# Modification of Carboxyl Groups in Bovine Carboxypeptidase A. II. Chemical Identification of a Functional Glutamic Acid Residue and Other Reactive Groups*

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ABSTRACT: The reaction of Woodward's reagent K with bovine carboxypeptidase A in the presence and absence of the competitive inhibitor β-phenylpropionate resulted in the differential labeling of the carboxyl group of $Glu_{270}$. After displacing the covalently bound reagent with [$^{14}$C]methoxamine, a dipeptide containing this group was isolated in 40% yield from a thermolytic digest of the inhibited enzyme. Other radioactive peptides contained the label on $Asp_{16}$, $Asp_{20}$, $Glu_{28}$, $Glu_{88}$, and $Glu_{302}$. Since these peptides were also isolated from fully active, modified enzyme prepared in the presence of β-phenylpropionate, it follows that these latter residues are neither involved in the catalytic activity nor in the preservation of the enzymatically active conformation. Two radioactive peptides containing the label on $Glu_{108}$ were isolated only from the β-phenylpropionate-protected enzyme, suggesting that modification of $Glu_{270}$ prevented reaction with $Glu_{108}$.

The reaction of bovine carboxypeptidase A with Woodward's reagent K results in the modification of carboxyl groups and concomitant loss of enzyme activity toward both peptide and ester substrates (Pétra, 1971). When the reaction is carried out in the presence of β-phenylpropionate or certain other competitive inhibitors, the enzyme is protected against inactivation but the reagent is nevertheless incorporated. The quantitative relationships of the protected and unprotected enzyme with Woodward's reagent K reported in the preceding paper form the basis of the present attempts to identify the single carboxyl group in the active site whose modification is responsible for enzyme inactivation. The investigation was extended to the identification of the additional carboxyl groups that are reactive with this reagent.

The experimental approach involved, first, displacement of the covalently bound reagent with [$^{14}$C]methoxamine, followed by degradation of the radioactively labeled protein with thermolysin. The purified radioactive peptides and the labeled residues were characterized by amino acid analysis and successive Edman degradations, and identified in the known amino acid sequence (Bradshaw *et al.*, 1969) and the three-dimensional model (Lipscomb *et al.*, 1968) of the enzyme.

## Experimental Section

### *Materials*

Chromatographically pure carboxypeptidase $A_\gamma^{Val}$[1] was prepared from carboxypeptidase A (Anson) according to Pétra and Neurath (1969). β-Phenylpropionic acid, Baker Analyzed Reagent, was recrystallized from an ethanol–water mixture. Woodward's reagent K was purchased from Aldrich. [$^{14}$C]*O*-Methylhydroxylamine was purchased from New England Nuclear and diluted with recrystallized, unlabeled reagent obtained from City Chemical Co. Carbobenzoxyglycyl-L-phenylalanine and sodium hippuryl-DL-phenyllactate were purchased from Cyclo Chemical Co. and Fox Chemicals, respectively. Pyridine and *N*-ethylmorpholine were redistilled from solid ninhydrin (1 g/l.) before use. Trifluoroacetic acid and phenyl isothiocyanate were purchased from Eastman Organic Chemicals and redistilled before use. Crystalline thermolysin was purchased from Daiwa Kasei K. K., Osaka, Japan.

### *Methods*

Measurements of protein concentration, ethylamine, enzymatic activity, and radioactivity were performed as previously described (Pétra, 1971).

*Preparation of the [$^{14}$C]Methoxamide Derivative of K-CPA-$_\gamma^{Val}$.* $CPA_\gamma^{Val}$ (140 mg) was inhibited with Woodward's reagent K as previously described (Pétra, 1971). The reaction was terminated after 10 min (80% inactivation) by the addition of 20 ml of 13.3 M sodium formate (pH 7.0), and the solution immediately cooled to 0°. Three such batches of protein were pooled and concentrated in the Diaflo to about 7 ml. After removing precipitated protein by centrifugation, the supernatant solution was added to 7 ml of 2 M methoxamine (250 μCi of [$^{14}$C]methoxamine, 36,000 dpm/μmole), and the solution allowed to stand for 15 hr at 37°, pH 6.4. At the end of this period, the protein was treated according to method 3 for the preparation of M-CPA (Pétra, 1971). Lyophilized powder (250 mg) was obtained (60% yield, 2.2 μmoles of [$^{14}$C]methoxamine/μmole of protein).

