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The Interaction of Polypeptides and Proteins with Apocarboxypeptidase A*

Thomas L. Coombs and Bert L. Vallee

ABSTRACT: A gel filtration technique has been employed to characterize the binding of protein and polypeptide substrates to apocarboxypeptidase A-δ and to compare their behavior with that of dipeptides. Tyrosyl residues of the enzyme facilitate the binding both of di- and polypeptides. The introduction of increasing proportions of charged lysyl or glutamic acid residues into a tyrosyl copolymer, however, reduces and eventually abolishes binding. Both polypeptides and proteins bind more firmly to apocarboxypeptidase than dipeptides. Acetylation of the apoenzyme with *N*-acetylimidazole, while destroying dipeptide binding, does not abolish the formation of polypeptide or protein complexes. These findings suggest that the macromolecules bind to groups on the surface of the apoenzyme, in addition to those concerned with dipeptide binding, resulting in greater stability of the apoenzyme with polypeptide and protein substrates. Further, acetylation both of the substrate and of the apoenzyme suggests that these additional groups are neither free tyrosyl nor lysyl residues but may be hydrophobic in nature. The poly-L-lysyl-L-tyrosyl copolymer employed was not hydrolyzed, even though it binds to the apoenzyme and presumably to the metalloenzyme, in accord with previous observations with certain dipeptide substrates. These observations also suggest the existence of binding sites on the surface of the enzyme in addition to those employed for hydrolysis of dipeptides. The characterization of such polyamino acid– and protein–enzyme complexes is continuing.

The specificity characteristics and mode of action of carboxypeptidase A have been elucidated largely through kinetic studies of the hydrolysis of synthetic dipeptides and their analogs (Bergmann and Fruton, 1942; Neurath and Schwert, 1950; Smith, 1951; Neurath, 1960). Under physiological conditions, the enzyme is also likely to act on polypeptides and proteins, but technical problems have discouraged the kinetic study of their hydrolysis. However, where comparisons have been possible, significant differences in the kinetics of hydrolysis of dipeptides and of substrates with higher molecular weight have already been demonstrated (Green and Stahmann, 1952; Neurath *et al.*, 1954; Harris and Knight, 1955; Davie, 1956; Davie *et al.*, 1959; Miller, 1964; Katchalski *et al.*, 1964; Lehrer *et al.*, 1965; Schechter and Berger, 1965; Schechter *et al.*, 1965; Slobin and Carpenter, 1963a,b, 1966).

Equilibrium methods employing gel filtration and isotope exchange have contributed further insight into the mechanism of dipeptide hydrolysis. The reversible binding of $^{65}Zn^{2+}$ to metal-free apocarboxypeptidase has permitted a separation of the initial, substrate binding step from the subsequent catalytic event (Coleman and Vallee, 1962a,b, 1964). Peptide substrates bind to apocarboxypeptidase and prevent the otherwise instantaneous restoration of zinc carboxypeptidase; peptides which are not hydrolyzed do not prevent the restoration of the metalloenzyme and presumably do not bind. Acetylation of the "free" tyrosyl residues of carboxypeptidase abolishes the capacity of apocar-

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boxypeptidase to bind dipeptide substrates (Vallee, 1964a,b; Coleman *et al.*, 1964, 1966), accounting, at least in part, for the loss of peptidase activity on acetylation (Simpson *et al.*, 1963).

The gel filtration technique has now been employed to compare the binding of proteins and polypeptides with that of dipeptides. A preliminary report has been given (Coombs and Wacker, 1965).

Materials and Methods

Beef Pancreas Carboxypeptidase [*(CPD)Zn*].[1] Four-times-recrystallized zinc carboxypeptidase A-δ was prepared from beef pancreas acetone powder by the procedure described by Allan *et al.* (1964). The proteolytic coefficient (*C*) of this preparation ranged from 25 to 30 at pH 7.5, 0°. The zinc:protein ratio was 2050 μg/g or 1.1 g-atoms/34,600 mol wt (Bargetzi *et al.*, 1963).

Metal-Free Apocarboxypeptidase (*CPD*) was prepared by dialyzing the native enzyme against 1,10-phenanthroline as described (Coombs *et al.*, 1964). The final preparations contained from 20 to 40 μg of zinc/g of protein, or less than 2% of the original zinc content, and the peptidase and esterase activities were proportionately low. The addition of Zn^{2+}, equivalent to 1 g-atom/mole of apoenzyme, fully restored both activities.

Apoacetylcarboxypeptidase (*AcCPD*) was prepared from carboxypeptidase which had been acetylated with *N*-acetylimidazole according to the procedure of Simpson *et al.* (1963). The acetylated enzyme was dialyzed first against 1 M NaCl–0.02 M Veronal buffer, pH 7.5, in order to remove excess *N*-acetylimidazole and reaction products, and then against 1,10-phenanthroline, as described for apocarboxypeptidase. The final preparations contained less than 2% of the original zinc content and had less than 2% of the esterase activity of the original acetyl enzyme. On adding Zn^{2+}, equivalent to 1 g-atom/mole of apoacetyl enzyme, about 85% of the esterase activity of the acetyl enzyme was restored, reflecting a greater lability of apoacetylcarboxypeptidase compared to apocarboxypeptidase.

Ribonuclease A, lyophilized, phosphate-free (Worthington Biochemical Corp.), *lysozyme*, twice crystallized (Worthington Biochemical Corp.), and *α-lactalbumin*, once crystallized (a gift of Dr. W. G. Gordon, Eastern Regional Research Laboratories, Philadelphia, Pa.), were found to be free of contaminating zinc and were all used without further purification.

