The Zinc-binding Groups of Carboxypeptidase A *

THOMAS L. COOMBS,† YOSHIMORI OMOTE,‡ AND BERT L. VALLEE

From the Biophysics Research Laboratory, Division of Medical Biology, Department of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston

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The zinc atom at the active site of γ- and δ-carboxypeptidase A is bound to the thiol group of the sole cysteine residue and the α-amino group of the N-terminal asparagine. This has been established by means of differential interaction of reagents, specific for these groups, with the zinc-containing and zinc-free enzyme. Ag^+, *p*-mercuribenzoate, ferricyanide, and oxidation were employed to identify the thiol group, and phenylisothiocyanate and 1-fluoro-2,4-dinitrobenzene were used to modify the α-amino group. Spectral studies with pyridoxal phosphate also indicate the participation of both an amino and a thiol group in binding zinc. The spectrum of the pyridoxal phosphate–apocarboxypeptidase complex closely resembles that of the pyridoxal phosphate–cysteine or –penicillamine complexes known to accompany thiazolidine formation. The pyridoxal phosphate–carboxypeptidase complex does not exhibit this spectrum. Pyridoxal phosphate apparently acts as a site-specific bifunctional reagent with respect to apocarboxypeptidase by linking the thiol group of its cysteine and the α-amino group of its N-terminal asparagine residues. Successive Edman degradations established the N-terminal sequence of γ-carboxypeptidase as asparaginyl-tyrosyl-alanyl. Photooxidation in the presence of methylene blue failed to implicate a histidyl residue in zinc binding.

The reversible removal of the functional metal atom of a metalloenzyme permits the chemical identification of those groups which comprise the metal-binding site. On removal of the metal, these groups can be modified by site-specific and selective reagents (Vallee, 1961); their chemical modification should interfere with the restoration both of the metal and—consequently—of enzymatic activity. Moreover, by interacting with these groups the metal atom itself acts as a site-specific reagent; thereby it may protect them specifically from subsequent modifications.

These principles have now been applied to carboxypeptidase A[1] where the functional zinc atom can be removed reversibly to form a stable, enzymatically inactive, metal-free apoenzyme (Vallee *et al.*, 1958, 1960b, 1963). The data here presented indicate that zinc is bound to the sulfhydryl group of a cysteine and to the α-amino group of the N-terminal asparagine residue of δ-carboxypeptidase. Simultaneously, the N-terminal peptide sequence of γ-carboxypeptidase has been established as asparaginyl-tyrosyl-alanyl-.

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‡ Fellow of the Kettering Foundation, Dayton, Ohio, 1960–1962.

[1] In this paper "carboxypeptidase" refers to carboxypeptidase A only. The nomenclature of the various chemical species of carboxypeptidase is in accord with that suggested by Bargetzi *et al.* (1963). Alanine is the N-terminal residue of α-carboxypeptidase (Cox *et al.*, 1964). Serine is the N-terminal residue of β-carboxypeptidase; asparagine is the N-terminal residue of γ-carboxypeptidase (Anson, 1937) identical with that of δ-carboxypeptidase (Allan *et al.*, 1964). The designation "active site" will refer specifically to the nitrogen-metal-sulfur bond, essential for hydrolysis. "Active center" will refer to all those features of primary, secondary, and tertiary structure of the enzyme, including the "active site," which are required for substrate binding, specificity, or hydrolysis of the substrate.

MATERIALS AND METHODS

Beef Pancreas Carboxypeptidase.[2]—Two preparations of eight-times-crystallized δ-carboxypeptidase were prepared from beef pancreas acetone powder (Allen *et al.*, 1964). Solutions of the final crystals in 1.0 M NaCl, 0.1 M Tris buffer, *p*H 7.5, were homogeneous in the ultracentrifuge and by moving-boundary electrophoresis when examined in LiCl buffers at *p*H values from 6.6 to 10.5, $\mu = 0.3$. The proteolytic coefficient, *C*, of these preparations ranged from 25 to 30 at *p*H 7.5, 25°, and from 6 to 7 at *p*H 7.5, 0°. The esterase activities, expressed as a zero-order rate constant, *k*, ranged from 1.0 to 1.2 $\times 10^3$ μeq H^+/min per mg protein N at *p*H 7.5, 25°. The zinc-to-protein ratios ranged from 1855 to 1950 μg/g of protein, 0.98–1.03 g-atoms/mole, respectively, based on a molecular weight of 34,300 for the protein (Smith and Stockell, 1954; Vallee and Neurath, 1955).

For some experiments a five-times-crystallized γ-carboxypeptidase preparation was employed (Worthington Biochemical Corp., Freehold, N.J.). It was 95% homogeneous by ultracentrifugation and had a zinc-to-protein ratio of 1830 μg/g of protein, corresponding to 0.97 g-atom/mole. The proteolytic coefficient, *C*, was 26.5 at 25°.

All preparations contained insignificant amounts of all other metals when analyzed spectrographically (Vallee, 1955).

Apocarboxypeptidase.—Apocarboxypeptidase was prepared by dialyzing the native enzyme against 1,10-phenanthroline at *p*H 7.0; precautions were taken against contamination by metal ions (Coleman and Vallee, 1960; Vallee *et al.*, 1960b). The preparations of "metal-free" apocarboxypeptidase used in these experiments contained between 26 and 39 μg of zinc per g of protein, or less than 2% of the original zinc

[2] The abbreviations used are in formulations only and when required for differentiation: [(CPD)Zn], zinc carboxypeptidase with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc or other metals substituting for it; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-β-phenyllactic acid; Tris, tris(hydroxymethyl)aminomethane; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; PTC, phenylisothiocyanate; PTH, phenylthiohydantoin; pyridoxal-P, pyridoxal phosphate.

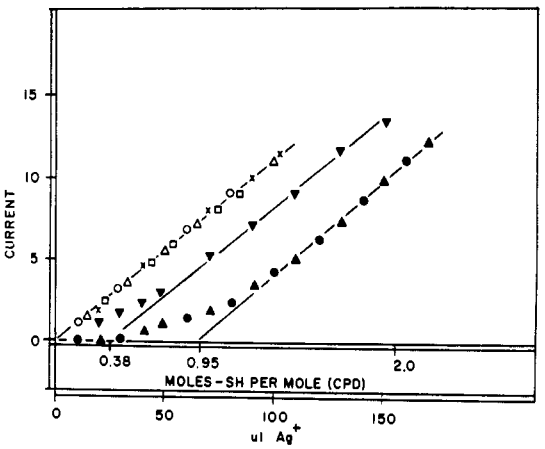

FIG. 1.—Argentometric titration of: δ-carboxypeptidase, O; apo-δ-carboxypeptidase, ●; apo-δ-carboxypeptidase + 1 g-atom Zn^{2+}, △; apo-δ-carboxypeptidase + HPLA, ▲; apo-δ-carboxypeptidase + CGP, ×; apo-δ-carboxypeptidase + *p*-mercuribenzoate, ▼; HPLA or CGP, □. Protein (0.1 μmole) ± 0.1 g-atom Zn^{2+}, 300 μmoles CGP, 150 μmoles HPLA or 0.2 μmole *p*-mercuribenzoate were titrated with Ag^+ in Tris, $NaNO_3$, KCl, *p*H 7.5, 4° as described (Benesch *et al.*, 1955). Apocarboxypeptidase and CGP or HPLA were preincubated for 5 minutes at 4°, in 0.1 M Tris, 1 M NaCl, *p*H 7.5 and apocarboxypeptidase and *p*-mercuribenzoate were preincubated for 1 hour at 4° in 0.1 M Tris, 1 M $NaNO_3$, *p*H 6.2 prior to titration with Ag^+.

content; correspondingly they had about 2% of the original activity.

Buffers and Metals.—Standard solutions of Zn^{2+}, Hg^{2+}, and Ag^+ were prepared from the spectrographically pure metal or salt (Johnson Matthey Co., Ltd., London). All other reagents including buffers were of analytical grade and were purified as described (Coleman and Vallee, 1960).

