A Zinc-binding Thiol Group in the Active Center of Bovine Carboxypeptidase B*

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ABSTRACT: Bovine pancreatic carboxypeptidase B contains a single thiol group which binds zinc to the apoenzyme. This thiol group does not react with sulfhydryl reagents unless zinc is first removed. This can be accomplished either by exposure to chelating agents such as 1,10-phenanthroline or by denaturation. When zinc is removed by exposure to 1,10-phenanthroline, readdition of the metal to the apoenzyme restores both peptidase and esterase activities to the degree expected from the fraction of free thiol present in the apoenzyme. Partial blocking of the thiol group, by reaction with iodoacetamide, prevents reconstitution of the holoenzyme to the degree predicted from the partial modification prior to the addition of metal. Specific substrates, such as benzoylglycyl-L-arginine, or specific inhibitors, such as ε-aminocaproic acid, prevent alkylation of the thiol of the apoenzyme and, hence, restoration of esterase activity upon the addition of zinc. Reaction of monocarboxymethylcarboxypeptidase B with sulfite leads to the formation of three additional thiol groups reactive with mercuribenzoate, indicating that in native carboxypeptidase B six of the seven half-cystines are paired in three cystine residues, and one occurs as a metal-mercaptide.

Bovine pancreatic carboxypeptidases A (Neurath and Schwert, 1950) and B (Wintersberger *et al.*, 1962) differ in substrate specificity and in amino acid composition but resemble each other in several physicochemical characteristics. In particular, bovine carboxypeptidase B (Cox *et al.*, 1962), like the comparable enzyme from porcine pancreas (Folk *et al.*, 1960) and bovine carboxypeptidase A (Vallee and Neurath, 1955), contains 1 g-atom of zinc per mole of enzyme (mw approximately 34,500). The metal is essential for the catalytic function of each of these enzymes. Removal of the zinc atom has resulted in the identification of the metal-binding site of carboxypeptidase A (Vallee *et al.*, 1960a). A number of approaches all led to the conclusion that the sole free cysteine residue (Vallee *et al.*, 1960b) and a nitrogen donor, probably the α-amino group of the N-terminal residue (Coleman and Vallee, 1961; Coombs *et al.*, 1964), bind the zinc atom to the apoenzyme of carboxypeptidases γ and δ. The zinc-binding cysteinyl peptide has been isolated (Walsh *et al.*, 1962) and a preliminary report of its amino acid sequence has been published (Sampath Kumar *et al.*, 1963). It was therefore of interest to ascertain whether the zinc-binding site of bovine carboxypeptidase B shared some of the features of that of carboxypeptidase A. The presence of seven cysteic acid residues in the acid hydrolysate of the performic acid–oxidized enzyme (Cox *et al.*, 1962) lends added interest to such an investigation, since, potentially, more than one cysteinyl residue could participate in the binding of the metal atom. The present experimental findings show, however, that six of the seven cysteic acid residues occur as disulfide-bonded cystines; this leaves only one cysteinyl residue as a potential ligand for zinc, and this is, in fact, its function in the native enzyme. The isolation and structure of the cysteinyl peptide will be reported in the companion paper (Wintersberger, 1965).

Materials and Methods

Carboxypeptidase B. A twice-crystallized enzyme was prepared from aqueous extracts of beef pancreas acetone powders as described (Wintersberger *et al.*, 1962). The final crystals contained 1760 μg Zn/g protein (0.94 g-atom/mole) and had a specific activity of at least 0.20 mmole HPLA[1] hydrolyzed per minute per mg enzyme protein (Wintersberger *et al.*, 1962). Spectro-

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[1] Abbreviations used in this work: HPLA, hippuryl-DL-phenyllactate; BGA, benzoylglycyl-L-arginine; CGP-carbobenzoxyglycyl-L-phenylalanine; NEM, *N*-ethylmaleimide; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DDPS, *N*-(4-dimethylamino-3,5-dinitrophenyl)succinimido; IAA, iodoacetamide; OP, 1,10-phenanthroline; SDS, sodium dodecyl sulfate; [(CPD)Zn], native carboxypeptidase B; (CPD), apocarboxypeptidase; CMB, *p*-mercuribenzoate; *S*-CM, *S*-carboxymethyl-.

graphic analysis (Vallee, 1955) indicated insignificant amounts of all other metals.

