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Chemical Evidence of a Disulfide Bond in Bovine Carboxypeptidase A*

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ABSTRACT: Cystinyl peptides have been isolated in 55% yield from a peptic digest of carboxypeptidase A, indicating that a single disulfide bond exists between Cys^{138} and Cys^{161} in the native enzyme. In addition, reduction and partial alkylation of the enzyme, using an isotope dilution technique, have indicated that either cysteinyl residue could be alkylated without inhibition of the enzyme and that alkylation of one thiol precludes alkylation of the other. These data are considered in relation to our previous report of an apparently selective alkylation. A model of carboxypeptidase containing a disulfide bond is consistent with the alkylation experiments. These conclusions are in accord with the already established identification of the zinc ligands as His^{69}, Glu^{72}, and His^{196} rather than of a cysteinyl residue.

Bovine carboxypeptidase A contains two "half-cystinyl" residues, but their state of oxidation has not been unambiguously resolved (Vallee and Riordan, 1968; Neurath *et al.*, 1968; Lipscomb *et al.*, 1968). In the zinc holoenzyme, prepared by the method of Allan *et al.* (1964), no thiol group can be detected (Vallee *et al.*, 1960) even under conditions which disrupt the three-dimensional structure (Walsh *et al.*, 1962; Coombs *et al.*, 1964). The apoenzyme reacts stoichiometrically with certain thiol reagents such as silver ion, *p*-mercuribenzoate, or ferricyanide, but each reagent reacts differentially with the zinc-containing and zinc-free enzyme. These observations have been interpreted as evidence for a sulfur–zinc ligand at the active site and support for this hypothesis was obtained from complexometric titrations of the apoenzyme with zinc and certain other metal ions, from the spectral characteristics of cobalt carboxypeptidase, and from the order and magnitude of the stability constants of various metallocarboxypeptidases (Williams, 1960; Vallee *et al.*, 1961; Vallee, 1964).

In other respects, however, the behavior of the "half-

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cystinyl" residues was atypical for thiol groups. No alkylation occurred during reaction of the apoenzyme with iodoacetamide, *N*-ethylmaleimide, and related alkylating reagents even after denaturation of the enzyme with urea or guanidine hydrochloride. However, various alkylating reagents reacted stoichiometrically with 1 equiv of cysteine/mole of carboxypeptidase if the enzyme was first treated with a reducing agent in the presence of the competitive inhibitor β-phenylpropionate (Walsh *et al.*, 1962; Sampath Kumar *et al.*, 1963). Since catalytic activity was retained, it was concluded that this represented a single nonessential cysteinyl residue which was unavailable for alkylation prior to reduction. Subsequent complete reduction yielded a second equivalent of cysteine whose alkylation resulted in complete loss of enzymatic activity. This second cysteine was therefore concluded to provide the zinc-binding thiol, and it was isolated and identified in a peptide sequence (Sampath Kumar *et al.*, 1963).

It was thus concluded that carboxypeptidase A contained one zinc-binding cysteine and a second nonessential cysteine, both of which were chemically unreactive in the native enzyme. Doubt concerning this interpretation appeared when isotope dilution experiments labeled both cysteinyl residues although only 1 equiv of alkylating agent had been introduced (Neurath *et al.*, 1968). In addition, Reeke *et al.* (1967) have interpreted the electron density map of carboxypeptidase A (Cox) to indicate that a single disulfide bond of cystine occurs in the protein and that neither half-cystine was near the zinc atom. In view of these conflicting data we have reevaluated and extended the chemical measurements and have isolated a unique cystinyl-containing peptide from a peptic digest of the enzyme under acidic conditions known to preclude disulfide exchange or oxidation.

Materials and Methods

Crystalline carboxypeptidase A (Anson) was obtained from Worthington Biochemical Corp. (lot No. COA-9 AB), as were L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone–trypsin and pepsin. Carboxypeptidase A^{Val} (Cox) was prepared according to Cox *et al.* (1964) from the pancreatic juice of a single animal.[1] β-Phenylpropionate was obtained from the Baker Chemical Company, anti-foam B from Dow–Corning, sodium borohydride from Metal Hydrides, Inc., [1-^{14}C]iodoacetamide from Volk. β-Mercaptoethanol was redistilled. Tris was purified according to the method of Fossum *et al.* (1951) and crystallized from methanol–water solution. Iodoacetamide (Mann Research) was recrystallized from heptane just prior to use, and *N*-ethylmorpholine was redistilled.