*[$^{14}$C]Methoxamide Derivative of K-CPA Prepared in the Presence of β-Phenylpropionate.* The reaction was carried out as described above except that $2 \times 10^{-3}$ M β-phenylpropionate was present before adding Woodward's reagent K. Enzymatic activity was fully retained. The label was displaced with methoxamine at 37°, pH 6.4, for 15 hr (250 μCi of [$^{14}$C]methoxamine, 31,110 dpm/μmole). Lyophilized powder (290 mg)

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[1] The following abbreviations are used: CPA, carboxypeptidase A; $CPA_\gamma^{Val}$ and $CPA_\gamma^{Leu}$ are the valine and leucine allotypes of the γ form of carboxypeptidase A (Pétra *et al.*, 1969); K, Woodward's reagent K; K-CPA, derivative produced by the reaction of K with CPA; M-CPA, methoxamide derivative of K-CPA; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactate.

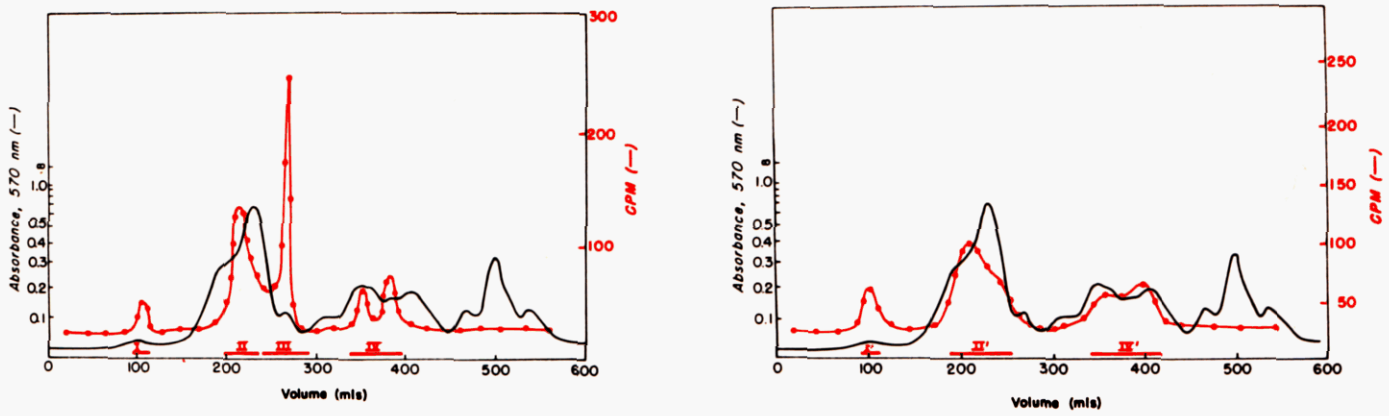



FIGURE 1: Chromatography of thermolytic digests of modified carboxypeptidase A on a 1.5 × 80 cm column of SE-Sephadex C-25 at 55°. The column was equilibrated with 0.05 M pyridine acetate (pH 2.4), pumped from the bottom of the column using a flow rate of 37 ml/hr. After application of the sample (8 ml), the column was developed using the following double-linear gradient: 200 ml of 0.05 M pyridine acetate (pH 2.4) to 200 ml of 0.5 M pyridine acetate (pH 3.75) and 200 ml of 0.5 M pyridine acetate (pH 3.75) to 200 ml of 2.0 M pyridine acetate (pH 5.0). Fractions of 3.5 ml were collected at a flow rate of 37 ml/hr. The column was monitored at 570 nm with an automatic Technicon peptide analyzer (Walsh *et al.*, 1970). Samples were removed for radioactivity measurements. Fractions were pooled as indicated by the solid bar. Eighty-five per cent of the radioactivity applied to the column was recovered. (A, left) Chromatography of 3.7 μmoles of M-CPA$\gamma^{val}$. (B, right) Chromatography of 3.5 μmoles of M-CPA$\gamma^{val}$ derived from K-CPA$\gamma^{val}$ prepared in the presence of β-phenylpropionate.

was obtained (70% yield, 1.3 μmoles of [$^{14}$C]methoxamine/μmole of protein).