Poly-L-*lysine*, *poly*-L-*lysyl*-L-*tyrosine* (24:1), *poly*-L-*glutamyl*-L-*lysyl*-L-*tyrosine* (9:6:1), and *poly*-L-*glutamyl*-L-*tyrosine* (9:1) were obtained from New England Nuclear Corp., Boston, and Pilot Chemical Co., Watertown, Mass. Analyses showed significant amounts of zinc contamination in these products, which was readily removed by dialysis, first against 1,10-phenanthroline followed by metal-free buffer.

Poly-L-*lysyl*-L-*tyrosine* (10:1) was synthesized from the *N*-carboxyamino anhydrides of ϵ-carbobenzoxy-L-lysine and *O*-carbobenzoxy-L-tyrosine mixed in the molar ratio indicated. Polymerization was initiated with sodium methoxide, and the protecting groups on the lysine and tyrosine side chains were removed with hydrogen bromide in glacial acetic acid after

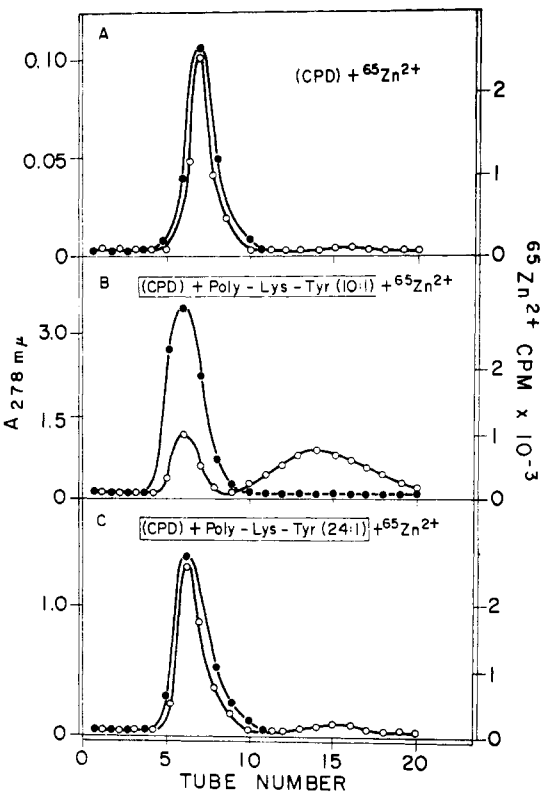

FIGURE 1: Binding of $^{65}Zn^{2+}$ to apocarboxypeptidase in the presence and absence of poly-L-lysyl-L-tyrosine (10:1 and 24:1). Samples (2 ml) of 6×10^{-6} M apocarboxypeptidase plus 6×10^{-6} M $^{65}Zn^{2+}$ (A); plus 3.7×10^{-3} M poly-L-lysyl-L-tyrosine (10:1) plus 6×10^{-6} M $^{65}Zn^{2+}$ (B); plus 4.8×10^{-3} M poly-L-lysyl-L-tyrosine (24:1) plus 6×10^{-6} M $^{65}Zn^{2+}$ (C), were passed over a 1 × 30 cm Sephadex G-25 column at a flow rate of 1 ml/min and eluted at 4° with 1 M NaCl–0.02 M Veronal, pH 7.5. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fraction (○) was measured. The enclosures, *e.g.* (CPD) + poly-Lys-Tyr (10:1), represent the order of additions. Thus, poly-L-lysyl-L-tyrosine (10:1) was added to apocarboxypeptidase first, then $^{65}Zn^{2+}$.

[1] The abbreviations used are in formulations only and when required for differentiation: [(CPD)Zn], zinc carboxypeptidase A, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc; [(AcCPD)Zn], zinc carboxypeptidase A acetylated with *N*-acetylimidazole; Ac_3RNAase, ribonuclease A, with the three "free" tyrosyl residues acetylated (Simpson and Vallee, 1966); CGP, carbobenzoxyglycyl-L-phenylalanine.

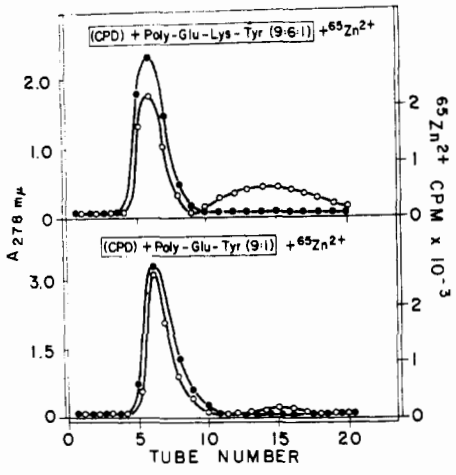

FIGURE 2: Binding of $^{65}Zn^{2+}$ to apocarboxypeptidase in the presence of poly-L-glutamyl-L-lysyl-L-tyrosine (9:6:1) and poly-L-glutamyl-L-tyrosine (9:1). Samples (2 ml) of 6×10^{-6} M apocarboxypeptidase plus copolymer, 20 mg/ml, plus 6×10^{-6} M $^{65}Zn^{2+}$ were passed over Sephadex G-25 at 4° with 1 M NaCl–0.05 M Tris, pH 7.5, as in Figure 1. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fraction (○) were measured. Enclosures as in Figure 1.