Substrates.—Carbobenzoxyglycyl-L-phenylalanine was obtained from the Mann Research Laboratories, New York. Hippuryl-DL-β-phenyllactate was a gift of Dr. H. Neurath.

Dialysis tubing was appropriately treated Visking-Nojax casing (Klotz and Hughes, 1957).

Enzymatic Activities.—Peptidase activity was determined with CGP as the substrate. The assays were performed at either 25° or 0° in buffers containing 1 M NaCl, *p*H 7.5, and the apparent proteolytic coefficient, *C*, was calculated from the linear portion of the first-order reaction plots observed when hydrolysis did not exceed 15% (Coleman and Vallee, 1960).

Esterase activity was determined with HPLA as the substrate as recently described (Simpson *et al.*, 1963). Activities are expressed as zero-order velocity constants, *k*, with units of μeq H^+/minute per mg of protein N.

Protein concentrations were measured either by precipitation with 10% trichloroacetic acid followed by drying at 104° (Hoch and Vallee, 1953) or by absorbance at 278 mμ (Bargetzi *et al.*, 1963; Simpson *et al.*, 1963). *Zinc* was determined both by a specific chemical colorimetric method (Vallee and Gibson, 1948) and by emission spectrography (Vallee, 1955).

Sulfhydryl Titrations.—Free sulfhydryl groups were determined using Ag^+ (Benesch *et al.*, 1955), *p*-mercuribenzoate (Sigma Chemical Co.) (Boyer, 1954), or ferricyanide (Mirsky, 1941). The interaction of Ag^+ with sulfhydryl groups was followed amperometrically in a supporting electrolyte containing 0.133 M Tris–0.01 M KCl and 0.113 M HNO_3, and was maintained at 4° in an ice bath during the titration; the final *p*H was 7.5 and the final volume was 15 ml. Microliter quantities of 10^{-3} M $AgNO_3$ were added through an SB-2 microburet (Micrometric Instruments). The platinum electrode was rotated at 1800 rpm with a synchronous motor, and the diffusion current in the presence of free Ag^+ was determined with a galvanometer (Leeds and Northrup Model 2420), the sensitivity of which was 0.025 μa/mm. After each addition of $AgNO_3$ no further increase in diffusion current occurred after 30 seconds.

The interaction of *p*-mercuribenzoate was followed spectrophotometrically at 255 mμ in 1 M Tris, 1 M $NaClO_4$ buffer, *p*H 7.5, using absorption cells of 1-cm path length (Boyer, 1954).

Phenylisothiocyanate reactions were carried out on paper strips (Fraenkel-Conrat *et al.*, 1955). The phenylthiohydantoin derivatives were separated and identified by paper chromatography (Sjoquist, 1953).

Spectrophotometric measurements were performed with a Beckman DU spectrophotometer or with a Cary recording spectrophotometer (Model 11). The cell-housing units of both instruments were thermostatically controlled. Absorbances were measured either in 1-cm standard path length cells or in special, 5-cm path length, small volume, rectangular quartz cells (Vallee, 1953) adapted to both spectrophotometers.

pH Measurements were made at 20° using either a Beckman Model A or a Leeds-Northrup *p*H meter equipped with standard glass and calomel electrodes.

RESULTS

Reaction of Native and Apo-δ-carboxypeptidase with Thiol Reagents.—REACTION WITH Ag^+.—On titration, native δ-carboxypeptidase does not take up Ag^+, indicating the absence of free reactive thiol groups. In contrast, 1 mole of the apo-δ-enzyme immediately reacts with 1 mole of Ag^+, revealing the presence of a single free thiol group (Fig. 1).

The addition of increasing amounts of Zn^{2+} to the apoenzyme immediately prior to titration with Ag^+ results in a progressive decrease in the mole fraction of free thiol titrated. On addition of 1 g-atom of zinc a free thiol group can no longer be titrated. Thus the sum of the mole fraction of zinc restored to the apoenzyme and the mole fraction of free thiol per mole of enzyme remains constant at unity (Table I). The complementarity between the zinc and thiol content of carboxypeptidase suggests that both Zn^{2+} and Ag^+ have a common point of interaction which occurs at this thiol group.

The instantaneous reaction between apocarboxypeptidase and Ag^+ can be prevented by other agents which can react with thiol groups. Incubation of

TABLE I

COMPLEMENTARITY OF TITRATABLE THIOL GROUPS AND ZINC CONTENT OF δ-CARBOXYPEPTIDASE[a]

Protein	Zn^{2+} Added (g-atom)	−SH Titer [moles/mole (CPD)]	Sum (−SH + Zn^{2+})
(CPD)	0.07	0.84	0.91
	0.27	0.63	0.90
	0.57	0.42	0.99
	0.77	0.35	1.12
	1.0	0	1.0
[(CPD)Zn] (native)	1.0[b]	0	1.0

[a] Increments of Zn^{2+} were added first to 0.1–0.2 μmole apocarboxypeptidase (CPD) and then the remaining free —SH was titrated with Ag^+ at 4°, Tris, NaCl, $NaNO_3$ buffer, *p*H 7.5. [b] Present in the native enzyme.

TABLE II

REACTION OF APO-δ-CARBOXYPEPTIDASE WITH *p*-MERCURIBENZOATE IN VARIOUS BUFFER SYSTEMS[a]

Buffer	Instantaneous[b] [moles —SH/mole (CPD)]	At Equilibrium [moles —SH/mole (CPD)]	Time for Equilibrium to be Reached (hours)
1. 0.1 M PO_4, 1.0 M NaCl	0	0	96
2. 0.1 M Tris, 1.0 M NaCl	0	0	96
3. 0.1 M Tris, 0.1 M NaCl	0	0.95	90
4. 0.1 M Tris, 0.1 M $NaNO_3$	0.19	0.75	72
5. 0.1 M Tris, 1.0 M $NaClO_4$	0.31	0.62	18
6. 1.0 M Tris, 1.0 M $NaClO_4$	1.06	1.06	0

[a] Apocarboxypeptidase (0.05 μmole) was incubated with 0.7 μmole *p*-mercuribenzoate at 4°, *p*H 7.5, and the increase in absorbance at 255 mμ was measured as described by Boyer (1954). [b] "Instantaneous" is defined as the earliest time at which the reaction could be measured.

apocarboxypeptidase with *p*-mercuribenzoate decreases the Ag^+ titer (Fig. 1). Incubation for 1 hour with a 2-fold molar excess of this agent in 0.1 M Tris, 1 M $NaNO_3$ buffer at *p*H 7.5, 4°, reduces the mole fraction of Ag^+ titratable groups from 0.91 to 0.48 per mole of enzyme and at *p*H 6.2 from 0.91 to 0.38 per mole enzyme (Fig. 1).

The interaction of Ag^+ with apocarboxypeptidase can also be prevented by prior addition of CGP. When 0.02 M CGP is first added to apocarboxypeptidase, uptake of Ag^+ does not occur on subsequent amperometric titration (Fig. 1); CGP itself has no effect on the diffusion current of the system (Fig. 1).

In contrast, and in accord with earlier observations on the enzyme-binding groups of substrates (Coleman and Vallee, 1962a), the ester substrate, HPLA, does not prevent the instantaneous reaction between apocarboxypeptidase and Ag^+ (Fig. 1).

REACTIONS WITH *p*-MERCURIBENZOATE AND FERRICYANIDE.—Neither *p*-mercuribenzoate nor ferricyanide reacts with the native enzyme. However, on removal of zinc, 1.06 moles of *p*-mercuribenzoate are taken up. After incubation in 4 M urea for 48 hours at *p*H 7.5, 4°, with a 36-fold molar excess of ferricyanide, 1.22 moles of ferricyanide react per mole of apoenzyme. Under optimal conditions 1 mole of the thiol group of glutathione reacted with 1 mole of either reagent, constituting satisfactory validation of the methods.