*Digestion by Thermolysin.* The digestion procedure described by Bradshaw (1969) was used with some modifications. The lyophilized powder (100–150 mg) was suspended in water (10–12 mg/ml) containing 0.001 M calcium acetate. The pH was adjusted to 10 with 0.1 N NaOH, and the turbid solution stirred for 5 min. When the pH was lowered to 7, the protein precipitated. The suspension was then placed in a bath of boiling water for 10 min. After cooling of the solution, the pH was adjusted to 9 and 1 ml of thermolysin solution (1 mg/ml in 0.001 M calcium acetate–0.01 M Tris-Cl, pH 8.0) was added to a final concentration of 0.05 mg/ml of thermolysin. Digestion was continued for 2 hr at pH 9 and 37° in the pH-Stat. Another milliliter of thermolysin solution was added and digestion continued for another hour. The digest was adjusted to pH 2 with 6 N HCl and centrifuged. The insoluble core was washed twice with 0.01 N HCl, suspended in 0.001 M calcium acetate, and again digested as described above. The process was repeated six times until most of the core was solubilized. The soluble digests were lyophilized. Small aliquots were removed to determine the extent of digestion by high-voltage electrophoresis at pH 6.5. The powders were stored at −20°.

*Peptide and Amino Acid Aanlyses.* Soluble peptides derived from the thermolytic digests were separated on a 1.5 × 80 cm column of SE-Sephadex C-25 at 55° (Walsh *et al.*, 1970). Each radioactive pool was subsequently fractionated on a column of Dowex 1-X2 (Bio-Rad) at 37° and the radioactive peptides were further purified by paper electrophoresis. The columns were monitored for radioactivity and for ninhydrin color at 570 nm using an automatic Technicon peptide analyzer (Hill and Delaney, 1967) as modified by R. A. Bradshaw.[2] Subtractive Edman degradations were carried out according to Konigsberg and Hill (1962) as modified by Shearer *et al.* (1967). The ethyl acetate extracts were evaporated to dryness and counted to determine radioactivity.

Amino acid compositions of peptides were determined by hydrolyzing samples at 110° for 15–20 hr in evacuated tubes. Analyses of acidic and neutral amino acids were performed by an accelerated system using buffers containing 1% dimethyl sulfoxide (R. R. Granberg *et al.*, 1971, in preparation).

Paper electrophoresis at 2000 V was carried out on prewashed 3MM paper at pH 2.1, 3.75, or 6.5 for 2 hr. Peptides were located on the paper by staining guide strips with ninhydrin. The peptides were eluted from the paper with 30% acetic acid. Each guide strip was cut into 1.5-cm sections, including areas which were ninhydrin negative, and placed in vials with 1 ml of water and 10 ml of scintillation fluid to locate the radioactive peptides.

## Results

*Isolation and Characterization of a Labeled Peptide of the Active Site.* The thermolytic digests of carboxypeptidase, modified in the presence and absence of β-phenylpropionate, respectively, were chromatographed under identical conditions on SE-Sephadex as shown in Figure 1A,B. The only significant difference in the elution profiles other than minor experimental variations in column chromatography is the presence of fraction III in the digest of the unprotected enzyme (Figure 1A).

FRACTION III. This fraction contained about 35% of the total radioactivity applied to the SE-Sephadex column and was further purified by chromatography on Dowex 1-X2. Fraction III was pooled, evaporated to dryness, and dissolved in 4.2 ml of 3% pyridine (pH 8.8). Figure 2 shows the elution profile of chromatography on a column of Dowex 1-X2 previously equilibrated with 3% pyridine at 37°. The single radioactive fraction (Th-III-1)[3] contained about 85% of the radioactivity applied to the Dowex column, and after evaporation to dryness, was further purified by high-voltage electrophoresis

[2] Personal communication.