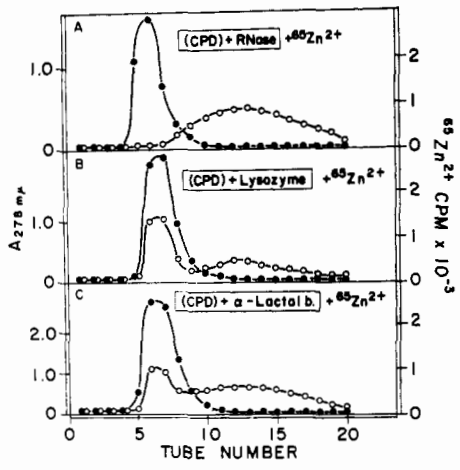

FIGURE 3: Binding of $^{65}Zn^{2+}$ to apocarboxypeptidase in the presence of ribonuclease, lysozyme, and α-lactalbumin. Samples (2 ml) of 6×10^{-6} M apocarboxypeptidase plus 3.6×10^{-4} M ribonuclease plus 6×10^{-6} M $^{65}Zn^{2+}$ (A); plus 3.6×10^{-4} M lysozyme plus 6×10^{-6} M $^{65}Zn^{2+}$ (B); plus 3.6×10^{-4} M α-lactalbumin plus 6×10^{-6} M $^{65}Zn^{2+}$ (C), were passed over Sephadex G-25 eluting at 4° with 1 M NaCl–0.05 M Tris, pH 7.5, as in Figure 1. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fraction (○) were measured. The radioactivity measurements shown for ribonuclease and α-lactalbumin were corrected for $^{65}Zn^{2+}$ binding to the substrate, as described in the text. Enclosures as in Figure 1.

polymerization (Katchalski, 1957). The polymer was dialyzed exhaustively against metal-free distilled water to remove low molecular weight components. The degree of polymerization was determined from equilibrium sedimentation runs in the Spinco Model E ultracentrifuge. The sedimentation studies indicated a weight-average molecular weight of 7700 ± 100 and a small degree of polydispersity.[2] Analysis of the final, dialyzed product showed it to be free of zinc and to contain the correct ratios of lysine and tyrosine.

$^{65}Zn^{2+}$ Standards. Spectrographically pure zinc rod (Johnson Matthey Co., Ltd., London, England) was dissolved in dilute metal-free hydrochloric acid and diluted to the required concentration with the buffers used to equilibrate the gel filtration columns (*vide infra*). $^{65}Zn^{2+}$ (U. S. Atomic Energy Commission) was added such that the radioactivity in the final 6×10^{-6} M solution of $^{65}Zn^{2+}$ employed in the gel filtration experiments was approximately 10,000 cpm/2 ml. The concentration of $^{65}Zn^{2+}$ added did not significantly alter the concentration of the initial Zn^{2+} solutions.

Buffers and other reagents were freed of contaminating metals by extraction with dithizone in carbon tetrachloride, and precautions were taken against zinc contamination from glassware, water, and reagents (Coombs *et al.*, 1964).

Protein concentration of carboxypeptidase was measured from the absorbance at 278 mμ. The molar absorptivity of native carboxypeptidase is 6.42×10^4 M^{-1} cm^{-1} and that of acetylcarboxypeptidase is 5.9×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Peptidase and esterase activities were determined as previously described using carbobenzoxyglycyl-L-phenylalanine and hippuryl-*dl*-β-phenyllactic acid as substrates, respectively (Coleman and Vallee, 1962a).

Zinc analyses were performed by means of atomic absorption spectrophotometry (Fuwa *et al.*, 1964).

Gel Filtration. A column of Sephadex G-25 (Pharmacia, Uppsala), 1 × 30 cm, was first washed with 2 N HCl and then with zinc-free distilled water in order to remove metal contamination. It was then equilibrated at 4° either with 1 M NaCl–0.05 M Tris, or 1 M NaCl–0.02 M Veronal buffers, pH 7.5. The flow rate of approximately 1 ml/min was controlled by gravity and 2-ml fractions were collected by an automatic fraction collector (Gilson Medical Electronics). Carboxypepti-

[2] The exclusion of the poly-L-lysyl-L-tyrosine copolymers by Sephadex G-25 (Figure 1) and cellulose dialysis membranes would indicate a mol wt >7700, the number obtained from sedimentation experiments performed by Dr. J. L. Bethune to whom we are indebted for these determinations. Charged oligomers, such as poly-L-lysyl-L-tyrosine, however, are known to behave as rigid rods and be refractory to penetration of pores in dialysis membranes or polydextran gel grains, thus acting anomolously as apparently high molecular weight compounds (H. Sober, personal communication). For this reason, the average molecular weights obtained from sedimentation were assumed to be applicable and used for calculation of the polymer concentrations.

dase and apocarboxypeptidase pass through this column without retardation and appear in the early fractions (4–10). $^{65}Zn^{2+}$, however, is retained and emerges in later fractions (10–20), consistent with the behavior of ions or small molecules on such columns. On addition of $^{65}Zn^{2+}$, both apocarboxypeptidase and apoacetylcarboxypeptidase instantaneously form the respective ^{65}Zn carboxypeptidases; these complexes are stable and do not dissociate either spontaneously or on passage through the Sephadex column (Figure 1A). Depending on their size, substrate molecules emerge either with carboxypeptidase or in later fractions. Protein and substrate molecules were detected by their absorbance at 278 mμ. The presence of a substrate, such as poly-L-lysine, which does not absorb appreciably in the 278-mμ region, was detected by the color at 570 mμ, developed after heating an aliquot of each fraction with ninhydrin (Moore and Stein, 1948). Zinc was assayed by counting the radioactivity of each fraction in a well-type scintillation counter (Tracer Laboratory).

Mixtures (2 ml) of apoenzyme, substrate, and $^{65}Zn^{2+}$ were prepared for the column as follows. Apoenzyme was dissolved in the buffer employed to equilibrate the column to yield a final concentration of 6×10^{-6} M and was then mixed for 30 sec with substrate of known concentration dissolved in the same buffer *followed* by addition of $^{65}Zn^{2+}$ to yield a final concentration of 6×10^{-6} M. The 2-ml sample containing all components was mixed further for 30 sec, applied to the column, and then eluted.

