweaver–Burk plots for inhibition of CbzGlyGly-L-Phe hydrolysis by phenylacetic acid, upper panel, and indole-3-acetic acid, lower panel. Essentially the same inhibition constant for phenylacetate was also obtained from $V_i/V_0$ plots for six substrate concentrations in the range of $1.6 \times 10^{-3}$ to $1.6 \times 10^{-2}$ M.

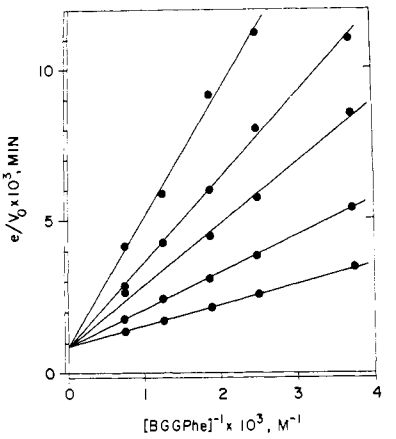


FIGURE 6: Inhibition of BzGlyGly-L-Phe hydrolysis by glycyl-L-tyrosine. Inhibitor concentrations were 0.67. 1.67, 2.65, and $4 \times 10^{-4}$ M. Conditions of assay: 1.0 M NaCl–0.05 M Tris (pH 7.5) and 25°.

hippuryl-β-DL-phenyllactic acid inhibition of the native enzyme. Actually, the velocity *vs.* substrate curves are displaced toward higher substrate concentrations (Riordan *et al.*, 1967; Vallee *et al.*, 1968).

The kinetic anomalies observed with substituted dipeptides may be attributable to the fact that the binding sites of the enzyme are "designed" to accept larger substrates. Although kinetic studies have been made on a wide variety of both peptide and ester substrates differing in N-terminal blocking groups and C-terminal residues, these substrates have all been dipeptides, acylamino acids, and their ester analogs. It is well known that carboxypeptidase can bind and hydrolyze synthetic substrates larger than the dipeptides or esters commonly employed (Coombs and Vallee, 1966; Schechter and Berger, 1966) and its action on proteins has long served as an accepted procedure for determining their C-terminal residues. A systematic study of the effect on $K_m$ and $k_{cat}$ of increasing the chain length of peptides and varying their N-terminal blocking groups has shown that changes in substrate composition are discernible over a range of five amino acid residues (Schechter and Berger, 1966; Abramowitz *et al.*, 1967). If the dimensions of the active center were to offer more potential points of contact than available in a given substrate chosen for catalysis, the binding of the substrate could result in multiple interactions, not necessarily consistent with a simple Michaelis–Menten mechanism. The observation of substrate and product inhibition and activation of dipeptides and their ester analogs is consistent with this view (Davies *et al.*, 1968a,b).

Increasing the length of the substrate or bulk of the blocking group would place more constraints on its binding and some forms of nonproductive binding might be obviated. Hence, in such a case more normal Michaelis–Menten kinetics would be expected. Indeed, the hydrolysis of the peptides resulting from internal chain extension of CbzGly-L-Phe and BzGly-L-Phe by one glycine unit no longer display substrate activation toward any of the active metalloenzymes (Figure 1).

The elimination of substrate activation from peptide hydrolysis when tripeptides are employed as substrates facilitates studies of the effect of modifiers. The activation of BzGly-L-Phe and CbzGly-L-Phe by the products BzGly and CbzGly as well as by a number of compounds with analogous properties to these N-terminal blocking groups has been documented (Davies *et al.*, 1968a). These same compounds inhibit the hydrolysis of hippuryl-β-DL-phenyllactic acid suggesting differences in the modes of ester and peptide binding.