Apocarboxypeptidase B. The apoenzyme was prepared by dialyzing 2-ml volumes of carboxypeptidase B (6–8 mg/ml) for 48 hours at 4° against three changes of 400 ml of 0.01 M acetate buffer, *p*H 5.5, containing 1×10^{-3} M 1,10-phenanthroline, and the 1,10-phenanthroline was then removed during an additional 48 hours of dialysis against four changes of acetate buffer. The small amount of denatured protein which precipitated during dialysis was removed by centrifugation and the clear supernatant was then stored at 4° under a toluene atmosphere to prevent bacterial contamination. The final product gave approximately 80% yield and contained 150 μg Zn/g protein (0.08 g-atom/mole).

Carbobenzoxylglycyl-L-phenylalanine and *ε-aminocaproic acid* were purchased from the Mann Research Laboratories, New York. *Benzoylglycyl-L-arginine* was a gift of Dr. Roger Roeske of the Lilly Research Laboratories. *Hippuryl-DL-β-phenyllactic acid* was prepared by Dr. William O. McClure of the Department of Biochemistry, University of Washington. *Iodoacetamide* (Mann Research Laboratories), *N-ethylmaleimide* (Schwarz Bioresearch Inc.), *N-4-(dimethylamino-3,5-dinitrophenyl)maleimide* (Aldrich Chemical Corp.), *p-mercuribenzoate* (California Corp. for Biochemical Research), and *β-mercaptoethanol* (Eastman Kodak) were all used without further purification.

Buffers and reagent solutions, prepared from the reagent-grade salts, were purified from contaminating zinc by extraction with dithizon in carbon tetrachloride (Coleman and Vallee, 1960), and water was purified by passage over a mixed cation-anion resin bed (Barnstead Still and Sterilizer Corp., Boston, Mass.) as previously described (Coleman and Vallee, 1960). *Dialysis tubing* was appropriately treated Visking-Nojax casing (Hughes and Klotz, 1956).

Protein concentrations were determined from the absorbance at 280 mμ, $E^{1\%}_{280\ m\mu} = 21$ (Cox *et al.*, 1962).

Enzymatic Activities. Carboxypeptidase B activity toward the substrate HPLA (0.01 M) was determined in 0.045 M NaCl–0.005 M Na-barbital buffer, *p*H 7.5 (Wintersberger *et al.*, 1962). Activity toward the basic dipeptide, BGA, was determined in 0.001 M solutions of the substrate, containing 0.045 M NaCl–0.005 M Na-barbital, *p*H 7.5, 25°. Activities toward the substrate, CGP (0.025 M), were measured in 0.1 M NaCl–0.02 M Na-barbital, *p*H 7.5, 25°, as described previously (Wintersberger *et al.*, 1962). *Zinc determinations* were carried out using either a microchemical dithizon method (Vallee and Gibson, 1948), an atomic absorption procedure (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964), or by means of an isotopic technique (*vide infra*).

Equilibrium dialyses at varying *p*H using radioactive labeled [^{65}Zn]carboxypeptidase B were carried out in a manner similar to that described for carboxypeptidase A (Coleman and Vallee, 1960).

Sulfhydryl Titrations. Free sulfhydryl groups were determined using Ag^+ (Benesch *et al.*, 1955), or *p*-mercuribenzoate (Boyer, 1954) as modified by Coombs *et al.* (1964).