The accurate quantitative analysis of the thiol content of apocarboxypeptidase by *p*-mercuribenzoate depends critically on the nature of the buffer and salts in which titrations are performed. High concentrations of phosphate, chloride, or nitrate either completely prevent the reaction or cause a time-dependent and variable recovery (Table II, lines 1 to 4), consistent with findings in model systems (Boyer, 1954). The reaction in perchlorate, however, is instantaneous and complete (Table II, line 6). In addition, Tris appears to facilitate the reaction; 1.0 M Tris, 1.0 M sodium perchlorate was optimal (Table II, lines 5 and 6).

Iodo- and bromoacetate, iodoacetamide, and *N*-ethyl maleimide failed to react with either native carboxypeptidase or the apoenzyme under a variety of conditions.

Functional Effects of Thiol Reagents.—These analytical results correlate directly with changes in enzymatic function. When apo-δ-carboxypeptidase is preincubated with an excess of Ag^+ or *p*-mercuribenzoate, the instantaneous and complete restoration of peptidase activity observed on readdition of 1 g-atom of Zn^{2+} per mole of apoenzyme is almost entirely blocked: only 17 and 10% of the activity is restored, respectively (Table III, lines 3 and 6). On the other hand, addition of Zn^{2+} to the apoenzyme prior to incubation with Ag^+ and *p*-mercuribenzoate results in a fully active enzyme (Table III, lines 4 and 7). When apocarboxypeptidase is aerated before readdition of Zn^{2+}, only 44% of the activity is restored (Table III, line 9). The single reactive thiol on the apoenzyme, therefore, has a crucial functional role through its bonding with zinc. When this group is modified either by complexation with Ag^+ or *p*-mercuribenzoate or by prior oxidation, the restoration of zinc, and consequently of activity, is prevented.

TABLE III

EFFECT OF THE ORDER OF ADDITION OF SULFHYDRYL REAGENTS AND Zn^{2+} ON RESTORATION OF ACTIVITY TO APO-δ-CARBOXYPEPTIDASE[a]

Order of Addition	Peptidase Activity (%)
1. (CPD) + Zn^{2+}	100
2. (CPD) + Ag^+	0
3. [(CPD) + Ag^+] + Zn^{2+}	17
4. [(CPD) + Zn^{2+}] + Ag^+	100
5. (CPD) + CMB[b]	0
6. [(CPD) + CMB] + Zn^{2+}	10
7. [(CPD) + Zn^{2+}] + CMB	100
8. (CPD) + O_2	0
9. [(CPD) + O_2] + Zn^{2+}	44
10. [(CPD) + Zn^{2+}] + O_2	100

[a] Ag^+ (2.2×10^{-5} M) or 3.7×10^{-3} M *p*-mercuribenzoate was added to 1.1×10^{-5} M apocarboxypeptidase either before or after the addition of 1.1×10^{-5} M Zn^{2+}. The enclosures, e.g. [(CPD) + Ag^+] + Zn^{2+}, represent the order of additions. Thus, in line 3, Ag^+ was added to apocarboxypeptidase first, then Zn^{2+}, while in line 4, Zn^{2+} was added first and then Ag^+. For the oxidation experiments, filtered air was bubbled through the apoenzyme solutions for 5 hours at 0°. Peptidase activity was measured at 4°, *p*H 7.5, using 0.02 M carbobenzoxyglycyl-L-phenylalanine as substrate in 0.1 M Tris, 1 M NaCl buffer. The native enzyme, [(CPD)Zn], and the restored enzyme, (CPD) + Zn^{2+}, both had proteolytic coefficients, *C*, of 8.0. After dialysis against 1,10-phenanthroline, the apoenzyme contained 0.03 g-atom zinc and the proteolytic coefficient was 0.25. [b] CMB = *p*-mercuribenzoate.

The capacity of apo-δ-carboxypeptidase to be reactivated by Zn^{2+} slowly and progressively decreases on storage (Table IV, lines A 1–3) suggesting slow oxidation of the reactive thiol group in a manner similar to that produced by aeration (Table III). The rate of this inactivation is markedly faster for apo-γ-carboxypeptidase (Table IV, lines B 1 and 2). It has not proved possible thus far to prepare apo-γ-carboxypeptidase which can be fully reactivated by Zn^{2+} using means which necessitate long exposure to air, such as dialysis against 1,10-phenanthroline. Removal of zinc from γ-carboxypeptidase with 1,10-phenanthroline followed by gel filtration, however, has yielded an apoenzyme which can be reactivated, provided Zn^{2+} is added to the protein solution immediately after elution (Table IV, lines C 1–5).

Effects of Denaturing Agents.—Amino acid analyses of γ- and δ-carboxypeptidases subsequent to performic acid oxidation reveal *two* cysteic acid residues (Smith and Stockell, 1954; Walsh *et al.*, 1962), but only one thiol group in the apoenzymes and none in the native enzymes (Vallee *et al.*, 1960a). The failure of this

TABLE IV
EFFECT OF MODE OF ZINC REMOVAL BY 1,10-PHENANTHROLINE (OP) AND OF STORAGE OF THE ENZYME ON THE THIOL TITER AND THE CAPACITY OF APO-γ- AND APO-δ-CARBOXYPEPTIDASE TO REGAIN ENZYMATIC ACTIVITY BY THE ADDITION OF Zn^{2+}

No.	Protein	Peptidase Activity (C) $-Zn^{2+}$	Peptidase Activity (C) $+Zn^{2+}$	Restoration (%)	—SH Titer [mole/mole (CPD)]
(A)	Apo-δ-carboxypeptidase: Zn and OP removed by dialysis;[a] total time of procedure, 4 days				
1.	[(CPD)Zn][b]		8.0		0
2.	(CPD), 4 days	0.6	8.0	100	0.95
3.	(CPD), 6 weeks	0	3.0	39	0.41
(B)	Apo-γ-carboxypeptidase: Zn and OP removed by dialysis;[a] total time of procedure, 4 days				
1.	[(CPD)Zn][b]		7.6		0
2.	(CPD), 4 days	0	0	0	0
(C)	Apo-γ-carboxypeptidase: Zn and OP removed by Sephadex;[c] total time of procedure, 50 minutes				
1.	[(CPD)Zn][b]		7.6		0
2.	(CPD), 50 minutes	1.0	7.1	94	0.80
3.	(CPD), 5 hours	0.8	6.2	82	0.65
4.	(CPD), 1 day	0.8	3.9	51	0.40
5.	(CPD), 4 days	0	2.0	26	0.20

[a] Apocarboxypeptidase, (CPD), was prepared from the native zinc enzyme, [(CPD)Zn], by dialysis for 2 days against 3 changes of 100-fold volume excess of 2×10^{-3} M 1,10-phenanthroline, OP, in 0.1 M Tris, 1 M NaCl, buffer, pH 7.5. The excess 1,10-phenanthroline and zinc phenanthrolinates were removed by further dialysis for 2 days against 4 changes of 0.1 M Tris, 1 M NaCl buffer alone. Total time of procedure, 4 days. The apoenzyme was then stored at 4°, pH 7.5, in 0.1 M Tris, 1 M NaCl for the times indicated. [b] Control. [c] Apocarboxypeptidase, (CPD), was prepared by incubating the native zinc enzyme for 30 minutes at 4° with 2×10^{-3} M 1,10-phenanthroline in 0.1 M Tris, 1 M NaCl buffer, pH 7.5. The excess 1,10-phenanthroline and zinc phenanthrolinates were removed rapidly by passage over a 2 × 35-cm Sephadex G-25 column which had been equilibrated at 4° with the Tris, NaCl buffer and had been treated with 25 ml of a 2×10^{-3} M 1,10-phenanthroline solution in 0.1 M Tris, 1 M NaCl, pH 7.5, immediately prior to the addition of the enzyme-1,10-phenanthroline incubation mixture. The column was eluted with the Tris, NaCl buffer and 5-ml fractions were collected. Apocarboxypeptidase appeared in fractions 12–16 after 20 minutes of elution. Total time of procedure, 50 minutes. The apoenzyme was then stored at 4°, pH 7.5, in 0.1 M Tris, 1 M NaCl for the times indicated. Titratable thiol was determined argentometrically as described in Fig. 1. Peptidase activity was determined at 4°, pH 7.5, using 0.02 M carbobenzoxyglycyl-L-phenylalanine as substrate in 0.1 M Tris, 1 M NaCl buffer.