[3] Nomenclature used for peptides: Roman numerals indicate pool numbers of SE-Sephadex columns. Arabic numbers indicate pool numbers of Dowex 1-X2 columns. The letters indicate anionic mobility of the peptide on paper electrophoresis (*i.e.*, a > b > c, etc.). Th- denotes a thermolytic peptide; for example, a pure peptide would have the notation Th-II-3a. Primed numbers refer to peptides derived from chemically modified enzymes prepared in the presence of β-phenylpropionate (*e.g.*, Th-II′-3a).

TABLE I: Amino Acid Composition of Radioactive Peptides.

| Amino Acid | Th-III-1b | Th-II-2f | | Th-II-3a | | Th-II-3b | | Th-II-3c | | Th-IV-1b | | Th-IV-1c | | Th-IV-2d | | Th-I′-c | Th-I′-d |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | +[a] | −[a] | + | − | + | − | + | − | + | − | + | − | + | − | + | + |
| Aspartic acid | | 2.2 | 2.0 | 2.2 | 1.9 | 2.0 | 2.0 | 2.2 | 2.0 | | | | | | | 2.0 | 2.2 |
| Threonine | | 1.0 | 0.9 | | | | | 0.9 | 0.7 | | | | | 1.0 | 1.0 | 1.0 | 1.0 |
| Serine | | 1.0 | 1.0 | | | | | | | | | | | | | 1.2 | 0.6 |
| Glutamic acid | 1.1 | 2.0 | 2.4 | 1.0 | 1.3 | 1.0 | 1.2 | 2.0 | 1.9 | 2.0 | 2.0 | 2.2 | 2.0 | 1.1 | 1.1 | 1.1 | 1.2 |
| Proline | | 1.2 | 0.9 | | | | | | | 1.2 | 1.2 | 1.0 | 1.0 | | | 0.7 | 0.8 |
| Glycine | | 1.3 | 0.9 | | | | | 1.2 | 1.1 | | | | | | | 1.1 | 1.4 |
| Alanine | | | | | | | | | | 1.0 | 1.0 | 1.0 | 0.7 | | | | 1.0 |
| Valine | | | | | | | | | | 1.2 | 1.0 | 0.9 | 0.9 | | | 0.4[e] | 0.7[e] |
| Methionine | | | | | | | | | | | | | | 0.9 | 1.0 | | |
| Isoleucine | | | | 0.9 | 1.1 | 1.0 | 1.0 | | | | | | | 1.0 | 0.9 | 0.6[e] | 0.6[e] |
| Leucine | | | | 1.0 | 1.1 | 0.9 | 1.1 | | | 1.0 | 1.0 | | | | | 0.7 | 0.8 |
| Tyrosine | | 1.0 | 0.9 | 0.6 | 0.9 | 0.8 | 0.9 | 0.8 | 1.0 | | | | | | | 0.7 | 0.4 |
| Phenylalanine | 0.9 | 1.0 | 1.0 | | | | | 0.8 | 0.8 | | | | | | | | 0.8 |
| Histidine | | | | | | | | | | 0.9 | 0.7 | 1.0 | 0.8 | 0.8 | 1.0 | | |
| [$^{14}$C]Methoxamine[b] | 0.8 | 1.3 | 0.8 | 0.4 | 0.4 | 0.8 | 0.8 | 1.1 | 0.9 | 0.7 | 0.8 | 1.0 | 1.0 | 0.8 | 0.9 | 0.4 | 0.4 |
| % Yield[c] | 40 | 25 | 25 | 10 | 10 | 10 | 10 | 20 | 20 | 20 | 20 | 10 | 10 | 10 | 10 | 8 | 8 |
| % Total radioactivity[d] | 35 | 15 | 10 | 5 | 5 | 10 | 8 | 10 | 5 | 13 | 8 | 9 | 5 | 13 | 10 | 4 | 6 |

[a] (+) represents modification in the presence of β-phenylpropionate; (−) in its absence. [b] Based on the specific radioactivity of the peptide. [c] Based on the recovery after paper electrophoresis. 30–70% loss of peptide is encountered during this last step. [d] 85 and 86% of the total amount of radioactivity incorporated into the protein were recovered from the SE-Sephadex column for the β-phenylpropionate-protected and unprotected enzyme, respectively. [e] Recovery is based on 24-hr hydrolysis.

at pH 3.75. Three major ninhydrin-stained bands and two minor ones were observed, but only one of them, peptide Th-III-1b,[3] was radioactive.