In contrast to its effect on dipeptide hydrolysis, CbzGly competitively *inhibits* the hydrolysis of CbzGlyGly-L-Phe and BzGlyGly-L-Phe (Figure 3). The inhibition constant, $2.7 \times 10^{-2}$ M, is close to that obtained for the competitive inhibition of CbzGly on the hydrolysis of cinnamoyl-β-phenyllactic acid ($K_i = 1.6 \times 10^{-2}$ M), an ester which does not exhibit substrate activation (Awazu *et al.*, 1967). However, the products of the longer peptides CbzGlyGly and BzGlyGly inhibit their hydrolysis only slightly, although they activate CbzGly-L-Phe and BzGly-L-Phe hydrolysis as well as CbzGly. Furthermore, cyclohexanol, a good activator of a number of dipeptides,[6] did not affect any of the oligopeptides tested here. Therefore, the slight difference in the inhibition constants for CbzGly, when present in excess during tripeptide as compared with that during cinnamoyl-β-phenyllactic acid hydrolysis, may not be due to differences in experimental conditions or of enzyme used, but to different modes of binding of substrate, of CbzGly or both. If dipeptide substrates are bound in multiple modes, smaller products such as CbzGly or similar sized compounds might bind in equal or greater numbers. Therefore, CbzGly or other "modifiers" might have no effect, inhibit, or activate the hydrolysis of the substrate being investigated, depending on the mode of binding of a given substrate. Correlations between binding data at equilibrium, kinetics of activation of dipeptide and inhibition of oligopeptides hydrolysis, presently under study, should provide answers for some of these questions.

The noncompetitive inhibition of CbzGlyGly-L-Phe hydrol-

[6] Cyclohexanol (0.05 M), causes a 300% activation of the hydrolysis of $10^{-3}$ M CbzGly-L-Phe, CbzGly-L-Tyr, CbzGly-L-Trp, CbzGly-L-Leu, BzGly-L-Phe, and AGLeu.

ysis by indole-3-acetate, phenylacetate, and β-phenylpropionate is also striking since the latter two competitively inhibit the hydrolysis of the ester hippuryl-L-mandelate (Kaiser and Carson, 1965). Similarly, β-phenylpropionate noncompetitively inhibits the hydrolysis of BzGly-L-Phe and BzGlyGly-L-Phe while it competitively inhibits hippuryl-β-DL-phenyllactic acid hydrolysis.

The $K_i$'s for β-phenylpropionate and phenylacetate inhibition are in good agreement with past data obtained both by direct binding over this concentration range (Coleman and Vallee, 1964) and by kinetics of competitive inhibition of ester hydrolysis (Kaiser and Carson, 1965). The close correspondence between $K_i$ values for peptide and ester inhibition by these aromatic acids suggests similar or identical enzyme inhibitor complexes, but since the type of inhibition differs, it would seem that the overall modes of hydrolysis of these peptides and esters are different.

Based on direct binding studies with [$^{14}$C]β-phenylpropionate and metal ion exchange data, Coleman and Vallee (1962, 1964) concluded that the binding of 1 mole of β-phenylpropionate required the presence of the metal and thus likely involved a metal–carboxyl group interaction. In addition, gel filtration studies showed that in contrast to peptides neither ester substrates nor the inhibitor β-phenylpropionate prevented restoration of zinc to the apoenzyme. Nuclear magnetic resonance studies (Shulman *et al.*, 1966) indicated that β-phenylpropionate displaces a water molecule from manganese carboxypeptidase and crystallographic studies have shown that *p*-iodo-β-phenylpropionate binds to four distinct regions, two of which are near the zinc atom. Binding of these latter two molecules was thought to be mutually exclusive and the carboxyl group was thought to be a ligand of the zinc atom (Steitz *et al.*, 1967) in agreement with suggestions both from chemical binding and nuclear magnetic resonance studies. Both X-ray crystallography (Lipscomb *et al.*, 1968) and chemical modification (Vallee and Riordan, 1968) have suggested that an arginyl residue may be a binding group for the C-terminal carboxyl group of peptide substrates.

However, chemical modifications of arginine in carboxypeptidase, while diminishing the rate of CbzGly-L-Phe hydrolysis, actually increase that of hippuryl-β-DL-phenyllactic acid (Vallee and Riordan, 1968). Thus, binding of this ester does not seem to require the integrity of the arginyl residue shown to be involved in peptidase activity, a finding consistent with the "dual-site" model of carboxypeptidase (Vallee *et al.*, 1968).