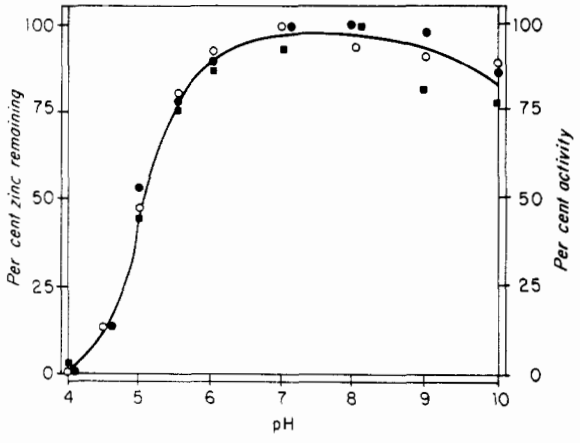

FIGURE 1: Dependence of zinc content and esterase activity of carboxypeptidase B on *p*H. Aliquots (1 ml) containing 0.1 μmole of native carboxypeptidase B labeled with ^{65}Zn were dialyzed at 4° versus 100 ml of 0.05 M Tris–0.05 M acetate–1 M NaCl buffer adjusted to the *p*H values indicated. One ml of the buffer alone in a separate, similar dialysis bag was dialyzed together with each enzyme solution. During the dialysis the radioactivity inside each bag was increased and the ^{65}Zn remaining bound to the protein was calculated from the radioactivity measurements as described (Coleman and Vallee, 1960). After equilibrium was reached, the dialysis bags were opened and the total zinc content, radioactivity, and esterase activity of the solutions inside were measured as described in the text. Zinc content at equilibrium: (a) radioactivity measurements, (○—○); (b) atomic absorption spectrometry, (■—■); esterase activity, (●—●).

Spectrophotometric measurements were performed with a Beckman DU spectrophotometer with photomultiplier attachment. *p*H *measurements* were carried out at room temperature with a Radiometer Type PHM 22p *p*H meter with a glass electrode.

Results

Correlation of Zinc Content and Activity with pH. The effect of *p*H on both zinc binding and esterase activity of carboxypeptidase B was determined by dialyzing an enzyme, which had been labeled with ^{65}Zn (Coleman and Vallee, 1960), against zinc-free buffers of varying *p*H. The rate of zinc removal was monitored by measuring the radioactivity of the solutions inside the dialysis membrane as a function of time. When equilibrium was achieved (20 hours for the dialysis at *p*H 4.0 and 100 hours for dialysis at *p*H 6.0–10.0), the final amount of zinc remaining firmly bound to the protein was determined both from the radioactivity of the solutions inside the dialysis membranes and from their total zinc content (Figure 1). All esterase activities of these

TABLE I: Effect of Chelating and Denaturing Agents on the Reaction of Carboxypeptidase B with Iodoacetate.[a]

Agent Added to Carboxypeptidase	*S*-CM-cysteine (residues/mole protein)	Zinc (g-atom/mole)	Sum (Zn + SH)	Per Cent Activity
None	0	1.00		100
β-Mercaptoethanol and IAA	7.0	0		0
OP and IAA	0.8	0.18	0.98	20
Urea and IAA	0.8	0.20	1.00	15
Urea, OP, and IAA	0.9	0.18	1.08	17
Guanidine, OP, and IAA	0.7	0.02	0.72	0
SDS, OP, and IAA	0.6	0.19	0.79	0

[a] Iodoacetamide (3 mg) was incubated at 25° for 2 hours with 0.1 μmole carboxypeptidase B, dissolved in 1 ml of 0.1 M Tris–1 M NaCl, *p*H 8.0, containing either 8 M urea alone, 2×10^{-3} M OP alone, or 2×10^{-3} M OP with 8 M urea, 5 M guanidinium chloride, or 0.4% SDS. To reduce the protein, 0.1 μmole carboxypeptidase B was preincubated with 10 μmoles β-mercaptoethanol before reaction with the IAA as described in the methods section. Each modified protein was precipitated after carboxymethylation, then hydrolyzed, and the hydrolysates were analyzed for *S*-CM-cysteine as described in the methods section. The zinc content of the carboxymethylated carboxypeptidases was determined in duplicate after dialysis of the proteins against three 100-ml volumes of zinc-free Tris-NaCl buffer, *p*H 8.0. The esterase activity of the dialyzed proteins was measured using 0.01 M HPLA as the substrate in 0.045 M Veronal–0.2 M NaCl, *p*H 7.5, 25°.

solutions equilibrated at a given *p*H were determined on HPLA *at p*H *7.5*.

The amount of zinc remaining bound to the protein after equilibration at each *p*H and the correspondent enzymatic activity assayed at *p*H 7.5, constituting the *p*H-stability curve (Coleman and Vallee, 1960; Bethune *et al.*, 1964), are shown in Figure 1. There are no significant losses either of zinc or of activity between *p*H 7.0 and 9.0. However, both zinc and esterase activity are lost concomitantly below *p*H 7.0 and above *p*H 9.0. With decreasing *p*H, both the extent and the rate at which zinc is lost increase such that, at *p*H 5, 50%, and at *p*H 4, all of the zinc and activity are lost. Both at *p*H 4.5 and 4.0 irreversible protein precipitation was noted.