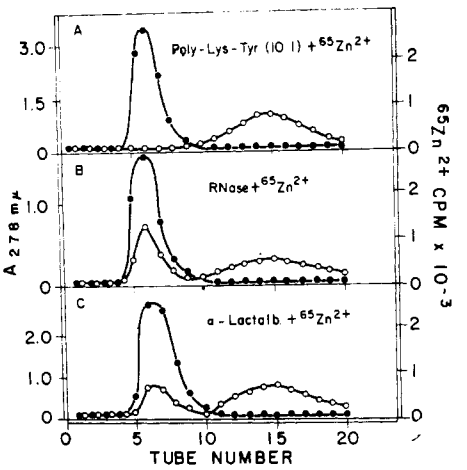

FIGURE 4: Binding of $^{65}Zn^{2+}$ to poly-L-lysyl-L-tyrosine (10:1), ribonuclease, and α-lactalbumin. Samples (2 ml) of 6×10^{-6} M $^{65}Zn^{2+}$ plus 3.7×10^{-3} M poly-L-lysyl-L-tyrosine (10:1) (A); plus 3.6×10^{-4} M ribonuclease (B); plus 3.6×10^{-4} M α-lactalbumin (C), were passed over Sephadex G-25, as in Figure 1, and eluted at 4° with 1 M NaCl–0.02 M Veronal, pH 7.5, for (A) and 1 M NaCl–0.05 M Tris, pH 7.5, for (B) and (C). Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fraction (○) were measured.

Results

Prevention of $^{65}Zn^{2+}$ Binding to Apocarboxypeptidase by Polypeptides. Carboxypeptidase readily hydrolyzes dipeptides containing an aromatic C-terminal amino acid. Hence, water-soluble polypeptides containing tyrosine were examined first. An L-lysyl-L-tyrosine copolymer in a molar ratio of 10:1 prevents the binding of $^{65}Zn^{2+}$ to the apoenzyme (Figure 1B). A 3.7×10^{-3} M solution of the polymer was the highest concentration that could be employed, since the viscosity of higher concentrations interfered with elution through the Sephadex column. At this concentration of the polymer, 50% of the radioactivity placed on the column is eluted in the position characteristic of free Zn^{2+}, and the radioactivity associated with apocarboxypeptidase in fractions 4–10 corresponds to 0.5 g-atom of $^{65}Zn^{2+}$ bound/mole of enzyme. The poly-L-lysyl-L-tyrosine copolymer emerges in fractions 4–10, together with apocarboxypeptidase. Under similar conditions, the poly-L-lysyl-L-tyrosine copolymer itself does not bind $^{65}Zn^{2+}$, as demonstrated in a control experiment in the absence of apocarboxypeptidase (Figure 4A).

Neither a copolymer of L-lysyl-L-tyrosine in a molar ratio of 24:1 nor poly-L-lysine prevents the binding of $^{65}Zn^{2+}$. Virtually all of the radioactivity is found in fractions 4–10, bound to apocarboxypeptidase (Figures 1A and C). A similar concentration of poly-L-glutamyl-L-lysyl-L-tyrosine tricopolymer (9:6:1) partially prevents the $^{65}Zn^{2+}$ binding; approximately 0.7 g-atom of $^{65}Zn^{2+}$ is bound/mole of apoenzyme (Figure 2).

An L-glutamyl-L-tyrosine copolymer (9:1) does not prevent binding of $^{65}Zn^{2+}$ to apocarboxypeptidase. The results are indistinguishable from those in the absence of substrate (Figures 1A and 2). Under the conditions of the experiments, none of these polypeptides binds significant quantities of $^{65}Zn^{2+}$.

Prevention of the Binding of $^{65}Zn^{2+}$ to Apocarboxypeptidase by Proteins. Proteins, known to be hydrolyzed by carboxypeptidase, were also studied. Ribonuclease, lysozyme, and α-lactalbumin all prevent the binding of $^{65}Zn^{2+}$ to the enzyme, though to varying degrees (Figures 3A–C). Ribonuclease, 3.6×10^{-4} M, completely prevents the formation of zinc carboxypeptidase, whereas in the presence of the same concentration of α-lactalbumin and lysozyme, 0.27 and 0.46 g-atom of $^{65}Zn^{2+}$, respectively, are bound to apocarboxypeptidase. The radioactivity measurements for ribonuclease and α-lactalbumin shown in Figures 3A and C have been corrected for the fact that these proteins themselves bind significant quantities of $^{65}Zn^{2+}$ at the high concentrations of substrate required to prevent formation of the zinc enzyme completely (Figures 4B and C), thereby rendering the results of the procedure ambiguous under these conditions. Lysozyme, however, does not bind $^{65}Zn^{2+}$ significantly.

Ovalbumin, 3.6×10^{-4} M, which is not hydrolyzed by carboxypeptidase (Steinberg, 1954) behaves like

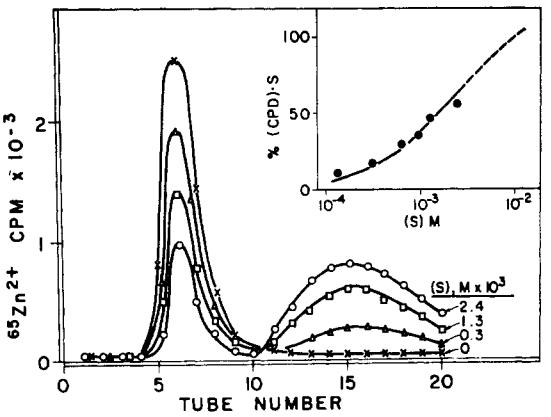

FIGURE 5: Prevention of $^{65}Zn^{2+}$ binding to apocarboxypeptidase by poly-L-lysyl-L-tyrosine (10:1) as a function of substrate concentration: 5×10^{-4} M (△), 1.5×10^{-3} M (□), 3.7×10^{-3} M (○); the upper curve represents a control without substrate (×). The elution over Sephadex G-25 was described in Figure 1. Absorbance at 278 mμ, not shown in this diagram, and $^{65}Zn^{2+}$ in cpm/2-ml fraction were measured. The percentage apoenzyme–substrate complex, determined from the areas under the effluent curves (see the text), as a function of the substrate concentration is shown in the inset. The total substrate concentration can be set equal to the free substrate concentration; the apoenzyme-bound substrate is negligible compared with the total substrate concentration since the apoenzyme concentration is 6×10^{-6} M throughout. Insolubility of the polypeptide prevented the use of concentrations higher than 3.7×10^{-3} M as indicated by the dashed section of the curve.