second "sulfur-containing" residue to be detected by thiol reagents, either in the native or in the apoenzyme, suggested that the second thiol group may not be free. The hypothesis that internal bonding to another amino acid residue might account for the findings was investigated. Argentometric titrations of native δ-carboxypeptidase and its apoenzyme were performed in the presence of urea, guanidine, Tween 80, saponin, Sterox, dimethylformamide, and sodium dodecylsulfate. None of these agents increased the free thiol titers observed originally. In fact, preincubation with 8 M urea and Tween 80 progressively decreased the thiol titer of apocarboxypeptidase. Representative data are shown in Table V. Hence such alterations in secondary or tertiary structure as are induced by these agents do not render the thiol group of the second

TABLE V
EFFECT OF DENATURING AGENTS ON THE TITRATABLE THIOL GROUPS OF δ-CARBOXYPEPTIDASE AND APO-δ-CARBOXYPEPTIDASE[a]

Denaturing Agent	Incubation Time	Titratable —SH [moles/mole [(CPD)Zn]]	Titratable —SH [moles/mole (CPD)]
Control		0	0.91
8 M Urea	0	0	0.84
	2 minutes	0	0.50
	1 hour	0	0
	24 hours[b]	0	0
2.5% Tween 80[c]	1 hour	0	0.51
	24 hours	0	0.41

[a] Carboxypeptidase and apocarboxypeptidase were incubated at pH 7.5, 4°, in 0.1 M Tris, 1 M NaCl containing the denaturing agent. The zero-time reading was taken in the absence of the agent, while the 2-minute reading represents the earliest time at which the —SH titration on an aliquot of the incubation mixture could be completed. Free —SH was determined argentometrically as described, except the agent was present in the titration solutions. The denaturing agents alone reduced the slope of the amperometric titration curve slightly but did not affect the end-point determination for a simple thiol such as glutathione. [b] Solution became cloudy. [c] 2.5% Sterox (Monsanto Chemical Co.), saponin (Baker Chemical Co.), dimethylformamide (Matheson Co., Inc.), 0.01% sodium dodecylsulfate, and 6 M guanidine-HCl were similarly added and did not produce changes in the —SH titer of carboxypeptidase.

cysteine residue free. Presumably intramolecular bonding does not account for the failure of the residue to be titrated. This is in accord with the data of Walsh *et al.* (1962) which demonstrate that reduction with mercaptoethanol is required to make this thiol available and reactive; it does not exist as a cysteinyl residue in native carboxypeptidase.

Reaction of Native and Apocarboxypeptidase with Nitrogen Group Reagents

Complexometric titrations and the sequence and magnitude of the stability constants of different metallocarboxypeptidases in comparison to those of simple ligands suggested that the metal atom is bound both to a sulfur and to a nitrogen atom (Coleman and Vallee, 1961; Vallee *et al.*, 1960a). Therefore experiments analogous to those employed for the identification of the thiol group were carried out using reagents which have been found to interact with amino and imidazolium groups, since the existent data best fit the hypothesis than an α-amino, an ε-amino, or a histidine nitrogen might be part of the metal-binding site.

Amino-Nitrogen Group Reagents.—REACTION WITH PHENYLISOTHIOCYANATE.—Phenylisothiocyanate, the Edman reagent, reacts to different degrees with native and apo-δ-carboxypeptidase. The R_F value of the phenylthiohydantoin isolated from the apoenzyme corresponds exactly to that of a standard PTH-asparagine sample. Very little product is obtained from interaction with the native enzyme (Fig. 2A).

Small amounts of a decomposition product (dashed circles, Fig. 2A) are present in the standard and in each sample.

Chromatography of these samples in the alternative solvent systems of Sjoquist (1953) confirmed the identity of the spots as PTH-asparagine, in agreement with earlier analyses (Thompson, 1954).

The difference in reactivity shown by the native and apo-δ-carboxypeptidases was determined quantitatively from the ultraviolet-absorption spectra of ether-alcohol extracts measured prior to chromatog-

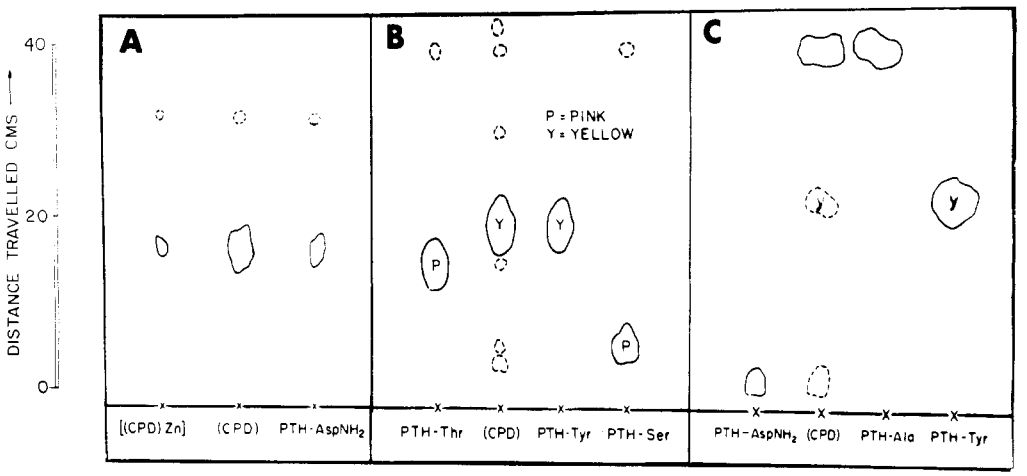

FIG. 2.—Chromatograms of PTC derivatives from three successive Edman degradations of carboxypeptidase. (A) 0.1 μmole δ-carboxypeptidase or apo-δ-carboxypeptidase were reacted with PTC on paper strips (Fraenkel-Conrat *et al.*, 1955). The final ether-alcohol extracts from the first degradations together with a marker of the phenylthiohydantoin of asparagine (PTH-AspNH$_2$) were then chromatographed on Whatman No. 1 paper using 40:40:20; heptane, 1-butanol, formic acid as solvent (Sjoquist, 1953). (B), The protein left on the paper strips from the first degradation, (A), was reacted once again with PTC. Chromatography of the alcohol-ether extracts was carried out in 30:60:5, heptane-ethylenedichloride–75% formic acid. The phenylthiohydantoins of threonine, tyrosine, and serine, PTH-Thr, PTH-Tyr and PTH-Ser, respectively, were employed as markers. (C) The protein left on the paper strips from the second degradation, (B), was reacted once again with PTC. Chromatography of the alcohol-ether extracts was carried out in 30:60:5, heptane, ethylenedichloride, 75% formic acid. The phenylthiohydantoins of asparagine, PTH-AspNH$_2$, alanine, PTH-Ala, and tyrosine, PTH-Tyr, served as markers. Major spots on each chromatogram are outlined with solid lines, minor components with dashed lines. The symbols P and Y denote the pink or yellow colors which develop when PTH-Thr and PTH-Ser or PTH-Tyr have been treated with starch iodine.