The protein concentration and esterase activity were determined as described by Bargetzi *et al.* (1963). Aliquots of fractions from column chromatography were analyzed after alkaline hydrolysis by ninhydrin in a Technicon Autoanalyzer (Schroeder and Robberson, 1965).

Radioactivity. Radioactivity measurements were carried out in a Packard Tricarb scintillation counter (Model 3003) with 1 ml of aqueous solution in 10 ml of a scintillation fluid containing 12.5% naphthalene, 0.75% 2,5-diphenyloxazole, and 0.0375% 1,4-bis-2-(5-phenyloxazoyl)benzene. Corrections for quenching were applied using [^{14}C]toluene standard. Protein and peptide concentrations in aliquots of the same solution were determined in acid hydrolysates by amino acid analysis.

The specific radioactivity of [1-^{14}C]iodoacetamide was established by measuring the specific radioactivity of purified [^{14}C]carboxymethylcysteine formed from the particular batch of iodoacetamide employed in the carboxypeptidase experiment. The radioactive carboxymethylcysteine was formed by reacting 10.8 μmoles of cysteine in 1 ml of 0.1 M *N*-ethylmorpholine, pH 9.0, with 2.16 μmoles of [^{14}C]iodoacetamide in 0.1 ml of 2.0 M NaCl, 0.20 M Tris, pH 8.0. The mixture was incubated under nitrogen at room temperature for 3 hr and then diluted with an equal quantity of 12 N HCl and hydrolyzed *in vacuo* at 105° for 16 hr. The hydrolysate was dried, dissolved in a minimum volume of water, and streaked on a 10-cm line on Whatman No. 3MM paper and a chromatogram developed for 13 hr with 1-butanol–acetic acid–water (3:1:1). The dried chromatogram was radioautographed for 24 hr using Kodak Medical X-Ray film "no-screen," the radioactive material corresponding to carboxymethylcysteine with a R_F of 0.48 was eluted, and separate aliquots were analyzed for carboxymethylcysteine and ^{14}C content in triplicate. Carboxymethylcysteine prepared in this manner had a specific radioactivity of 8.59×10^5 dpm/μmole.

Protein and Peptide Analysis. Amino acid analyses were carried out on Beckman-Spinco analyzers by the method of Spackman *et al.* (1958) after 20-hr hydrolysis in 6 N HCl at 105° *in vacuo*. Subtractive Edman degradation was carried out by the method of Konigsberg and Hill (1962) as modified by Shearer *et al.* (1967).

Peptides were oxidized by the method of Hirs (1956) using 0.2–1.0 ml of performic acid at 0° for 3 hr, then diluted with 6–30 ml of cold water and lyophilized.

Aliquots of alkylated protein were precipitated in 5% trichloroacetic acid and washed twice with water before adding 6 N HCl. To prevent oxidative losses, nitrogen was then bubbled through all solutions containing alkylation products and frozen prior to a 20-min evacuation during thawing of the samples. Disulfide-containing peptides in column effluents were detected with a Technicon Autoanalyzer by an adaptation of the method of Zahler and Cleland (1968) developed for this purpose (Walsh *et al.*, 1970).

Reduction and Alkylation of Carboxypeptidase A^{Val} (Cox). Conditions of reduction and alkylation to introduce 1 equiv of [^{14}C]iodoacetamide followed by a second equivalent of [^{12}C]iodoacetamide were adopted from those described by Walsh *et al.* (1962) and Sampath Kumar *et al.* (1963). The modification of 1 equiv of cysteine was accomplished as follows: 297 mg (8.6 μmoles) of carboxypeptidase A^{Val} (Cox) was dissolved in 119 ml of 2 M NaCl, 2 M Tris, 0.15 M β-phenylpropionate, pH 8.0. After adding a few drops of Dow–Corning anti-foam B, nitrogen was bubbled through the mixture for 5 min at 0° and 29.7 ml of $NaBH_4$ (5% in 2.0 M NaCl, 2.0 M Tris, 0.2 M β-phenylpropionate, pH 8.0) was added with stirring. After 17 hr, 878 μmoles of [^{14}C]iodoacetamide reagent (identical with that used for preparing the standard carboxymethylcysteine) was added and alkylation allowed to proceed for 6 hr at 0°. The reduced and alkylated mixture was dialyzed successively against three changes of 2-l. volumes of 2 M NaCl, 2 M Tris, 0.1 M β-phenyl-

[1] The nomenclature and nature of these species of carboxypeptidase A are discussed by Pétra and Neurath (1969).