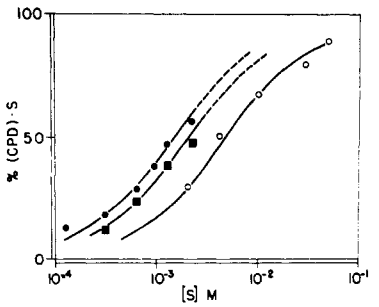

FIGURE 6: Binding of poly-L-lysyl-L-tyrosine (10:1) to apocarboxypeptidase and apoacetylcarboxypeptidase and of carbobenzoxyglycyl-L-phenylalanine to apocarboxypeptidase. Semilogarithmic plots of the percentage apoenzyme–substrate complex formed as a function of the substrate concentrations. The percentage apoenzyme–substrate complex was determined from the areas under families of effluent curves similar to those in Figure 5, using the particular substrate and apoenzyme described: poly-L-lysyl-L-tyrosine (10:1)–apocarboxypeptidase (●); poly-L-lysyl-L-tyrosine (10:1)–apoacetylcarboxypeptidase (■); CGP-apocarboxypeptidase (○). Insolubility of the polypeptide prevented the use of concentrations higher than 3.7×10^{-3} M as indicated by the dashed sections of the curves. The curve for CGP-apocarboxypeptidase was reconstructed from the data in Coleman and Vallee (1962a).

lysozyme and partially prevents the formation of zinc carboxypeptidase resulting in the formation of approximately 50% ^{65}Zn carboxypeptidase. However, like ribonuclease and α-lactalbumin, ovalbumin also binds significant quantities of $^{65}Zn^{2+}$, requiring correction.

Number of Moles of Poly-L-lysyl-L-tyrosine (10:1) Binding to Apo- and Apoacetylcarboxypeptidases and Apparent Binding Constant, K_s'. By varying the concentration of poly-L-lysyl-L-tyrosine (10:1) added to apocarboxypeptidase, restoration of zinc carboxypeptidase can be prevented to varying degrees (Figure 5). From the areas under the resultant family of curves, an apoenzyme–substrate binding curve can be constructed (Coleman and Vallee, 1962a) (Figure 5 inset and Figure 6). Assuming equilibrium

$$(CPD)_{free} + n(S) \rightleftharpoons (CPD \cdot S_n) \quad (1)$$

then

$$K_s' = \frac{(CPD)_{free}(S)^n}{(CPD \cdot S_n)} \quad (2)$$

The concentration of $(CPD)_{free}$ is assumed equivalent to the amount of $[(CPD)^{65}Zn]$ formed in the presence of the varying concentrations of poly-L-lysyl-L-tyrosine and hence the concentration of $CPD \cdot S_n$ will be $(CPD)_{total} - ([(CPD)^{65}Zn])$.

The formation curve of the apoenzyme–substrate complex encompasses 3 log decades of substrate concentration with a $K_s' = 1.6 \times 10^{-3}$ M, quite similar to though smaller than that obtained previously for carbobenzoxyglycyl-L-phenylalanine where $K_s' = 5 \times 10^{-3}$ (Figure 6) (Coleman and Vallee, 1962a). The binding curves can be treated as Langmuir isotherms (Kistiakowsky and Shaw, 1953) where p(S) is plotted as a function of log ϕ and where

$$\phi = \left(\frac{100}{\%(CPD)_{free}} - 1\right)$$

The average number of moles of substrate bound per mole of apoenzyme, $\bar{n}$, can be calculated from the slope of the resultant plot and K_s' from the p(S) value where $\phi = 0$. For poly-L-lysyl-L-tyrosine (10:1) with apocarboxypeptidase $\bar{n} = 0.94$ and $K_s' = 1.6 \times 10^{-3}$ M, in agreement with the value determined from the binding curve (Figure 6). The analogous binding curve for the apocarboxypeptidase–carbobenzoxyglycyl-L-phenylalanine complex gives $\bar{n} = 0.92$, while $K_s' = 5.0 \times 10^{-3}$ M, *i.e.*, 1 mole of either

polypeptide or dipeptide binds to 1 mole of apoenzyme (Figure 7).

Binding curves for poly-L-lysyl-L-tyrosine (10:1) were also obtained with apoacetylcarboxypeptidase. The apoacetylenzyme binds poly-L-lysyl-L-tyrosine (10:1) in a manner very similar to that of apocarboxypeptidase (Figures 6 and 7). The slopes of the binding curves are identical. For poly-L-lysyl-L-tyrosine–apoacetylcarboxypeptidase $\bar{n}$ = 0.95, and the apparent binding constant (K_s') = 2.2 × 10^{-3} M. K_s', though slightly greater than for the unmodified apoenzyme, is not altered substantially. This finding contrasts with similar studies of carbobenzoxyglycyl-L-phenylalanine (Vallee, 1964a,b; Coleman *et al.*, 1964, 1966).