TABLE VI

δ-CARBOXYPEPTIDASE: COMPLEMENTARITY OF ZINC REMOVAL AND N-TERMINAL AMINO ACID INTERACTION WITH PHENYLISOTHIOCYANATE[a]

Sample	Zn Present (g-at./mole)	PTH-Asp·NH$_2$ (mole/mole)	Sum of (Zn^{2+} + PTH-Asp·NH$_2$) (mole/mole)
[(CPD)Zn] + PTC			
2 minutes	0.72	0.28	1.00
15 minutes	0.44	0.47	0.91
30 minutes	0.38	0.69	1.07
(CPD)			
20 minutes	0	1.00	1.00

[a] Conditions of reaction: Stage I. δ-Carboxypeptidase (0.7 μmole) or 0.3 μmole apo-δ-carboxypeptidase was supported in quadruplicate on 1 × 5-cm Whatman No. 1 paper strips and reacted with PTC in a 1:1:1 pyridine-dioxane-water atmosphere at 22° for the times shown. Prior to application of the proteins the paper was treated with dithizone in CCl_4 until zinc could no longer be detected in the ashed papers. Stage II. Each strip was reacted for 12 hours, 22°, 100 mm Hg pressure in a glacial acetic acid–HCl atmosphere. Firmly bound zinc was determined after dialysis of pairs of the paper strips at the end of Stage I against three changes of 100-ml volumes of 0.1 M Tris, 1 M NaCl buffer, *p*H 7.5 which had been made zinc free. The firmly bound zinc was removed by treatment of the dialyzed paper strips with 2 N HCl and determined by the dithizone method as described (Vallee and Gibson, 1948).

raphy (Fig. 3). The molar absorptivities at 269 mμ correspond to 1.0 and 0.6 mole of PTH-asparagine for the apo- and the native enzyme, respectively. Apparently zinc prevents complete reaction of PTC with the N-terminal asparagine, similarly to the protection of the thiol group by the metal ion.

Moreover, the yield of PTH-asparagine from the native enzyme increased in direct proportion to the time allowed for its reaction with PTC in the first stage of the Edman degradations. As the yield of PTH-asparagine increases, the amount of zinc remaining firmly bound to the protein at the end of this stage decreases (Table VI).

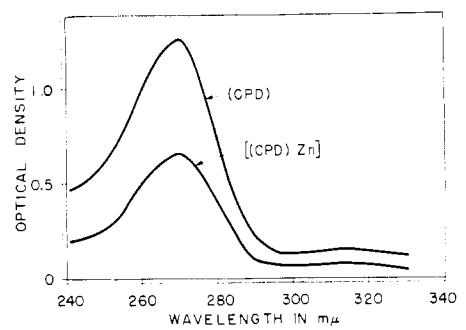

FIG. 3.—Ultraviolet-absorption spectra of the phenylthiohydantoin amino acids derived from the first Edman degradation of δ-carboxypeptidase, [(CPD)Zn], and apo-δ-carboxypeptidase, (CPD). The absorption spectra of ether alcohol extracts from identical quantities of each protein, 0.3 μmole, are shown before chromatography but after reaction with PTC. Beckman DU spectrophotometer 1-cm path length cells, reference solution, ether alcohol.

THE N-TERMINAL SEQUENCE OF CARBOXYPEPTIDASE.—A part of the N-terminal sequence, close to the metal-binding N-terminal asparagine, was determined by successive Edman degradations on γ-carboxypeptidase. After a second degradation using PTC, 0.9 mole/mole PTH-tyrosine was isolated and identified (Fig. 2B). The major spot from this second degradation, with chromatographic behavior identical to that of a PTH-tyrosine standard, developed the characteristic yellow color associated with PTH-tyrosine after treatment with starch-iodine (Fraenkel-Conrat *et al.*, 1955).

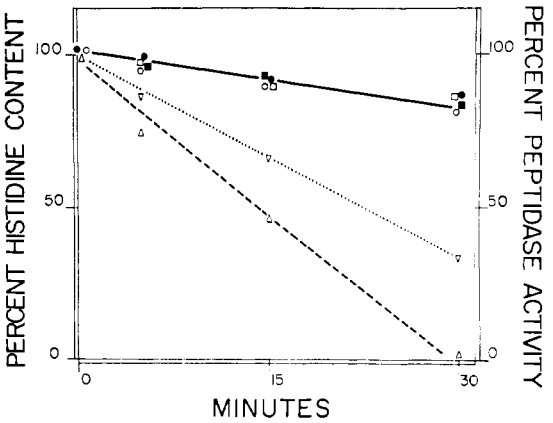

FIG. 4.—Irradiation of δ-carboxypeptidase, apo-δ-carboxypeptidase, and histidine in the presence of methylene blue. Protein (0.1 μmole) or L-histidine ± a mole equivalent of Zn^{2+} in 0.1 M Tris, 1 M NaCl, *p*H 7.5, were irradiated at 25° using a 500-w "Photoflood" lamp (General Electric Corp.) 18 inches from the sample. Peptidase activity was determined at *p*H 7.5, 25°, using 0.02 M CGP as substrate in 0.1 M Tris, 1 M NaCl. The activity of apocarboxypeptidase was measured by addition of an equivalent of Zn^{2+} subsequent to irradiation but prior to measuring the peptidase activity. Histidine content was determined using the method of MacPherson (1942). Peptidase activity, carboxypeptidase, ●–●; peptidase activity, apocarboxypeptidase + 1 g-atom Zn^{2+}, ■–■; histidine content, carboxypeptidase, O–O; histidine content, apocarboxypeptidase, □–□; histidine, control, △– –△; histidine + a mole equivalent of Zn^{2+}, control, ▽···▽.

After the second degradation traces of serine and threonine were additionally isolated (Fig. 2B). These could be further identified and differentiated from PTH-tyrosine by their pink color and differing R_F values (Fraenkel-Conrat *et al.*, 1955). Traces of these amino acids were observed variably also after the reaction with FDNB as described by Thompson (1953).

After a third degradation of γ-carboxypeptidase, 0.60–0.74 mole of PTH-alanine per mole of protein was isolated (Fig. 2C).

Additional spots correspond to traces of dehydro-PTH-alanine remaining close to the origin and to traces of PTH-asparagine and tyrosine, probably originating from incomplete reactions during the first and second degradations (Fig. 2C).

The N-terminal sequence for γ-carboxypeptidase, therefore, is asparaginyl-tyrosyl-alanyl-. Fourth and fifth degradations yielded multiple spots, rendering identification ambiguous.

REACTION WITH 1-FLUORO-2,4-DINITROBENZENE.—With FDNB 0.7 mole of DNP-aspartate, 17–18 moles of ε-DNP-lysine, and small amounts of DNP-serine and threonine per mole of native γ-carboxypeptidase have been demonstrated (Thompson, 1953). If, as is hypothesized from the PTC data (*vide supra*), zinc is bound to the N-terminal α-amino group, the recovery of only 0.7 mole of DNP-aspartate by means of FDNB might imply that, under the experimental conditions employed, zinc prevents the complete reaction of FDNB.

Thompson's (1953) early experiments were repeated with γ-carboxypeptidase (Table VII, line 5). Peptidase activity and residual zinc were measured before acid hydrolysis. The activity was completely lost and 0.7 mole of DNP-aspartate was detected, confirming Thompson's results. Moreover, 0.23 g-atom of zinc remained firmly bound to the enzyme; the sum of DNP-aspartate removed and zinc still bound was 0.93 (Table VII, line 5). Native and apo-δ-carboxypeptidase were reacted with FDNB at *p*H 7.0. A known fraction of the apocarboxypeptidase was converted to the metalloenzyme by addition of Zn^{2+}. As the amount of Zn^{2+} added increased from 0.28 to 0.77 g-atom the yield of DNP-aspartate decreased from 0.7 to 0.25 mole/mole of enzyme (Table VII, lines 2–4). The molar sum of DNP-aspartate formed and of zinc remaining firmly bound to the apoenzyme equalled one. No imidazole-DNP-histidine or *S*-DNP-cysteine could be detected in any of the acid hydrolysates.