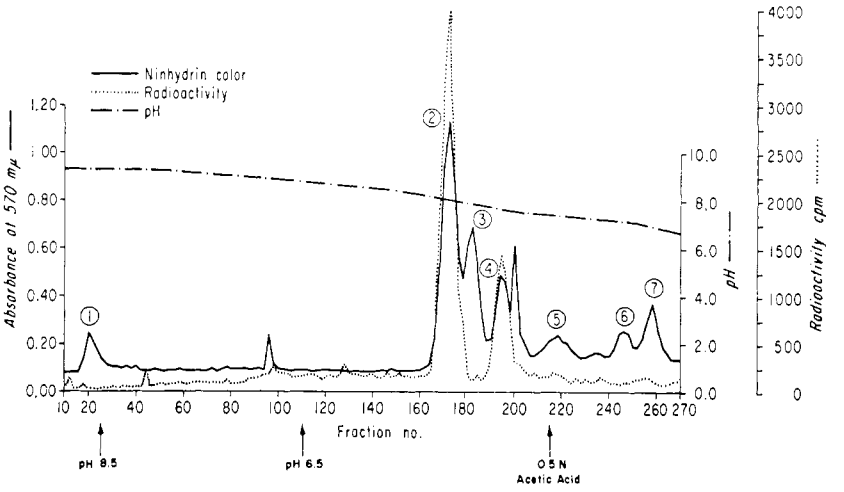

FIGURE 1: Chromatography of tryptic peptides of reduced and alkylated carboxypeptidase A^{Val} on Dowex 1-X2. The buffers used (pH 9.4, 8.5, and 6.5) are described by Schroeder and Robberson (1965). The sample, a lyophilized fraction from a Sephadex G-50 column, was applied in the pH 9.4 buffer to a pre-equilibrated column (at 38°) and eluted with pH 9.4 buffer in a constant volume mixer of 135 ml (Schroeder *et al.*, 1962). At the arrows on the figure, another buffer or acetic acid was introduced. Aliquots (1.5 ml) were collected at 45 ml/hr. Alkaline hydrolysates of 0.1-ml aliquots of alternate fractions were analyzed with ninhydrin; 0.05-ml aliquots of alternate fractions were monitored in the scintillation counter for [^{14}C]*S*-carboxamidomethylcysteinyl peptides. Fractions corresponding to the radioactive peaks 2 and 4 were pooled and lyophilized.

propionate, pH 8.0, then three changes of 7-l. volumes of 1 M NaCl, 0.005 M phosphate, pH 7.5, until no more [^{14}C]iodoacetamide could be detected in the dialysate. Aliquots were taken for amino acid analysis, radioactive measurements, and enzymatic assay. The esterase activity of this alkylated enzyme was identical with native protein, and the modified protein contained 8.61×10^5 dpm/μmole of protein.

The second equivalent of nonradioactive iodoacetamide was introduced in the following manner. The dialyzed, monoalkylated carboxypeptidase A (8.28 μmoles in 194 ml) was treated with 19.4 ml of 1 M Tris, pH 8.0, 19.4 ml of 10^{-2} M *o*-phenanthroline, 61.5 g of recrystallized urea, and 0.97 ml of redistilled 2-mercaptoethanol. Reduction proceeded at 0° for 3 hr under nitrogen, then 7.76 g of nonradioactive iodoacetamide was added, the pH readjusted to 8.0, and alkylation allowed to proceed for 2 hr. Five volumes of 50% trichloroacetic acid were added and the precipitated protein was washed three times to extract the trichloroacetic acid.

Tryptic Digestion of Carboxymethylated Carboxypeptidase and Isolation of Carboxymethylcysteinyl Peptides. The washed trichloroacetic acid precipitate of the reduced and dialkylated carboxypeptidase (7.1×10^6 dpm) was dissolved in 21 ml of 84% trifluoroacetic acid. After 24 hr at room temperature, an equal volume of 8 M recrystallized urea was added. This mixture was dialyzed against four changes each of 4 l. of 0.001 M sodium borate, pH 9.2, at 4°. The resulting denatured protein was adjusted to pH 9.0 in a pH stat and a total of 7.2 mg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone–trypsin was added in three equal aliquots over a 24-hr period. The tryptic digest was then adjusted to pH 5.0 with 1 M HCl and lyophilized.