After establishment of equilibrium, reversibility of the reaction was measured by adding the equivalent of 2 g-atoms of zinc per mole to the enzyme, adjusting the *p*H to 7.5, and then determining esterase activities. Activity was not restored at *p*H 4.0 and 4.5 where protein precipitation was observed. On adding Zn^{2+} at *p*H 5.0, activity increased from 50 to 80% of that of the native enzyme control. Under these conditions activity was fully restored at *p*H 5.5, 6.0, and 10.0.

Reactive Thiol Groups of Native and Apocarboxypeptidase B. Oxidation of carboxypeptidase B with performic acid followed by acid hydrolysis yields seven residues of cysteic acid per molecule (Cox *et al.*, 1962). Conversely, complete reduction of the enzyme with β-mercaptoethanol in 8 M urea, followed by carboxymethylation of the thiol groups with iodoacetamide, yields seven residues of *S*-carboxymethylcysteine per molecule (Table I).

When prior reduction is *not* carried out, carboxymethylation of carboxypeptidase B yields from 0 to 0.9 g-residue of *S*-carboxymethylcysteine per mole of carboxypeptidase B, depending on the conditions employed (Table I). There is no detectable formation of *S*-carboxymethylcysteine on incubation of the native enzyme with iodoacetamide alone, and neither zinc nor enzymatic activity is lost. When, however, at *p*H 8 either 2×10^{-3} M 1,10-phenanthroline or 8 M urea is added alone or 1,10-phenanthroline in conjunction with 8 M urea, 5 M guanidine hydrochloride, or 0.4% sodium dodecyl sulfate, from 0.6 to 0.9 g-residue of *S*-carboxymethylcysteine per mole of enzyme can be obtained after treatment with iodoacetamide. Urea alone is as effective as 1,10-phenanthroline in permitting the carboxymethylation of close to one cysteine residue of carboxypeptidase B. Thus, without prior reduction, the maximum yield of *S*-carboxymethylcysteine was always slightly less than but could not be made to exceed 1 g-residue per mole, a circumstance which was maintained even after complete digestion of the modified enzyme with chymotrypsin (*vide infra*).

Zinc analyses were performed subsequent to incubation of the enzyme with iodoacetamide in the presence of the reagents described, followed by dialysis against zinc-free buffer. In all instances the decrease in activity was directly proportional to the amount of zinc removed and inversely proportional to the amount of *S*-carboxymethylcysteine obtained, and the sum of the mole fraction of zinc remaining bound and that of *S*-carboxymethylcysteine formed is close to 1 (Table I).

When *apocarboxypeptidase* B was exposed to NEM or Ag^{+}, close to 1 mole of thiol per mole of enzyme reacted. Incubation of the native enzyme with DDPM at *p*H 4.5, known to remove zinc from the active center,

brings about the same result (Table II). DDPM and iodoacetamide compete for this group of the apoenzyme, as is apparent when the protein is reacted first with DDPM at *p*H 4.6 followed by reaction with iodoacetamide at *p*H 8.0. The final hydrolysate contained 0.8 g-residue of *S*-succinocysteine and 0.15 g-residue of *S*-carboxymethylcysteine per mole of enzyme (Table II).

TABLE II: Reaction of Apocarboxypeptidase B with Thiol Reagents.

Reaction	—SH Reacted (mole/mole protein)
(CPD) + Ag^{+}[a]	0.95
(CPD) + DDPM[b]	0.90
(CPD) + NEM	0.70
(CPD) + DDPM + IAA[c]	0.8 (*S*-succinocysteine)
	0.15 (*S*-CM-cysteine)