TABLE VII

γ- AND δ-CARBOXYPEPTIDASE: COMPLEMENTARITY OF N-TERMINAL DNP–ASPARTIC ACID FORMATION AND ZINC CONTENT[a]

No.	Sample	Zn [g-at./mole (CPD)]	DNP–Aspartic Acid [mole/mole (CPD)]	Sum of (Zn^{2+} + DNP-Asp) (mole/mole)
1.	[(CPD)Zn] control	1.0	0	1.0
2.	[(CPD)Zn]	0.77	0.25	1.02
3.	(CPD) + 0.5 g-at. Zn^{2+}	0.47	0.52	0.99
4.	(CPD)	0.28	0.65	0.93
5.	[(CPD)Zn][b]	0.23	0.70	0.93

[a] Protein (0.4 μmole) was reacted with 0.1 ml FDNB at 25°, *p*H 7.0, for 2 hours in 0.005 M Tris, 1 M NaCl buffer keeping the *p*H constant by means of a Radiometer *p*H-stat. Excess unreacted FDNB was removed by centrifugation at 500 rpm followed by freezing at −5° for 10 minutes. The modified protein supernatant suspension was then dialyzed at *p*H 7.5, 4°, against 4 changes of 100-ml volumes of 0.1 M Tris, 1 M NaCl buffer, made zinc free. After dialysis 0.2 μmole protein was analyzed for zinc and protein content as described (Vallee and Gibson, 1948; Hoch and Vallee, 1953) while the remaining 0.2 μmole protein was acid-hydrolyzed and the resulting DNP-aspartate quantitatively separated chromatographically and determined as described (Fraenkel-Conrat *et al.*, 1955) correcting for losses in hydrolysis and chromatography (Fraenkel-Conrat *et al.*, 1955). [b] 0.4 μmole γ-carboxypeptidase was reacted with 0.1 ml FDNB in a saturated sodium bicarbonate–60% ethanol mixture at 25°, shaking the mixture gently for 3 hours (Thompson, 1953). Separation and determination of DNP-aspartate, zinc, and protein was then carried out as described.

Reaction with an Imidazolium-Nitrogen Reagent.—PHOTOOXIDATION WITH METHYLENE BLUE.—The participation of an imidazole group in metal binding was examined by photooxidation of both native and apo-δ-carboxypeptidase in the presence of methylene blue (Weil *et al.*, 1953). Peptidase activities of the native enzyme and of the irradiated apoenzyme, to which zinc had been restored, were measured as a function of time of irradiation. Simultaneously, the histidine content was determined (MacPherson, 1942). The nonirradiated proteins, as well as L-histidine and L-histidine plus Zn^{2+}, served as controls.

As irradiation proceeds, both the native and the apoenzyme are progressively inactivated but at similar rates (Fig. 4) which vary as a function of time, intensity of irradiation, buffers, salt, or *p*H, but the rate at which the histidine content of carboxypeptidase decreases is not affected by the presence or absence of zinc. Importantly, the rate of destruction of histidine in the enzyme is much slower than that observed for the L-histidine control. However, addition of Zn^{2+} to L-histidine significantly decreases

the rate of oxidation of this amino acid, suggesting that Zn^{2+} can protect histidine against irradiation (Fig. 4).

Reactions of Native and Apocarboxypeptidase with a Nitrogen-Sulfur Group Reagent

Pyridoxal-P reacts with amino thiols, such as cysteine and penicillamine, by incorporating the nitrogen and sulfur atoms of these compounds into a thiazolidine ring (Matsuo, 1957; du Vigneaud *et al.*, 1957; Buell and Hansen, 1960; Braunstein, 1960). This reaction, which proceeds at a measurable rate, is accompanied by characteristic changes in the ultraviolet spectrum. Spectral changes have also been observed when pyridoxal-P and muscle phosphorylase interact (Krebs and Fischer, 1962). The spectral changes incident to thiazolidine formation might render pyridoxal-P suitable as a site-specific reagent for the identification of a nitrogen-sulfur site, such as that hypothesized to exist in carboxypeptidase.

The ultraviolet spectra of native and apocarboxypeptidase were examined in the presence and absence of pyridoxal-P (Fig. 5A), and L-cysteine was reacted with it for comparison (Fig. 5B).

Pyridoxal-P alone exhibits a single absorption band between 320 and 450 mμ with a maximum at 388 mμ. The interaction of L-cysteine with this agent produces a new maximum near 330 mμ, which increases in intensity as a function of the time of reaction. The corresponding decrease in the intensity of the maximum at 388 mμ with an isosbestic point at 350 mμ (Fig. 5B) follows second-order kinetics (Buell and Hansen, 1960). The difference spectrum shows the essential features of these changes (Fig. 5D). The molar absorptivity at 330 mμ for the cysteine-pyridoxal-P complex is $3.2 \times 10^3\ M^{-1}\ cm^{-1}$.

On exposure of native carboxypeptidase to pyridoxal-P, the absorbance at 388 mμ decreases simultaneously with a small increase between 430 and 450 mμ; but new maxima do not appear between 325 and 350 mμ (Fig. 5A, C). In contrast, exposure of apocarboxypeptidase to this agent generates a new maximum at 325 mμ. Its intensity progressively increases with time of reaction while the absorbance of the maximum at 388 mμ decreases correspondingly; there is an isosbestic point at 344 mμ. An initial, small increase in absorbance in the range from 420 to 450 mμ remains constant on incubation for as long as 24 hours (Fig. 5A, C). The difference spectrum obtained by subtracting the absorbance of pyridoxal-P from that of the reaction mixture containing both pyridoxal-P and apocarboxypeptidase is very similar to that accompanying the reaction of cysteine and pyridoxal-P (Fig. 5C, D).

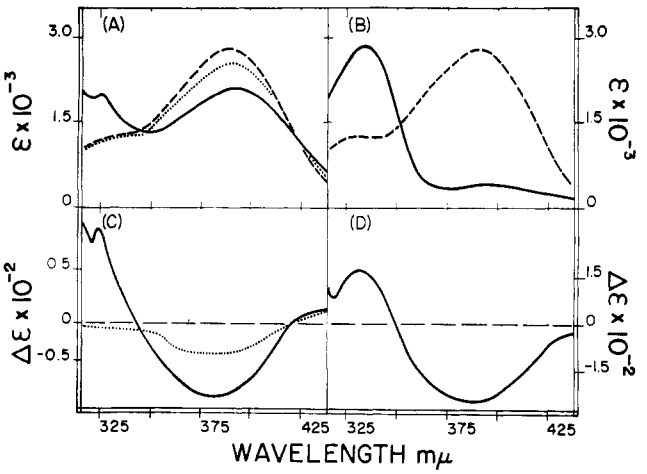

FIG. 5.—Absorption spectra of pyridoxal-P with δ-carboxypeptidase and apo-δ-carboxypeptidase (A), and with L-cysteine (B). Pyridoxal-P alone (---------); pyridoxal-P plus δ-carboxypeptidase, (. . . . . .); pyridoxal-P plus apo-δ-carboxypeptidase, (———), (A); pyridoxal-P plus L-cysteine (———), (B). Difference spectra of pyridoxal-P plus δ-carboxypeptidase versus pyridoxal-P, (. . . . . .) and pyridoxal-P plus apo-δ-carboxypeptidase versus pyridoxal-P (———), (C); pyridoxal-P plus L-cysteine versus pyridoxal-P (———), (D). Protein (0.1 μmole) was incubated with 2 μmoles pyridoxal-P and 2.0 μmoles L-cysteine with 1.0 μmole pyridoxal-P at 4° in 0.02 M Veronal, 1 M NaCl buffer, *p*H 7.5, for 24 hours and 1 hour, respectively. Cary recording spectrophotometer, 1 cm path length cells.

Discussion

When the removal of a metal atom from a metalloenzyme, with concomitant loss of activity, is fully reversible, as in carboxypeptidase, the chemical identification of the ligand site of the protein becomes possible (Vallee, 1961, 1964). This circumstance renders feasible the identification of the uncovered metal-binding groups by virtue of the difference in reactivity of the metal-containing and metal-free enzyme with site-specific selective reagents. This general approach underlies the experiments here reported.