The lyophilized digest was suspended in 50% acetic acid and centrifuged to yield a clear supernatant. Gel filtration on a column (2.5 × 95 cm) of Sephadex G-50 was carried out in 1 M acetic acid at 24 ml/hr. Aliquots (50 μl) of 2-ml fractions were monitored for ^{14}C. The radioactive fractions were pooled, lyophilized, and suspended in 3 ml of a pH 9.4 buffer (see legend to Figure 1). This suspension was applied to a column (0.9 × 100 cm) of Dowex 1-X2 equilibrated with the same buffer and developed with a series of gradients as indicated in Figure 1.

Peptic Digestion of Carboxypeptidase A (Anson). Carboxypeptidase A (Anson) (220 mg, 6.3 μmoles) was dissolved in 9 ml of 5% formic acid and a total of 17 mg of pepsin added in two portions over a period of 2 hr at 37°. Upon cooling the incubation mixture a gel was formed, which was broken by adding 5 ml of 5% formic acid and centrifuging at 15,000 rpm for 30 min. The precipitate was resuspended in 5 ml of 5% formic acid and centrifuged, and the supernatants were combined. An aliquot of the supernatant was oxidized and an analysis for cysteic acid yielded 10.5 μmoles, indicating that 83% of the half-cystinyl peptides were soluble in 5% formic acid.

Results

Isolation of Cystinyl Peptides from a Peptic Digest of Carboxypeptidase A (Anson). The soluble peptic peptides from 6.3 μmoles of carboxypeptidase A were applied to an SE-Sephadex C-25 column (1.5 × 51 cm) and developed as described in Figure 2. Four fractions were pooled as indicated in Figure 2 and aliquots removed for oxidation to determine cysteic acid recovery. Oxidized pools I through IV contained 0, 2.2, 1.3, and 1.7 μmoles of cysteic acid, respectively. Since a maximum of 4.75 μmoles of cystine (9.5 μmoles of cysteic acid) could theoretically be recovered after subtracting the mechanical losses associated with the disulfide monitoring system and the ninhydrin analysis, this represents a total recovery of 55% of the applied half-cystine. Each pool was lyophilized and oxidized, and these oxidized pools were separately rechromatographed on freshly prepared SE-Sephadex columns identical with the original column. In principle, each cystinyl peptide should yield two cysteic acid containing peptides, each eluting ahead of contaminating noncystinyl peptides. In practice this was observed for both pools II and III (OX-II and OX-III).

Rechromatography of these oxidized pools is illustrated in Figure 2. Oxidized pool II yielded two major peptides (OX-II-1 and OX-II-2), a minor peak (3), and a major peak with mobility similar to the unoxidized starting pool. The latter did not contain cysteic acid after oxidation and represents the noncystinyl peptides in the original pool II. Peak OX-II-1 corresponds in amino acid composition to a mixture of 0.34