[a] For Ag^{+} titrations, 0.1 μmole apocarboxypeptidase B was titrated at 4° in Tris-KCl-$NaNO_3$ buffer, *p*H 7.5, as previously described (Coombs *et al.*, 1964). [b] Carboxypeptidase B (0.1 μmole) in either 0.1 M acetate buffer, *p*H 4.6, containing 2 μmoles DDPM, or in 0.1 M acetate, *p*H 4.6, containing 2×10^{-3} M OP and 5 μmoles NEM were incubated overnight at 37°. The modified proteins were then precipitated with an equal volume of 10% trichloroacetic acid, washed with water and acetone, and finally hydrolyzed with acid. The hydrolysates were analyzed for *S*-succinocysteine as described above. [c] One-tenth μmole of the DDPS–carboxypeptidase B obtained after incubation with DDPM as described above was dissolved in 0.1 M Tris–1 M NaCl, *p*H 8.0, containing 8 M urea, and incubated with 3 mg IAA, as described in Table I. The doubly treated protein was then precipitated, washed, and hydrolyzed, and the hydrolysate was analyzed for *S*-succino- and *S*-CM-cysteine as described above. The enclosure (CPD) + DDPM indicates the order of the modification procedure.

Complementarity of Zinc Content, Activity, and Reactive Thiol. The addition of increasing fractional equivalents of Zn^{2+} to apocarboxypeptidase B prepared at *p*H 5.5 immediately prior to reaction of the apoenzyme with iodoacetamide progressively *decreases* the mole fraction of *S*-carboxymethylcysteine isolated after reaction, separation, and acid hydrolysis. On addition of 1 g-atom of Zn^{2+} no *S*-carboxymethylcysteine can be isolated. Thus the mole fraction of zinc restored to the apoenzyme complements the mole fraction of *S*-carboxymethylcysteine formed; their sum remains close to unity (Table III). Both peptidase and esterase activities, determined before reaction with iodoacetamide, are restored in direct proportion to the amount of Zn^{2+} added to the apoenzyme. The activity restored becomes equal to that of native carboxypeptidase B only when 1 g-atom Zn^{2+} has been readded to the apoenzyme.

Prior addition of the basic dipeptide substrate, BGA, or of the competitive inhibitor, ε-aminocaproic acid, prevents the reaction between apocarboxypeptidase B and iodoacetamide (Table III). Addition of Zn^{2+} after removal by dialysis of iodoacetamide and BGA or ε-aminocaproic acid, respectively, restores 86 and 78% of the esterase activity.

Reaction of the apoenzyme with Ag^{+} similarly demonstrated complementarity between the zinc content and reactive thiol of apocarboxypeptidase. When increasing amounts of Zn^{2+} are added to the apoenzyme prior to amperometric titration with Ag^{+}, the titer of free thiol groups varies inversely such that the sum of Zn^{2+} and free thiol remains close to unity (Table IV).

Preincubation with BGA completely prevents the interaction between apocarboxypeptidase B and Ag^{+}, while HPLA and CGP prevent it partially. The Ag^{+} titer of 0.87, the control, is reduced to 0.68 and 0.64 mole —SH per mole of enzyme, respectively. The protective effect of ε-aminocaproic acid could not be tested in this system, since it suppressed the amperometric current to values too small to permit differentiation.

Distribution of Half-Cystine Residues. In the absence of reducing conditions, maximally one thiol group of carboxypeptidase B reacts with iodoacetamide, DDPM, NEM, or Ag^{+} under conditions which remove the functional zinc atom and inactivate the enzyme. Reaction of iodoacetamide-treated apocarboxypeptidase B with sodium sulfite (Cecil and McPhee, 1955) established that the remaining six half-cystine residues occur as disulfides in the native enzyme. *S*-Carboxymethylcarboxypeptidase B does not react with an 8-fold molar excess of *p*-mercuribenzoate, as is apparent from the absence of any increase in absorbance at 255 mμ. However, after addition to *S*-carboxymethylcarboxypeptidase B of sodium sulfite in 15-fold molar excess over thiol, calculated on the basis of the amount of disulfide presumed to be present in the enzyme, *p*-mercuribenzoate now reacts, as indicated by an increase in the 255-mμ absorbance. The magnitude of the increase at 255 mμ is a function of the molar ratio of *p*-mercuribenzoate to *S*-carboxymethyl protein. A plot of the change in the absorbance at 255 mμ versus the molar ratio, *p*-mercuribenzoate/*S*-carboxymethyl protein, shows that the maximum change occurs at a ratio of 3.1 (Figure 2). This indicates that three thiol groups have now reacted: they may be presumed to have formed as a consequence of the reduction of three disulfide linkages in the *S*-carboxymethyl protein by sodium sulfite. Since chymotrypsinogen has been shown to react stoichiometrically with sulfite (Pechère *et al.*, 1958), this zymogen was chosen to serve as a control to validate the procedure. Five thiol groups were found to react (Figure 2), presumably originating from the five