Prevention of the Binding of $^{65}Zn^{2+}$ to Apo- and Apoacetylcarboxypeptidase by Ribonuclease and Acetylribonuclease (Ac$_3$RNAase). Acetylation of poly-L-lysyl-L-tyrosine with *N*-acetylimidazole renders this polymer insoluble in water, making this material unsuitable for further exploration of the role of tyrosyl residues in substrate binding. Attention was therefore given to water-soluble acetylribonuclease. The three "free" tyrosyl residues of ribonuclease can be acetylated with a 220 molar excess of *N*-acetylimidazole to yield Ac$_3$RNAase (Riordan *et al.*, 1965; Simpson, 1966) (Table I). Ribonuclease, 3.6 × 10^{-4} M, completely prevents the formation of zinc carboxypeptidase (Figure 8A); substitution of apoacetylcarboxypeptidase for apocarboxypeptidase reduces the binding of ribonuclease as evidenced by the formation of 20% zinc acetylcarboxypeptidase (Figure 8B). Addition of Ac$_3$RNAase, 3.6 × 10^{-4} M, to apocarboxypeptidase (Figure 8C) or to apoacetylcarboxypeptidase (Figure 8D) similarly decreases binding.

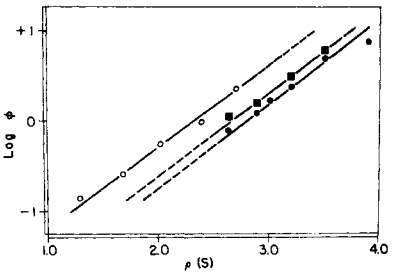

FIGURE 7: Determination of apparent binding constant and average number of moles of substrate bound per mole of apoenzyme for poly-L-lysyl-L-tyrosine (10:1) binding to apocarboxypeptidase and apoacetylcarboxypeptidase and for carbobenzoxyglycyl-L-phenylalanine binding to apocarboxypeptidase. Log ϕ is plotted as a function of minus the log of the substrate concentration, where ϕ = $(100/\%(CPD)_{free}) - 1$, as described in the text. The apparent binding constant, K_s', is the concentration of substrate where log ϕ = 0. The curve for CGP-apocarboxypeptidase was reconstructed from the data in Coleman and Vallee (1962a).

TABLE I: Acetylation of Free Tyrosyl and Amino Groups of Ribonuclease by *N*-Acetylimidazole.

Protein	*O*-Acetyltyrosine (M/M)	Free NH_2 (leu equiv/m)
Native RNAase (control)	0	9
Ac$_3$RNAase	2.9	2
Deacetylated Ac$_3$RNAase	0	2

Ribonuclease (29.2 μmoles) in 20 ml of 1 M NaCl–0.02 M Veronal, pH 7.5, was treated with a 220 molar excess of *N*-acetylimidazole, 25°, 1 hr. Excess reagent and products were dialyzed off. *O*-Acetyl groups were determined from the reduction of absorbance at 278 mμ as described by Riordan *et al.* (1965) and *N*-acetyl groups from the decrease of ninhydrin color as described in the text. *O*-Acetyl groups were deacetylated 1 hr at 25° by addition of an equal volume of 2 M hydroxylamine in 1 M NaCl–0.02 M Veronal, pH 7.5, followed by dialysis.

Acetylation of ribonuclease with a 220-fold molar excess of *N*-acetylimidazole acetylates both lysyl and "free" tyrosyl residues of this protein (Table I), though at lower molar excesses of reagent the reaction is more selective (Riordan *et al.*, 1965). In order to assess the contribution of tyrosyl residues of the substrate to apoenzyme–substrate binding, the *O*-acetyltyrosyl groups of Ac$_3$RNAase were deacetylated with hydroxylamine at pH 7.5, resulting in a return of the absorbance at 278 mμ to that characteristic of native ribonuclease (Simpson, 1966); but, as measured by ninhydrin, the number of free amino groups remains unchanged (Table I). Deacetylated Ac$_3$RNAase binds to apocarboxypeptidase (Figure 9) almost as well as does native ribonuclease (Figure 8A).

Hydrolysis of Poly-L-lysyl-L-tyrosine (10:1) by Native and Acetylated Carboxypeptidase. Since certain polypeptides and proteins bind to both apo- and apoacetylcarboxypeptidase (Figures 6–8), while some dipeptides bind only to the apoenzyme and not to the acetylated apoenzyme (Coleman and Vallee, 1966), the hydrolysis of poly-L-lysyl-L-tyrosine (10:1) by native zinc carboxypeptidase and acetyl zinc carboxypeptidase was examined. Incubation of the polypeptide both with native or with acetyl zinc carboxypeptidase did not yield a detectable increase in ninhydrin-positive material during 3 hr of incubation, indicating no hydrolysis.

Discussion

The separation of substrate binding from the subsequent catalytic step for dipeptide substrates by means