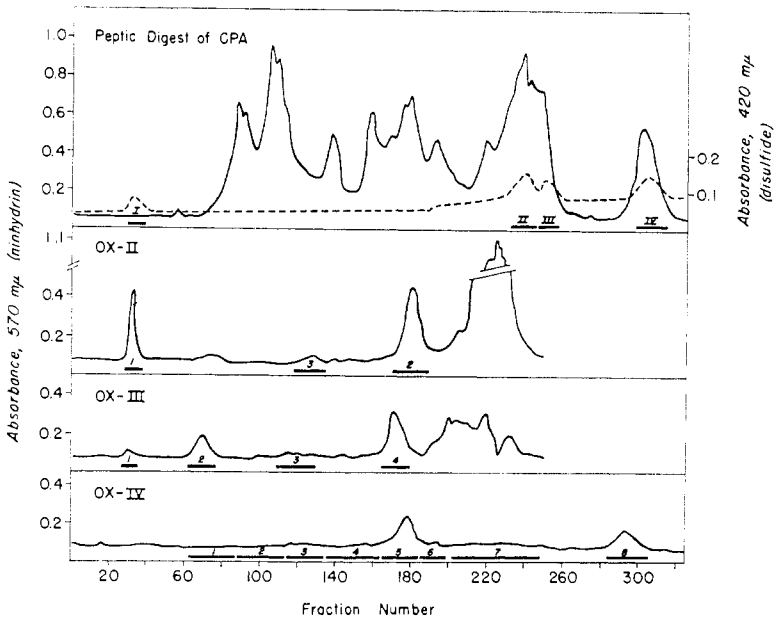

FIGURE 2: Chromatography of a peptic digest of carboxypeptidase A (Anson) on SE-Sephadex C-25 (1.5 × 51 cm) at 55°. After the sample was applied and washed in with 45 ml of 0.05 M pyridine (pH 2.4 with acetic acid), the chromatogram was developed with two successive linear gradients as follows: 200 ml of 0.05 M pyridine acetate (pH 2.4) to 200 ml of 0.5 M pyridine acetate (pH 3.75); then 230 ml of 0.5 M pyridine acetate (pH 3.75) to 230 ml of 2.0 M pyridine acetate (pH 5.0). Fractions (2 ml) were collected at a rate of 39 ml/hr. The effluent stream was divided at the bottom of the column with a Technicon pump and 3.0 ml/hr removed continuously for disulfide analysis by the method of Walsh *et al.* (1970). Alternate fractions (2.5%) were analyzed with ninhydrin after alkaline hydrolysis. The upper diagram traces the results of the ninhydrin analysis (solid line) and of the disulfide analysis (broken line). Fractions II through IV were pooled as indicated, separately oxidized as described in the text, and rechromatographed under identical conditions as OX-II through OX-IV (lower diagrams). The solid bars indicate the pooled fractions which are identified in the text according to the nomenclature on this figure.

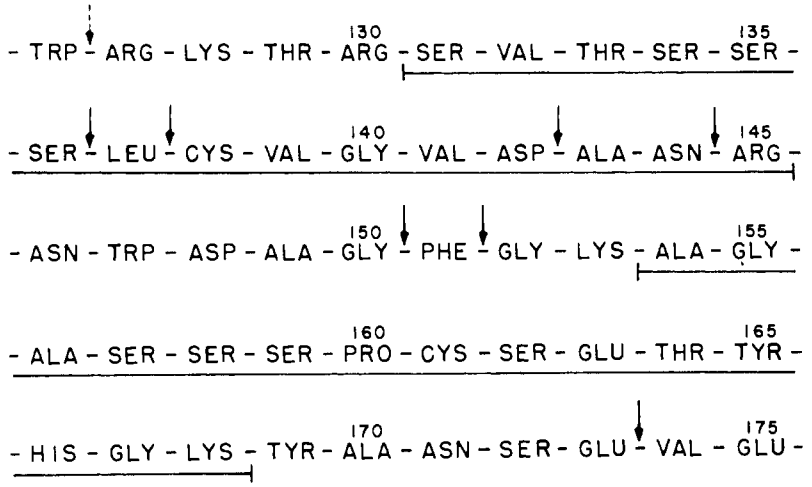

FIGURE 3: Sequence of bovine carboxypeptidase A surrounding the cystinyl residue (taken from Bradshaw *et al.*, 1969). The underlined peptides represent tryptic peptides isolated as [1-^{14}C]*S*-carboxymethylcysteine derivatives from carboxypeptidase A^{Val} (Cox). The arrows indicate sites of peptic attack of carboxypeptidase A (Anson).

μmole of the sequence 137–142 (Leu-Cys-Val-Gly-Val-Asn) (Bradshaw *et al.*, 1969) and 0.33 μmole of peptide 138–142 (Table I and Figure 3). Such ambiguity in peptic cleavage is commonly observed and complicates the interpretation of peptic digests (Smyth, 1967). Peak OX-II-2 corresponds in composition to 0.21 μmole of the sequence 151–173 (Phe-Gly-Lys-Ala-Gly-Ala-Ser-Ser-Ser-Pro-Cys-Ser-Glu-Thr-Tyr-His-Glu-Lys-Tyr-Ala-Asn-Ser-Glu) and 0.55 μmole of 152–173. In this case, pepsin has apparently cleaved on both sides of Phe^{151}. Thus Cys^{138} was recovered from OX-II in 66% yield and Cys^{161} in 74% yield.