TABLE III: Correlation of Zinc, Activity, and Iodoacetamide-reactive Thiol of Carboxypeptidase B [(CPD)Zn] and Prevention of Alkylation of Apocarboxypeptidase B (CPD) by BGA and ε-Aminocaproic Acid.[a]

Protein	Zn^{2+} Added (g-atoms)	Per Cent Activity Restored		*S*-CM-Cysteine (residues/mole)	Sum (*S*-CM-Cys + Zn^{2+})
		Esterase	Peptidase		
[(CPD)Zn] control		100	100	0	1.0
(CPD)	1.0	100	100	0	1.0
(CPD)	0.67	64	60	0.24	0.91
(CPD)	0.33	35	40	0.42	0.75
(CPD)	0	23	20	0.77	0.77
(CPD) + BGA	0	86[b]		0	
(CPD) + ε-NH_2-caproic	0	78[b]		0.10	

[a] Increments of Zn^{2+} were first added to 0.076 μmole apocarboxypeptidase B (CPD) dissolved in 0.5 μmole of 0.01 M sodium acetate, *p*H 5.5. Five-tenths ml of 0.2 M Tris–2 M NaCl, *p*H 8.0, containing 3 mg IAA, was then added and the mixture was incubated at 37° for 1 hour. The modified proteins were then precipitated, washed, and hydrolyzed with 6 N HCl, and the hydrolysates were analyzed for *S*-CM-cysteine as described above. Prior to incubation with IAA, esterase activity using 0.01 M HPLA as substrate and peptidase activity using 0.001 M BGA as substrate were measured at *p*H 7.5, 25°, in Veronal and NaCl buffers. Native carboxypeptidase B [(CPD)Zn], incubated under the same conditions, served as a control. Apocarboxypeptidase B (0.076 μmole), dissolved in 0.5 ml 0.1 M acetate, *p*H 5.5, containing either 0.02 M BGA or 0.05 M ε-aminocaproic acid, was treated with 3 mg IAA in Tris-NaCl, *p*H 8.0, as described above. [b] Esterase activity with 0.01 M HPLA was determined after dialyzing off all substrate and IAA with zinc-free Tris-NaCl buffer, *p*H 8.0, and then adding 1 g-atom Zn^{2+} to the dialyzed apoenzymes.

TABLE IV: Complementarity of Zinc Content and Ag^+-titratable —SH of Carboxypeptidase B [(CPD)Zn], and of Apocarboxypeptidase B (CPD).[a]

Sample	Zinc Added (g-atom/mole)	—SH (mole/mole)	Sum (Zn + —SH)
(CPD)	0.08	0.87	0.95
(CPD)	0.38	0.76	1.14
(CPD)	0.68	0.51	1.19
(CPD)	1.08	0	1.08
[(CPD)Zn][b]	0.94	0	0.94
(CPD) + 0.01 M BGA	0	0.07	
(CPD) + 0.01 M HPLA	0	0.68	
(CPD) + 0.02 M CGP	0	0.64	

[a] Increments of Zn^{2+} were added first to 0.1 μmole apocarboxypeptidase B (CPD), and the remaining free —SH was then titrated amperometrically with Ag^+ at 4°, Tris-KCl-$NaNO_3$ buffer, *p*H 7.5, as described. Apocarboxypeptidase (0.1 μmole) was preincubated in 0.1 M Tris–1 M NaCl, *p*H 7.5, 4°, containing 0.1 M BGA, 0.01 M HPLA, or 0.02 M CGP, and then titrated amperometrically with Ag^+ in Tris-KCl-$NaNO_3$, *p*H 7.5, 4°, as described. [b] Control.

disulfide linkages previously established to occur in this protein (Pechère *et al.*, 1958).