The apoenzyme reacts quantitatively with reagents which are specific for sulfur- and nitrogen-containing groups and which selectively modify the metal-binding groups at the active center (Vallee *et al.*, 1963). Ag^+, *p*-mercuribenzoate, and ferricyanide and, in ancillary fashion, oxidation served to identify the thiol group (Vallee *et al.*, 1960a). PTC, FDNB, and photooxidation with methylene blue were chosen to modify a postulated nitrogenous group (Coombs and Omote, 1962). Pyridoxal-P was employed with the intent of cross-linking the sulfur- and nitrogen-containing groups and to explore its suitability as a bifunctional reagent. Throughout, the zinc content and products of reaction derived from the native enzyme and from the apoenzyme were measured and compared. When possible, analytical data and enzymatic activities were correlated. The success or failure of restoring activity by addition of Zn^{2+} to apocarboxypeptidase, modified by these reagents, served as a useful functional criterion of site-specific alterations.

The interactions of Ag^+, *p*-mercuribenzoate, and ferricyanide demonstrate the presence of a free thiol group in the apoenzyme (Vallee *et al.*, 1960a). This thiol is blocked and becomes unreactive when zinc is present (Fig. 1, Tables I and II). Preincubation of the apoenzyme with an excess of Ag^+ or *p*-mercuribenzoate or oxidation of the thiol group prevents the binding of zinc and consequently the restoration of activity (Table III). The peptide substrate, CGP, prevents the binding both of Ag^+ (Fig. 1) and of Zn^{2+} to the apoenzyme (Coleman and Vallee, 1962a,b); both these metal ions bind to the thiol at the active site in close proximity to the binding sites of the substrate to the apoenzyme.

p-Mercuribenzoate is appreciated as a specific reagent for the analytical demonstration of thiol groups (Boyer, 1959; Cecil and McPhee, 1959; Cecil, 1963) and its effects on apocarboxypeptidase are in accord with prediction (Table II). When the apoenzyme is preincubated with *p*-mercuribenzoate the ensuing decrease in the Ag^+ titer confirms that both these agents react at the same site (Fig. 1). The data in Table II, however, emphasize the need for careful control of the ambient ionic environment of apocarboxypeptidase so that titrations with *p*-mercuribenzoate may be performed in a satisfactory manner.

(Apo)carboxypeptidase is not readily dissolved in aqueous solutions of low ionic strength and hence a careful investigation of the effect on thiol titrations of the salts and buffers required to dissolve the enzyme became necessary. Most of the systems studied interfered both with the rate and completeness of reaction, though to varying degrees. The reaction was instantaneous and complete only in 1 M Tris, 1 M perchlorate, yielding thiol titers analogous to those observed with Ag^+. Tris apparently facilitates the solubilization of *p*-mercuribenzoate (Table II, lines 5 and 6). The dependence of the reaction of *p*-mercuribenzoate with thiol groups on the ionic environment confirms the original data of Boyer (1954), and should be kept in mind as an explanation for possible discrepancies between Ag^+ and *p*-mercuribenzoate titrations in any system.

The partial oxidation of the thiol group (Tables III and IV) enlarges upon this approach. Oxidation of the thiol group may account for the decrease in the capacity of apocarboxypeptidase to be reactivated by Zn^{2+} on aging and storage (Table IV), though the reasons for the difference in the behavior of γ- and δ-carboxypeptidase in this regard are not as yet clear.

The high concentrations of NaCl (or LiCl) required to dissolve carboxypeptidase significantly affect the interpretation both of argentometric titrations and the effect of *p*-mercuribenzoate on the enzyme (*vide supra*). The complex of Hg^{2+} with sulfur is much more stable than is that of Zn^{2+}. Hence, at equilibrium, Hg^{2+} would be expected to displace zinc from carboxypeptidases. This prediction can be demonstrated experimentally when chloride ions are eliminated from dialyzing solutions (Coombs *et al.*, 1962).

Despite persistent efforts the thiol group of the apoenzyme could not be shown to react with iodoacetate, iodoacetamide, or *N*-ethylmaleimide to any significant extent. However, if carboxypeptidase was first denatured to render it susceptible to proteolytic degradation, and then digested with chymotrypsin, or if the native enzyme was treated with reducing agents, such as β-mercaptoethanol, the thiol group became fully reactive toward alkylating agents such as iodoacetate, iodoacetamide, or *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (Walsh *et al.*, 1962), a finding fully in accord with our earlier observations (Vallee *et al.*, 1960a). After identification of the thiol by selective labeling with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, a tetradecapeptide containing the cysteine residue of the active site was obtained (Walsh *et al.*, 1962, Sampath Kumar *et al.*, 1963b).

The sequence and magnitudes of the stability constants of a series of metallocarboxypeptidases are in the order $Hg^{2+} \gg Cd^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$, and their comparison to the same constants for simple bidentate ligands suggested that nitrogen is the second atom of the bidentate binding site of the apoenzyme (Vallee *et al.*, 1960a; Coleman and Vallee, 1961).

Complexometric titrations of apocarboxypeptidase with Zn^{2+} ions revealed apparent pK_a values of 7.7 and 9.1, supporting this interpretation. The pK_a of 9.1 was assigned to the thiol group while that of 7.7 was thought to be consistent with an α-amino group or, alternatively, an imidazole nitrogen (Coleman and Vallee, 1961). Hence, a series of investigations was undertaken to differentiate between these possible alternatives.

PTC reacts with N-terminal α-amino groups and FDNB reacts with both α- and ϵ-amino groups as well as with imidazole nitrogen. If the zinc is bound at the α-amino group the different yields from paired experiments on native zinc and apocarboxypeptidase should indicate the degree to which the metal prevents reaction, a general experimental plan quite analogous to that employed for the thiol group (Coombs and Omote, 1962; Vallee *et al.*, 1963). As in that instance, the experiments with PTC and with FDNB indicate that the sum of the mole fraction of derivatives plus the fraction of a gram-atom of zinc remaining on the protein is unity (Tables VI and VII, Figs. 2 and 3).

The conditions which must be employed to bring about cyclization, separation, and isolation of the products of these reactions destroy the structural integrity of the enzyme, and hence its activity. Therefore reactivation by Zn^{2+} ions could not be employed to confirm the analytical implications of the results by enzymatic means. It is significant, however, that our results with FDNB at *p*H 9 (Table VII) were identical to those of Thompson (1953) in yielding 0.7 M DNP-aspartate/mole enzyme; but zinc analyses, not previously performed, further demonstrated that 0.27 g-atom of zinc remains on the enzyme, fully accounting for the low yield of DNP-aspartate. Extension of these studies with FDNB at *p*H 7.0 document that the yield of DNP-aspartate is a direct function of the zinc content of the enzyme. Since the ϵ-amino groups of carboxypeptidase can be acetylated without affecting the activity (Coombs and Omote, 1962; Vallee *et al.*, 1963), the interaction of FDNB with these groups of the enzyme cannot easily be held accountable.

During the isolation of DNP-aspartate, traces of DNP-serine and -threonine were also found and thought to constitute the residues penultimate to the N-terminus, the only reasonable interpretation possible at that time (Thompson, 1953).

Similarly we have found DNP-serine and -threonine in some but not all of our analyses. Recent studies on procarboxypeptidase have resolved the problems posed by these findings. Neurath and co-workers (Bargetzi *et al.*, 1963, Sampath Kumar *et al.*, 1963a,b) have isolated three carboxypeptidases, identical in activity but differing in end groups. Alanine, serine, and asparagine are the N-termini of α-, β-, and γ-carboxypeptidase, respectively. In addition the prefix "δ" has been given to the enzyme isolated from acetone powder (Allan *et al.*, 1964), which also has an N-terminal asparagine but differs from γ-carboxypeptidase in solubility and, as here demonstrated, in reactivation of the apoenzyme by zinc; β-carboxypeptidase is found as a contaminant of γ- and δ-carboxypeptidases (Sampath Kumar *et al.*, 1963a), thus apparently accounting for the findings of Thompson (1953).

Edman degradation was also employed to demonstrate the N-terminal sequence as asparagine, tyrosine, and alanine. The discovery that two tyrosyl residues are involved in the action of the enzyme (Simpson *et al.*, 1963) lends potential significance to the findings of a penultimate tyrosyl residue in γ-carboxypeptidase.