Oxidized pool III yielded an analogous pattern and, in fact, OX-III-4 appears to be identical with OX-II-2 in both mobility and composition, accounting for 0.36 μmole of Cys^{161} (from 0.6 μmole of cystine). OX-III-1 corresponds to a small amount (0.1 μmole) of the same material as OX-II-1 and undoubtedly reflects the overlapping of peaks II and III in the original chromatogram. Peak OX-III-2 is more significant and corresponds in composition to 0.24 μmole of a 74/26 mixture of the sequence 138–151 (Cys) Val-Gly-Val-Asn-Ala-Asn-Arg-Asn-Trp-Asp-Ala-Gly-Phe- and the sequence 138–150. Tryptophan was not detected since the material had been oxidized. Again, the ratio of peptides containing Cys^{138} and Cys^{161} is unity.

Oxidized pool IV did not yield a clear product of the two half-cystinyl peptides. Only one major peak (OX-IV-5) is observed in the region of fractions 165–185 which corresponds in mobility to OX-II-2 and OX-III-4. Its amino acid com-

TABLE I: Amino Acid Compositions of Oxidized Peptide Pairs from OX-II, OX-III, and OX-IV.

μmoles Cys on to column	2.04				1.21						1.53	
Peptide in Figure 2	OX II-1		OX II-2		OX III-1		OX III-2		OX III-4		OX IV-5	
μmole of Peptide recovered	0.67		0.76		0.10		0.24		0.36		0.55	
	Initial	1st Turn	Initial	1st Turn	Initial	1st Turn	Initial	1st Turn	Initial	1st Turn	Initial	1st Turn
Cysteic acid	0.88	0.55	0.87	0.94	0.78	0.45	0.82	0.28	0.76	0.97	1.07	1.12
Asp	1.04	1.02	1.32	1.14	1.75	1.56	4.00	4.07	1.17	1.33	1.13	0.83
Thr			1.07	1.00					1.05	1.02	1.22	1.07
Ser			4.63	4.92			0.27	0.18	4.80	4.80	5.20	5.00
Glu			1.71	1.41					1.83	1.46	2.00	1.43
Pro			1.09	1.00					0.99	1.03	1.08	1.02
Gly	1.07	1.05	3.08	2.35	1.40	1.27	2.04	2.13	3.04	2.66	2.99	1.63
Ala	0.19	0.21	2.93	2.76	0.57	0.47	1.84	1.75	2.96	2.99	2.96	1.68
Val	2.05	1.93	0.39	0.10	2.00	2.00	1.86	2.13	0.41	0.16	0.20	0.23
Leu	0.51	—			0.36	—						
Tyr			Lost	1.63					1.54	1.67	1.32	1.27
Phe			0.28	0.08	0.15		0.70	0.77	0.29	0.06	0.11	
Lys			2.22	1.92					1.88	1.67	2.17	1.7
His			1.00	1.09					0.88	0.62	1.02	1.00
Arg							1.01	1.00				
Apparent identity (see Figure 3)	50% 137–142 50% 138–142		28% 151–173 72% 152–173		60% 138–144 40% 137–142		26% 138–150 74% 138–151		29% 151–173 71% 152–173		152–173 + Contaminants	

position, however, differed in showing contamination with arginine and leucine. Pooled peak OX-IV-5 was therefore subjected to paper electrophoresis at pH 6.5, 2000 V, for 2 hr to yield a minor neutral peptide and a major basic peptide. The latter showed the composition in Table I and appears to correspond to the sequence 152–173. The minor contaminant was not identified, but accounted for only 10% of the cysteic acid in OX-IV-5. Further examination of arbitrarily pooled fractions 1 through 8 revealed a background of cysteic acid at about 0.001–0.005 μmole/ml spread through fractions 100–240. Apparently half or more of the cysteic acid residues in OX-IV did not chromatograph as a discrete peak on this column but was distributed throughout the eluent volume. Amino acid analyses of the various pools were indicative of a larger peptide surrounding Cys^{138} (*e.g.*, 127–142), but no definitive identification could be made.