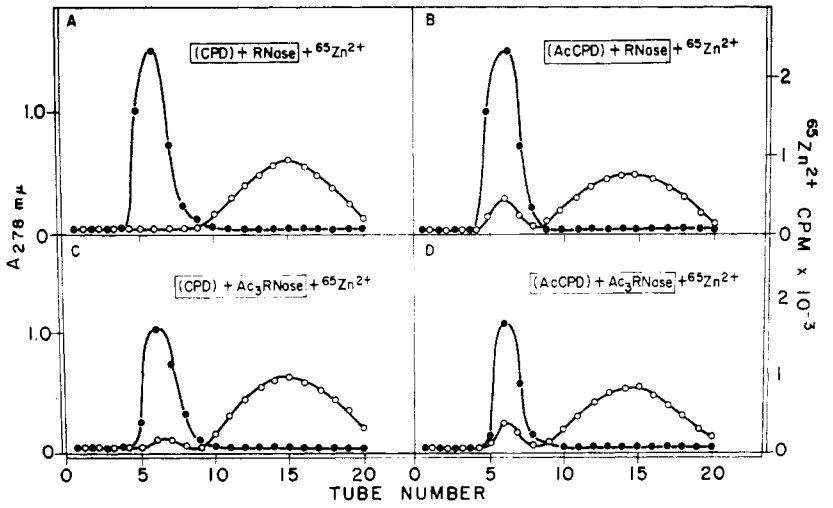

FIGURE 8: Effect of native and acetylated ribonuclease on the binding of $^{65}Zn^{2+}$ to apocarboxypeptidase and apoacetylcarboxypeptidase. Samples (2 ml) of 6×10^{-6} M apocarboxypeptidase plus 3.6×10^{-4} M ribonuclease plus 6×10^{-6} M $^{65}Zn^{2+}$ (A); 6×10^{-6} M apoacetylcarboxypeptidase plus 3.6×10^{-4} M ribonuclease plus 6×10^{-6} M $^{65}Zn^{2+}$ (B); 6×10^{-6} M apocarboxypeptidase plus 3.6×10^{-4} M Ac_3RNAase plus 6×10^{-6} M $^{65}Zn^{2+}$ (C); 6×10^{-6} M apoacetylcarboxypeptidase plus 3.6×10^{-4} M Ac_3RNAase plus 6×10^{-6} M $^{65}Zn^{2+}$ (D), were passed over Sephadex G-25 as in Figure 1 and eluted at 4° with 1 M NaCl–0.02 M Veronal, pH 7.5. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fractions (○) were measured. The radioactivity measurements were corrected for $^{65}Zn^{2+}$ binding to the substrate as described in the text. Enclosures as in Figure 1.

of gel filtration (Coleman and Vallee, 1962a,b) offered a means of studying differences in the *binding* to carboxypeptidase of dipeptides on one hand and of polypeptides and proteins on the other. Therefore, the present study was directed to the binding of high molecular weight substrates, rather than their hydrolysis.

The high susceptibility to hydrolysis of dipeptides containing a C-terminal aromatic residue indicated the use of polypeptides containing varying proportions of tyrosyl residues randomly distributed throughout the polypeptide chain. Poly-L-lysine, which is not hydrolyzed by carboxypeptidase, also does not prevent $^{65}Zn^{2+}$ restoration, indicating failure to bind, in analogy to the dipeptide benzoylglycyl-L-lysine which is neither bound to apocarboxypeptidase (T. L. Coombs, unpublished experiment) nor hydrolyzed by the holoenzyme (Neurath and Schwert, 1950). Introduction of a small percentage of tyrosyl residues into poly-L-lysine does not alter this; poly-L-lysyl-L-tyrosine (24:1) does not prevent $^{65}Zn^{2+}$ restoration. When the tyrosine content is increased further, however, as in the (10:1) poly-L-lysyl-L-tyrosine copolymer, prevention of $^{65}Zn^{2+}$ binding to the apoenzyme is now observed. This polymer binds to apocarboxypeptidase and presumably blocks the metal binding site (Coleman and Vallee, 1962a; Vallee, 1964a,b).

The interaction of poly-L-lysyl-L-tyrosine containing different proportions of tyrosine and lysine with apocarboxypeptidase (*vide supra*) extends the suitability of the gel filtration technique to the detection of apoenzyme–polypeptide complexes. The replacement of lysyl by glutamyl residues, as in poly-L-glutamyl-L-tyrosine (9:1), abolishes binding; the holoenzyme is fully formed. Even the presence of fewer negatively charged glutamyl residues, as in poly-L-glutamyl-L-lysyl-L-tyrosine (9:6:1), markedly changes the binding characteristics. The negative charge of glutamic acid might counteract hydrophobic forces which might be involved in the binding of tyrosyl residues. The failure of glutamyl polypeptides to bind is in accord with the failure of carboxypeptidase to hydrolyze polyglutamic acid (Miller, 1964). Similarly, the substitution of a glutamyl for the glycyl residue of carbobenzoxyglycyl-L-phenylalanine to yield carbobenzoxyglutamyl-L-phenylalanine results in a 20-fold decrease of hydrolysis (Bergmann and Fruton, 1942). Further, glutamyl residues have been noted to affect adversely the susceptibility of L-lysyl-L-tyrosine copolymers to hydrolysis by trypsin (Katchalski *et al.*, 1964). Since even a small proportion of glutamyl residues interferes significantly, the over-all charge of the polymer alone apparently does not determine binding. The relative contribution of charge distribution and configuration of polymers might be assessed by synthesis of copolymers with increasing proportions of glutamic acid, whereas the substitution of neutral for glutamyl residues might elucidate the role of free carboxyl side chains in binding.

The binding curve of the poly-L-lysyl-L-tyrosine (10:1)–apocarboxypeptidase complex demonstrates that, on the average, 1 mole of this substrate binds to 1 mole of apoenzyme; this binding curve is similar to

those of dipeptide–apoenyzme complexes (Figures 6 and 7). The similarity in the curves might suggest that the poly- and dipeptides are bound in analogous fashion. The apparent binding constant, K_a', for the poly-L-lysyl-L-tyrosine–apocarboxypeptidase complex, however, is significantly smaller than that for the carbobenzoxyglycyl-L-phenylalanine–apocarboxypeptidase complex indicating firmer binding for the polypeptide than for the dipeptide. This suggests that, in addition to the groups at the surface of the apoenzyme involved in binding dipeptides, yet additional ones are capable of interacting with polypeptides, thereby conferring added stability.

The experiments with apo*acetyl*carboxypeptidase, where only the free tyrosyl residues of the enzyme have been modified (Simpson *et al.*, 1963), support this hypothesis, since acetylation of the apoenzyme destroys dipeptide binding (Vallee, 1964a,b; Coleman *et al.*, 1964, 1966), whereas the polypeptide binding is reduced but little (Figures 6 and 7). Thus, in *dipeptide* binding, the "free" tyrosyl residues of carboxypeptidase appear to be critical while they appear to play an auxiliary role in *polypeptide* binding.