In this regard the values of $K_m$ for the ester, hippuryl-β-DL-phenyllactic acid, when hydrolyzed by a number of [(CPD)Me] derivatives, varies more than does the corresponding values of $k_{cat}$. In contrast, $k_{cat}$ varies more than does $K_m$ when the peptides BzGlyGly-L-Phe and CbzGlyGly-L-Phe serve as the standard substrates. Such data imply significant but perhaps varying participation of metals in peptide and ester catalysis, respectively. It will be important to extend these studies to ester analogs of oligopeptides and yet others with less complex substrate inhibition than hippuryl-β-DL-phenyllactic acid.

The identities or differences in binding of peptides and esters are important in relation to the mechanism of action of this enzyme toward these substrates. A number of chemical modification and kinetic studies have suggested that the hydrolytic mechanism for peptides and esters might differ (Vallee *et al.*, 1963, 1968, and references therein). Thus far, no investigation of this problem has resulted in data on *peptides* which would conclusively demonstrate that they are, indeed, hydrolyzed by a mechanism similar to that of esters (Kaiser and Carson, 1965; Bender *et al.*, 1965; Whitaker *et al.*, 1966). But the results of some kinetic studies of the effect of pH and inhibitors on the hydrolysis of esters have been inferred to suggest that the binding sites of peptides and esters must be very similar, if not the same (Kaiser and Carson, 1965; Carson and Kaiser, 1966). However, this conclusion was based on comparisons with peptide substrates which exhibit substrate activation and inhibition. Comparisons between these esters and peptides displaying Michaelis–Menten kinetics do reveal significant differences in the manner of their hydrolyses (Auld, 1969; D. S. Auld and B. L. Vallee, in preparation). Competitive inhibitors of the hydrolysis of such esters noncompetitively inhibit the hydrolysis of such peptides. In addition, steady-state kinetics of the hydrolysis of CbzGlyGly-L-Phe between pH 4.5 and 10 have shown that the pH profile for $k_{cat}$ is sigmoidal with an inflection point at pH 6.1 (Auld, 1969). In contrast, the ester acetylmandelate is characterized by a narrow bell-shaped pH profile for $k_{cat}$ (Kaiser and Carson, 1965).

It should be emphasized that any discussion of peptidase or esterase kinetics, based on studies of a particular substrate, need not pertain to all substrates. It has been pointed out that in such instances semantic laxity might inadvertently imply conclusions which data currently available do not allow (Vallee *et al.*, 1969).

It has been proposed that for peptide hydrolysis the Michaelis constant, $K_m$, could be a ratio of the dynamic constants of the consecutive reactions ($k_2$:$k_1$) rather than a thermodynamic equilibrium constant (Lumry *et al.*, 1951).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

However, no direct relationship between the values of $K_m$ and $k_{cat}$ is found when a series of metallocarboxypeptidases is studied while acting on one of these tripeptides or when one particular metallocarboxypeptidase is examined while acting on a series of tripeptides. Thus, for the hydrolysis of BzGlyGly-L-Phe, CbzGlyGly-L-Phe, and CbzGlyGly-L-Leu the $k_{cat}$ for [(CPD)CO] is 8–30-fold greater than that of [(CPD)Mn] while their respective $K_m$'s differ 2-fold.

The values of $k_{cat}$ and $K_m$ for tripeptides which differ only in their C- and N-terminal blocking groups are also of interest in this regard. On going from CbzGlyGly-L-Phe to CbzGlyGly-L-Leu to CbzGlyGly-L-Val, $K_m$ varies by a factor of nearly 100 while $k_{cat}$ is essentially constant. In contrast, holding the C-terminal residue constant (*e.g.*, Phe or Leu) and changing the N-terminal residue from carbobenzoxy- to benzoyl- changes $k_{cat}$ more than $K_m$. The marked effects of the side chains of the third and fourth amino acids on $k_{cat}$ have been noted previously (Abramowitz *et al.*, 1967). These findings are compatible with $K_m$ being a measure of the substrate's binding affinity ($k_{-1}/k_1$). In agreement with past conclusions based on studies of other substrates, the nature of the C-terminal residue would appear to be most important for good binding of the substrate (Neurath and Schwert, 1950). However, the marked effect on $k_{cat}$ of the N-terminal residue suggests that enzyme–substrate interactions at loci removed

from the site of the bond breaking may alter the conformation of the catalytic center of carboxypeptidase.

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