Substantiation of the hypothesis that zinc is bound to the α-amino nitrogen of the N-terminal asparagine on one hand and to the thiol group of the sole cysteine residue on the other would be greatly facilitated by the joint isolation of all of the components of the active site, which apparently are not in linear sequence In view of the difficulties of providing such experimental evidence by conventional means, alternative methods were sought to supplement that provided by titrations, by differential metal binding and by amino acid analysis of each of the two ligand sites (*vide supra*).

The spectra of the pyridoxal-P-cysteine, -homocysteine,-cysteinylglycine, and -penicillamine complexes which Dr. Edmond Fischer kindly brought to our atten-

tion (Matsuo, 1957; du Vigneaud *et al.*, 1957; Buell and Hansen, 1960; Braunstein, 1960) suggested that this compound might be suitable as a bifunctional reagent to cross-link the zinc-binding sites. Since the spectrum of the pyridoxal-P-apoenzyme complex is quite analogous to that of the cysteine complex, the reagent may interact with a thiol and an amino group of apocarboxypeptidase just as it apparently does with these aminothiols.

Reactions of pyridoxal-P with aminothiols such as cysteine, resulting in the formation of a thiazolidine by ring closure, are slow and follow a second-order rate when, as in Figure 5B and D, the aminothiol is in excess (Buell and Hansen, 1960). When the amino and thiol groups are separated further than in cysteine, as in homocysteine and cysteinylglycine, the rate of formation of the thiazolidine decreases even further.

The rate of formation of the pyridoxal-P-apocarboxypeptidase complex, like that of substituted aminothiols, is slower than that of cysteine; it is probably second order, as is apparent from the rate of change of the maxima at 325 and 388 mμ and from experiments now in progress. These circumstances mitigate the interaction of the reagent with simple thiols, such as mercaptoacetic acid, as a basis of the spectral changes. For such thiols the rate of formation is instantaneous, fully reversible, and hence only discernible when the monothiol is in vast excess (Bergel and Harrap, 1961). In the experiment with apocarboxypeptidase here reported, pyridoxal-P was added in excess, thus militating against the formation of a complex with a monothiol. The new maximum at 325 mμ constitutes the essential difference in the spectra of the apocarboxypeptidase and carboxypeptidase complexes with pyridoxal-P. Since the appearance of a free sulfhydryl group on removal of zinc differentiates the apo- and the native enzyme, it seems reasonable to interpret the spectral changes in the apoenzyme complex as consistent with thiazolidine formation.

The increase in absorbance at 325 mμ after 24 hours corresponds to the interaction of 0.6 mole of pyridoxal-P with 1 mole of apocarboxypeptidase, based on the cysteine model. Determinations of the molar absorptivity at the maximum of the pyridoxal-P-apoenzyme complex and of the precise time required to achieve equilibrium will likely alter this numerical approximation which is of the expected order of magnitude.

In addition to these spectral changes, the absorbance between 420 and 450 mμ of *both* the native and the apoenzyme rapidly increases on exposure to pyridoxal-P. These spectral changes differ both in kind and degree from those seen in the cysteine system. In accord with the interpretation of similar spectra in other instances they probably represent formation of Schiff's bases with the free ε-amino groups of the protein (Braunstein, 1960; Buell and Hansen, 1960; Krebs and Fischer, 1962).

The pK_a of 7.7 found on complexometric titration is consistent with the α-amino group as the most likely binding site, and mitigates the involvement of an ε-amino group.

The probability that an α-amino group can participate in binding zinc also receives support from studies of metal binding in other proteins and polypeptides where metal ions have been shown to bind to α-amino groups. Copper binds to the N-terminal residues of bovine serum albumin (Peters, 1960), oxytocin and 7-phenyloxytocin (Breslow, 1961), and of vasopressin (Campbell *et al.*, 1963). Zinc is bound at the N-terminal phenylalanine of insulin (Mills, 1953; Marker, 1960).

Photooxidation of the apoenzyme in the presence of methylene blue might be expected to destroy a histidine residue essential for zinc binding. Addition of Zn^{2+} to the irradiated apoenzyme would then fail to restore activity to the level of that exhibited by the native enzyme, irradiated as a control. However, experiment demonstrates that the native enzyme loses activity at the same rate at which the apoenzyme loses its capacity to be reactivated (Fig. 4). Moreover, there is no difference in the rates at which the histidine residues of the enzymes are oxidized. Zn^{2+} actually protects L-histidine against the effects of irradiation in the presence of methylene blue; the rate of destruction of the L-histidine-Zn^{2+} complex is much slower than that of L-histidine alone (Fig. 4). Thus the metal ion exhibits the postulated protective action for the amino acid in solution but it fails to protect the enzyme, giving no indication of the involvement of histidine in binding Zn^{2+}.

While such data do not finally eliminate a histidyl residue from consideration, they must be viewed in context. Bromo- and iodoacetate fail to carboxymethylate a histidine residue of the apoenzyme over a wide range of *p*H and over long periods of exposure (Pulido and Vallee, unpublished observations). Kinetic studies with these agents, moreover, have shown them to be strictly competitive inhibitors; irreversible inactivation was not observed. No imidazole-DNP-histidine could be detected after reaction of both zinc and apocarboxypeptidase with FDNB. Finally, no spectral evidence for the formation of acetyl-histidine or pyrydoxal-P-histidine complexes have been obtained. Thus all data presently available fail to implicate histidine as a part of the zinc-bonding site although they do not disprove it conclusively. It should be stated explicitly, however, that these remarks on zinc binding do not bear on the possible involvement of a histidine residue in the mechanism of either peptide or ester hydrolysis by carboxypeptidase (Vallee *et al.*, 1963.) The hypothesis that the zinc atom is bound to the thiol group of the sole cysteine residue and the α-amino group of the N-terminal asparagine of γ-carboxypeptidase is most consistent with the data available so far. Structural studies now in progress will likely elucidate this problem further.

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Transport of Glycine by Pigeon Red Cells*

GEORGE A. VIDAVER†

From the Department of Chemistry, Indiana University, Bloomington

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Glycine entry into pigeon red cells can be analyzed into two components, a sodium-independent one which is linear with glycine concentration, and a sodium-dependent one which fits Michaelis-Menten kinetics. A double reciprocal plot of the sodium-dependent component of glycine entry against $(Na^+)^2$ also gives a straight line, as though two sodium ions are cosubstrates with glycine at some step in the entry process. The K_m term (glycine concentration giving half-maximal entry rate) in the entry equation, rather than the V_{max} term (maximum entry rate), is governed by Na^+, implying a sodium effect on glycine binding by the transport mechanism rather than on its transfer across the membrane. The sodium dependence implies the existence of a complex containing two sodium ions and one glycine. The exit process also has a sodium dependent component. Glycine inside the cells does not appear to be bound. Glycine accumulation seems to be an energy-utilizing ("active") process arising from a sodium-accelerated glycine entry, not balanced by a corresponding glycine exit.

Many types of cells can accumulate amino acids (Christensen, 1962). The entry process could be considered as the result of two components, one obeying Michaelis-Menten kinetics and one resembling simple diffusion (Heinz, 1957; Akedo and Christensen, 1962; Helmreich and Kipnis, 1962). Amino acid influx was inhibited by metabolic inhibitors (Heinz, 1957). Amino acid loss from cells followed the kinetics of a passive process (Heinz and Walsh, 1958; Helmreich and Kipnis, 1962). Evidence has been presented that amino acid inside the cells is "free" (Christensen *et al.*, 1952c; Heinz, 1957).

Amino acid accumulation by Ehrlich ascites cells was inhibited when Na^+ in the medium was replaced by K^+ (Christensen *et al.*, 1952b) and it has recently been reported that Na^+ is required for glycine entry into Ehrlich ascites cells (Kromphardt *et al.*, 1963).

Studies so far made on amino acid transport have been hampered by the difficulty of modifying the composition of the cell interior. The reversibility of hemolysis of red cells suggested the use of lysed and restored red cells to circumvent this limitation, since during hemolysis materials exchange freely between the inside of the cells and the lysing solution (Hoffman, 1958).

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