Discussion

The common generic name of *carboxypeptidases* for enzymes which catalyze the hydrolysis of C-terminal peptide bonds in proteins and polypeptides, and of ester bonds in analogs of certain peptide substrates, suggests that they might operate by a common mechanism. Carboxypeptidase A and B, e.g., differ only in their specificity, i.e., the nature of the side chain which characterizes the amino acid or hydroxy acid which is released during hydrolysis. In the bovine enzyme, in fact, this specificity is not absolute, as bovine carboxypeptidase B is also active toward substrates of carboxypeptidase A (Wintersberger *et al.*, 1962), though carboxypeptidase A will not attack substrates of carboxypeptidase B. The specificity of the corresponding porcine enzymes is much more distinct (Folk and Gladner, 1958).

There is growing evidence for the belief that enzymes which perform similar functions may share chemical details of their active sites, and, beyond this, similar mechanisms of action. This view has been strengthened recently by a chemical comparison of the structures of bovine chymotrypsin and trypsin (Walsh and Neurath, 1964), of elastase (Smillie and Hartley, 1964), and of papain and ficin (Light *et al.*, 1964). The prominent features of the former group of endopeptidases is the

presence of two histidine residues and a serine residue in the active site, and of the latter group of proteinases, the thiol of a cysteine residue which needs to be free for enzymatic function.

Using the same criteria, the presence of 1 g-atom of zinc as an integral and functional component is the most prominent feature of carboxypeptidases A and B, whether of bovine (Vallee and Neurath, 1955; Cox *et al.*, 1962) or porcine (Folk *et al.*, 1960) origin. The single cysteine and presumably the N-terminal amino group form the bidentate ligand site for the metal in bovine carboxypeptidase A_γ and A_δ, the most thoroughly studied enzymes of the group (Vallee *et al.*, 1960b; Coleman and Vallee, 1961; Coombs *et al.*, 1964). The close correlation of loss of zinc and of enzymatic activities in carboxypeptidase B establishes the essential role of zinc in this enzyme through the functional consequences of its removal. Further, H^+ and Zn^{2+} ions compete in analogous fashion for the metal-binding sites of both carboxypeptidases A and B. As the *p*H is decreased both enzymes progressively lose zinc and activity *pari passu*. Fifty per cent of the zinc is removed at *p*H 5.0 for carboxypeptidase B (Figure 1) and at *p*H 4.7 for carboxypeptidase A (Vallee *et al.*, 1960a).

This similarity of the dependence of zinc binding on *p*H implies the participation of similar metal-binding groups in both apoenzymes. However, the zinc–apocarboxypeptidase B complex is more sensitive to OH^- competition than is the analogous complex of the apocarboxypeptidase A: carboxypeptidase B loses 20% of its original zinc at *p*H 10 (Figure 1) while even at *p*H 11 all of the zinc of carboxypeptidase A remains firmly bound (Coleman and Vallee, 1960). Since identity of the —SH donor group seems established, the explanation for the observed differences in metal binding must be sought in the nature of additional donor sites.

The stability of the respective apoenzymes and their capacity for reactivation through zinc binding constitute further differences between carboxypeptidases A and B. Carboxypeptidase B cannot be restored fully by readding Zn^{2+} after dialysis at *p*H 5.0, while carboxypeptidase A dialyzed even at *p*H 4.2 is fully reactivated on readdition of Zn^{2+} (Vallee *et al.*, 1960a). Apocarboxypeptidase A_δ is relatively stable and can be stored at 4° and neutral *p*H without any appreciable loss in its capacity to bind zinc (Coombs *et al.*, 1964). In contrast, apocarboxypeptidase B rapidly and irreversibly precipitates at neutral *p*H as zinc is removed; thus far this apoenzyme has been successfully prepared and stored only at *p*H 5.5 (*vide infra*). It seems that either the metal binding thiol group of apocarboxypeptidase B is oxidized readily, or the conformation of the apoenzyme changes to preclude zinc binding.