*Peptide Th-III-1b.* The peptide was eluted from the paper with 30% acetic acid and, after evaporation to dryness, subjected to amino acid and end-group analyses and to radioactivity measurements. The peptide contained phenylalanine and glutamic acid approximately in a 1:1 mole ratio. Radioactivity measurements gave 0.8 mole of [$^{14}$C]methoxamine/mole of dipeptide (Table I). Subtractive Edman degradation yielded 0.1 mole of phenylalanine/mole of glutamic acid, thus establishing the sequence as Phe-Glu. The phenylthiohydantoin of phenylalanine was also directly identified by gas–liquid chromatography after extraction with ethyl acetate.[4] Most of the radioactivity (70%) remained in the aqueous phase. Analysis of an unhydrolyzed aliquot in the amino acid analyzer revealed a new chromatographic peak. Hydrolysis in 5.7 N HCl for 15 hr at 110° completely converted this derivative into glutamic acid. These results indicate the presence of an acid-labile bond in the modified glutamic acid residue, probably the γ-methoxamide derivative of glutamic acid. Further evidence in support of this conclusion is given below.

Since there is only one Phe-Glu sequence in carboxypeptidase A (Bradshaw *et al.*, 1969) at positions 269–270, it follows that the radioactive label is located on $Glu_{270}$.

*Isolation and Characterization of Other Radioactive Peptides.* Besides fraction III, radioactive material was also associated with fractions I, II, and IV of thermolytic digests of carboxypeptidase A modified in the absence of β-phenylpropionate, and with the corresponding fractions I′, II′, and IV′ isolated from enzyme modified in the presence of this competitive inhibitor (see Figure 1A,B). The amino acid compositions and yields of peptides isolated from these fractions are included in Table I.

FRACTIONS I AND I′. These fractions emerged in the breakthrough volume of the SE-Sephadex column. No peptides were isolated from fraction I, suggesting that this small

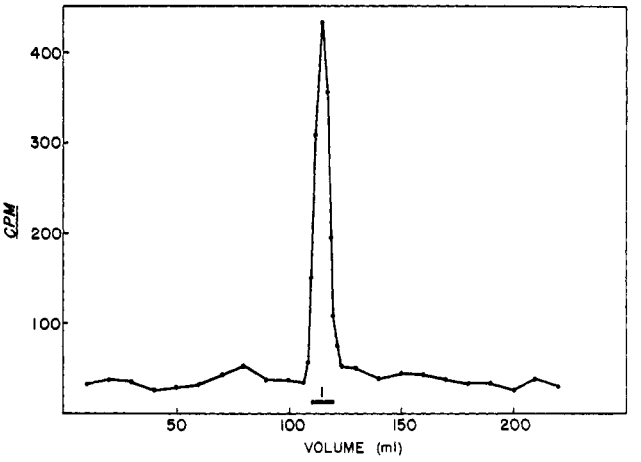



FIGURE 2: Chromatography of fraction III obtained from Figure 1A on a 0.9 × 60 cm column of Dowex 1-X2 at 37°. The elution profile was developed at a flow rate of 17 ml/hr using a four-chamber gradient (100 ml/chamber) of 3% pyridine (pH 8.8), 0.5 N pyridine acetate (pH 6.0), 1.0 N pyridine acetate (pH 6.5), and 2.0 N pyridine acetate (pH 5.0). Fractions of 1.7 ml were collected. Fifty microliters was removed for radioactivity assays. Peak 1 was pooled as indicated by the solid bar. Seventy to seventy-five per cent of the radioactivity applied to the column was recovered.