[^{14}C)Carboxamidomethylated Carboxypeptidase A^{Val} (Cox). Reduction of 8.6 μmoles of carboxypeptidase A^{Val}(Cox) and alkylation with [^{14}C]iodoacetamide yielded 1.1 equiv of *S*-carboxamidomethylcysteine as judged by amino acid analysis. The modified protein contained 8.61×10^5 dpm/μmole of protein. Since standard synthetic [^{14}C]carboxymethylcysteine contained 8.59×10^5 dpm/μmole, these data also indicate 1.0 equiv of *S*-carboxymethylcysteine/mole of protein. The specific esterase activity of the modified enzyme toward hippuryl-DL-phenyllactate remained identical with that of the native enzyme.

Further reduction and alkylation of the radioactive protein with cold reagent resulted in an inactive protein containing 1.9 equiv of carboxamidomethylcysteine as judged by amino acid analysis. No specific radioactivity was lost since this fully alkylated product contained 8.57×10^5 dpm/μmole of protein.

The soluble peptides from the tryptic digest of 8.1 μmoles of alkylated protein were partially purified on Sephadex G-50 as described and the radioactive material was further fractionated on Dowex 1-X2 as illustrated in Figure 1.

Amino acid analyses of the two radioactive peaks in Figure 1 are given in Table II. Peak 2 corresponds in chromatographic mobility and in composition to the tryptic peptide 154–168, whereas peak 4 corresponds to the tryptic peptide 131–145. The specific radioactivity of each peak was approximately 50% of that of synthetic *S*-carboxymethylcysteine made from the same preparation of [^{14}C]iodoacetamide. Thus, it can be deduced that the radioactive label had been distributed equally between Cys^{138} and Cys^{161} and that the cold reagent simply diluted the ^{14}C label of both of these two residues. This result clearly is at variance with the alternate expectation that a single cysteinyl residue would be alkylated with radioactive label and the other cysteinyl residue with cold reagent. These data thus indicate that at the stage of introduction of 1 equiv of radioactive label, there was a 50/50 mixture of two derivatives, one alkylated on Cys^{138} and the other on Cys^{161}. Furthermore, the undiminished enzymatic activity indicates that both monoalkylated species were fully active.

Bovine carboxypeptidase A exists in nature in two allotypic

TABLE II: Amino Acid Composition of [^{14}C]Carboxamidomethylcysteinyl Peptides from a Tryptic Digest of Reduced and Alkylated Carboxypeptidase A.

	Peak 2[a]	Theory 154–168[b]	Peak 4[a]	Theory 131–145[b]
Lysine	1.06	1		
Histidine	0.95	1		
Arginine			0.94	1
Aspartic acid	0.47		2.23	2
Threonine	1.15	1	1.05	1
Serine	4.18	4	3.95	4
Glutamic acid	1.29	1		
Proline	1.12	1		
Glycine	2.22	2	1.18	1
Alanine	2.22	2	1.31	1
Valine			3.36	3
Leucine	0.21		1.08	1
Tyrosine	0.73	1		
Carboxymethylcysteine	1.00	1	1.00	1
μmoles recovered	1.88		0.59	
Specific radioactivity (dpm/μmole)	4.65×10^5		4.65×10^5	
Specific radioactivity as % of that of synthetic [^{14}C]-CM-Cys[c]	54%		52%	

[a] From Figure 1. [b] See Figure 3. [c] [^{14}C]CM-Cys synthesized from the same preparation of [^{14}C]iodoacetamide reagent contained 8.59×10^5 dpm/μmole.

forms, the "valine" and the "leucine" type, each of which can occur as any of three activation products, α, β, or γ (Sampath Kumar *et al.*, 1964; Pétra *et al.*, 1969). No major differences in activity correlate with this heterogeneity. The particular species used in the experiment reported above is the "valine" allotype primarily in the α form as prepared by the method of Cox *et al.* (1964). However, similar results have been obtained with the mixed allotypes and with enzyme primarily in the γ form as previously reported by Neurath *et al.* (1968).

Discussion

The complete amino acid sequence of bovine carboxypeptidase A has been recently elucidated by Bradshaw *et al.* (1969). The portion of this sequence containing the two half-cystinyl residues is given in Figure 3 and the sites of enzymatic attack by pepsin are indicated by arrows. The half-cystines are separated by 22 residues and each is located within a unique amino acid sequence.