Ribonuclease, α-lactalbumin, and lysozyme served to study protein substrates which are hydrolyzed by carboxypeptidase A (Fraenkel-Conrat *et al.*, 1955). All of them bind to apocarboxypeptidase, though to varying degrees (Figure 3). At high concentrations these proteins bind $^{65}Zn^{2+}$ (Figure 4) and, hence, as the required corrections become larger the method becomes unsuitable (Figure 4), a problem which precluded the use of insulin for analogous studies (Slobin and Carpenter, 1963a,b, 1966). Nevertheless, gauging from the range of ribonuclease concentrations over which reliable data can be obtained, the concentration required for complete prevention of ^{65}Zn carboxypeptidase formation is smaller by an order of magnitude than that required for dipeptides to produce the same effect. These findings further imply the existence of additional binding sites in apocarboxypeptidase to permit interaction with high molecular weight substrates.

Though carboxypeptidase does not hydrolyze ovalbumin, it does form a complex. The protein, 3.6×10^{-4} M, partially prevents formation of ^{65}Zn carboxypeptidase in a manner quite similar to lysozyme.

Similarly, although poly-L-lysyl-L-tyrosine (10:1) binds to apocarboxypeptidase, the native or the acetylated zinc enzymes do not seem to hydrolyze this polymer. Thus, while binding of a potential substrate to the apoenzyme is a necessary requirement, this is not a sufficient index that catalysis will ensue. These conclusions are in accord with the inhibition of dipeptide hydrolysis by block copolymers of glutamic acid and tyrosine (Lehrer *et al.*, 1965).

The "free" tyrosyl residues of carboxypeptidase were acetylated with *N*-acetylimidazole in an effort to identify the additional binding groups of the enzyme. Acetylation of all seven of the "free" tyrosyl groups of carboxypeptidase with *N*-acetylimidazole merely reduces but does not abolish binding of poly-L-lysyl-L-tyrosine (10:1) (Figure 7). The binding curve is quite similar to that of the apocarboxypeptidase–poly-L-lysyl-L-tyrosine complex. The "free" tyrosyl residues of carboxypeptidase, therefore, are apparently not the only ones involved in binding of polypeptides.

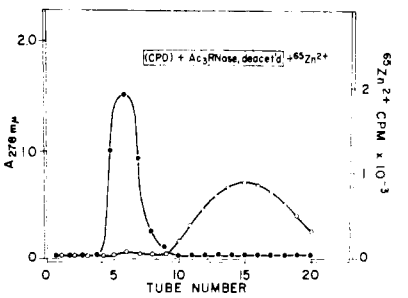

FIGURE 9: Effect of Ac_3RNAase, after deacetylation with hydroxylamine, on the binding of $^{65}Zn^{2+}$ to apocarboxypeptidase. A 2-ml sample of 6×10^{-6} M apocarboxypeptidase plus 3.6×10^{-4} M deacetylated Ac_3-RNase plus 6×10^{-6} M $^{65}Zn^{2+}$ was passed over Sephadex G-25 as in Figure 1 and eluted at 4° with 1 M NaCl–0.02 M Veronal, pH 7.5. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ in cpm/2-ml fraction (○) were measured. The radioactivity measurements were corrected for $^{65}Zn^{2+}$ binding to the substrate as described in the text. Significance of the enclosure as in Figure 1. Ac_3RNAase acetylated with a 200 molar excess of *N*-acetylimidazole, 1 hr at pH 7.5, 25°, 1 M NaCl–0.02 M Veronal, was then deacetylated with 1 M hydroxylamine as in Table I.

Since acetylation renders poly-L-lysyl-L-tyrosine insoluble, the effects of modification of substrate tyrosyl residues could not be tested with this polymer. Acetylation of ribonuclease was revealing, however, in this regard. Acetylation of the three "free" tyrosyl residues of ribonuclease decreases binding to apocarboxypeptidase but slightly. Similarly, acetylation of carboxypeptidase only decreases binding of native ribonuclease to a minor extent, as is apparent also for poly-L-lysyl-L-tyrosine. Acetylation both of apoenzyme and substrate does not additionally affect their interaction, further indicating the involvement of binding groups other than tyrosine.

In this regard the large excess of *N*-acetylimidazole required to acetylate all of the "free" tyrosyl residues also modifies the free lysyl residues of ribonuclease (Table I). In order to separate the potential contributions to binding of these two types of residues, the *O*-acetyltyrosyl groups of ribonuclease were deacetylated with hydroxylamine; *N*-acetylribonuclease binds as effectively to apocarboxypeptidase as does the native enzyme (Figure 9), indicating that the lysyl residues of ribonuclease do not contribute significantly to binding.

The data thus demonstrate that the binding of proteins and polypeptides to carboxypeptidase cannot be described fully in terms predicted from studies of di-

peptide substrates alone. It is conceivable that the systematic examination of higher molecular weight peptide substrates of carboxypeptidase may reveal that dipeptides and esters, preferred for their operational advantages in the past, are suboptimal in the examination of the mechanism of action of the enzyme. Recent studies by Schechter and Berger (1965, and personal communication) would tend to support this viewpoint. The action of carboxypeptidase A was investigated on peptides of L- and D-alanine, varying from di- to hexapeptides and containing D and L residues at defined positions with the aim of defining the binding site of the enzyme, based on activities. The hydrolytic rates measured with 35 peptides led to the conclusion that the substrate binding site of the enzyme is complementary to at least four amino acid residues. We are continuing to explore therefore the mode of binding and hydrolysis of larger substrates to identify more suitable approaches to the mechanism of action of carboxypeptidase.

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