Bovine carboxypeptidase B, though similar in molecular weight and with respect to the content of several of the constituent amino acids (Cox *et al.*, 1962), differs most notably from carboxypeptidase A in regard to the sulfur-containing amino acids. Compared with carboxypeptidase A it contains six rather than three methionine residues, and upon oxidation

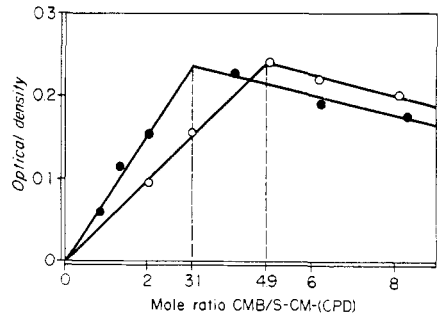

FIGURE 2: Titration of free —SH of sulfite-reduced *S*-carboxymethylcarboxypeptidase (●) and of sulfite-reduced chymotrypsinogen A (○) with *p*-mercuribenzoate (CMB) by the mole ratio method. Carboxypeptidase (9 mg) was dissolved in 3.5 ml of 0.1 M Tris, *p*H 8, containing 8 M urea and 2×10^{-3} M 1,10-phenanthroline. IAA (10 mg) was added and after incubation for 2 hours at 37° the solution was dialyzed against two changes of 250 ml 0.1 M Tris, *p*H 7, containing 8 M urea. Analysis for *S*-carboxymethylcysteine gave 0.9 g-residue/mole. Addition of a 15-fold molar excess of sodium sulfite over the amount of disulfide expected caused an increase in optical density (255 mμ) the magnitude of which depended on the molar ratio of *p*-mercuribenzoate to enzyme used. The maximum was at a ratio of 3:1. The curves defined by open circles correspond to the *p*-mercuribenzoate titration of several-times-recrystallized bovine chymotrypsinogen A which was previously similarly treated with sulfite.

with performic acid it yields seven instead of two cysteic acid residues per molecule (Cox *et al.*, 1962). While the odd number of cysteic acid residues is compatible with the presence of at least one cysteine residue in the native enzyme, the demonstration of the presence of a functional thiol in this enzyme became possible only after initial difficulties in the reversible removal of zinc, with restoration of full enzymatic activity, had been overcome. The preparation and storage of the metal-free apoenzyme at *p*H 5.5, rather than at *p*H 7.5 as in carboxypeptidase A (Vallee *et al.*, 1960a), was found to be of crucial importance for this purpose.

The single thiol group of *native* carboxypeptidase B does not react with sulfhydryl reagents such as iodoacetamide, or Ag^+ ions; it reacts only after zinc has been removed with 1,10-phenanthroline.

Unlike the thiol group of carboxypeptidase A, that of carboxypeptidase B also reacts merely after exposure of the enzyme to urea, guanidinium chloride, or sodium dodecyl sulfate; these denaturing agents labilize and remove the metal even in the absence of chelating agents. Addition of zinc to the apoenzyme prepared with 1,10-phenanthroline at *p*H 5.5 restores both peptidase and esterase activities to the degree expected from the mole fraction of thiol in the apoenzyme. Partial blocking of the thiol, by reaction with iodo-

acetamide, prevents reconstitution of the holoenzyme and restoration of activity to the degree to which the sulfhydryl group has become modified prior to the addition of the metal. In this regard, carboxypeptidase B behaves perfectly analogously to carboxypeptidase A.

Further similarity between the two enzymes can be demonstrated by the effects of prior addition of a specific substrate or of a competitive inhibitor on the reaction between the apoenzyme and the thiol reagent. Thus, the addition of the basic dipeptide substrate BGA, or the competitive inhibitor ϵ-aminocaproic acid (Folk, 1956), prevents alkylation and enhances restoration of esterase activity upon the subsequent addition of zinc. The functional and structural relationship between enzyme activity, zinc content, and thiol thus clearly indicates that the thiol group of a single cysteine residue is one of those involved in binding zinc to this enzyme.

This conclusion, that only one thiol is free in the apoenzyme, is strengthened further by the demonstration that, after its carboxymethylation, reaction with sulfite yields three additional groups capable of reacting with mercuribenzoate. This stoichiometry is strongly indicative of the presence of three disulfide bonds and fully accounts for the seven residues which give rise to cysteic acid after oxidation of the native enzyme.

The nature of the one or more groups to which the zinc is bound in carboxypeptidase B remains to be established. The chemical structure of the cysteinyl peptide isolated from carboxypeptidase B is described in the accompanying paper (Wintersberger, 1965).

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