The recovery of peptides corresponding in amino acid composition to a fragment of carboxypeptidase containing both half-cystinyl residues in 55% yield provides direct chemical evidence for the existence of these residues in a disulfide structure in the native enzyme. It should be noted that this evidence is consistent with a disulfide of the A–B type rather than that of the A–A or B–B type. These peptides were obtained in unusually high yields by taking advantage of new techniques of disulfide separation and analysis which were developed for this purpose and are described elsewhere (Walsh *et al.*, 1970).

The studies with radioactive iodoacetamide clarify the extent of alkylation of each specific half-cystinyl residue at the intermediate stage of alkylation when a single equivalent of alkylating agent was introduced. This single equivalent of radioactive iodoacetamide was introduced as the ^{14}C derivative. A second equivalent was not radioactive; thus the specific radioactivity of each peptide ultimately isolated gave a measure of the proportion of that peptide which had been labeled during the introduction of the first equivalent. The utility of this approach relates to the fact that the proportion of the peptides labeled can be determined independently of the final yield of the isolated peptide. The results indicate that each peptide was labeled to the extent of 50%. Only two interpretations seem to be possible. First, a transcarboxymethylation could conceivably have occurred; however, it is not a likely chemical event and since there is no precedent for such a reaction it will not be considered further. Second, alkylation of the two half-cystinyl residues in carboxypeptidase is mutually exclusive; thus, each is labeled to the extent of 50% during the introduction of the first equivalent of alkylating agent. This, of course, implies that the two half-cystinyl residues are in close juxtaposition after reduction, a conclusion consistent with the occurrence of a disulfide bond in the compact native enzyme as visualized in the three dimensional structure (Lipscomb *et al.*, 1968). Since a 50/50 mixture of the two alkylation products is fully active it follows that neither half-cystinyl residue can play any direct role in enzymatic function.

Conditions favoring the introduction of a single equivalent of alkylating agent included the competitive inhibitor β-phenylpropionate (Sampath Kumar *et al.*, 1963). At the time it was assumed to be protecting a functional thiol in the active site; however, this cannot be the case. The most probable explanation is that β-phenylpropionate binds to the enzyme and stabilizes a native conformation in which the cysteinyl residues are in the close proximity mentioned above. Removal of β-phenylpropionate and introduction of 4 M urea would alter the conformation and lead to both alkylation of the remaining cysteinyl residues and loss of enzymatic activity.

One inconsistency remains in the alkylation data: prolonged digestion of carboxypeptidase with chymotrypsin, followed by treatment with *o*-phenanthroline and iodoacetamide, has been reported to result in the formation of one carboxymethylcysteine per mole of enzyme (Walsh *et al.*, 1962). Since precautions were taken to minimize oxidation of sulfhydryls by air it can only be assumed that 60 hr at pH 8.0 at 37° resulted in hydrolytic or partial reductive cleavage of the disulfide structure. The chemical data of Vallee *et al.* (1960), which in fact first draw attention to the apparent thiol nature of the Zn ligand, were based on the interaction of metal-containing reagents with the enzyme in the absence of the metal, but not in its presence. The titration data, the stability constants, and,

to a lesser extent, the spectral data are indirect in nature and do not provide definitive proof of cysteine involvement.

The earlier alkylation data point clearly to the hazard of interpreting the functional consequences of chemical modification of proteins when identification of the modified site rests upon peptides obtained in low yield. Our previous identification of a unique cysteine "at the active center" had been based on the recovery of an alkylated peptide in a Nagarse digest (Walsh *et al.*, 1962; Sampath Kumar *et al.*, 1963). However, the yield was low and in retrospect it is obvious that the peptide containing Cys^{161} was recovered in considerably better yield than the peptide containing Cys^{138}. In the present experiments, by carrying out the initial modification with radioactive alkylating agent and then completing the alkylation of all cysteines with cold reagent, it is possible to define the percent modification independently of the final yield of peptide.

Taken together, the evidence for a disulfide bond in carboxypeptidase A seems compelling. This provides, of course, a ready explanation of the fact that the apoenzyme could not be alkylated and is consistent with the observation that alkylation of half-cystinyl residues required prior reduction. Direct evidence of the disulfide rests upon the electron density maps (Lipscomb *et al.*, 1968) and upon the present isolation of peptic fragments of carboxypeptidase containing the intact disulfide structure. Furthermore, these data are in accord with the already established identification of the zinc ligands as His^{69}, Glu^{72}, and His^{196} (Lipscomb *et al.*, 1968; Bradshaw *et al.*, 1969).

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