[4] The authors thank Dr. M. A. Hermodson for performing gas–liquid chromatographic analyses.

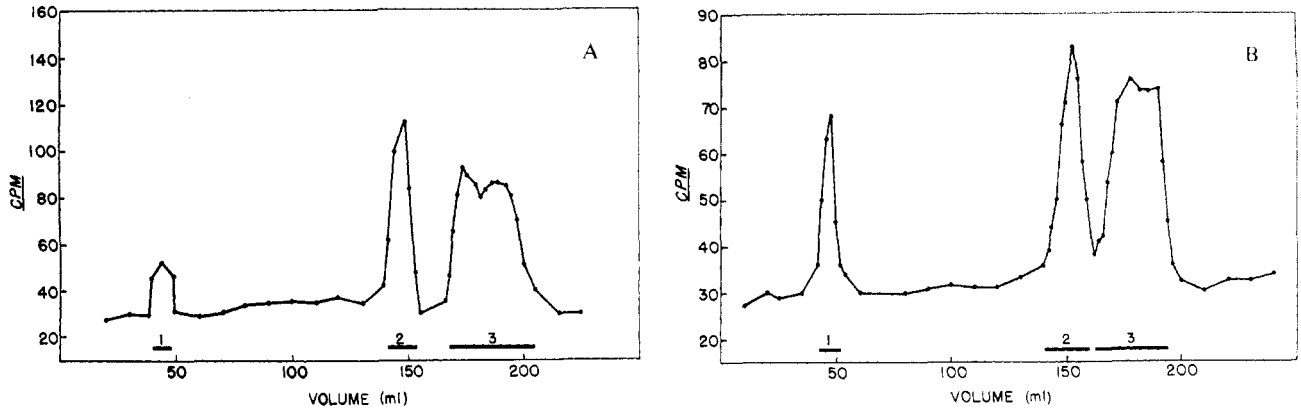



FIGURE 3: Elution profile of fractions II and II′ on a 0.9 × 60 cm column of Dowex 1-X2 at 37°. The column was developed with a linear gradient of 150 ml of 3% pyridine (pH 8.8) to 150 ml of 2 N pyridine acetate (pH 5.0) at a flow rate of 17 ml/hr. Fractions of 1.7 ml were collected and pooled as indicated by the solid bar. 90% of the radioactivity applied to the column was recovered. (A) Fraction II. (B) Fraction II′.

TABLE II: Amino Acid Sequence of Radioactive Peptides.

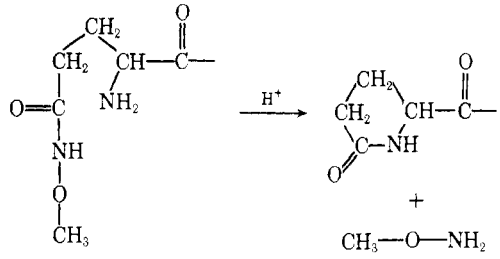
| Peptide | Sequence |
|---|---|
| Th-III-1b | Phe-$Glu_{270}$ |
| Th-II-3b[a] | Leu-$Asp_{16}$-(Glu,Ile,Tyr,Asp) |
| Th-II-3a[a] | Leu-Asp-Glu-Ile-Tyr-$Asp_{20}$ |
| Th-IV-1c[a] | Val-Ala-$Glu_{28}$-(His,Pro,Gln) |
| Th-IV-1b[a] | Leu-(Val,Ala,$Glu_{28}$,His,Pro,Gln) |
| Th-II-3c[a] | Phe-Thr-$Glu_{88}$-(Asn,Tyr,Gly,Gln,Asn) |
| Th-II-2f[a] | Phe-Thr-$Glu_{88}$-(Asn,Tyr,Gly,Gln,Asn,Pro,Ser) |
| Th-IV-2d[a] | Ile-Met-$Glu_{302}$-(His,Thr) |
| Th-I′-d | Leu-($Glu_{108}$,Ile,Val,Thr,Asn,Pro,Asn,Gly,Phe, Ala) |
| Th-I′-c | Leu-($Glu_{108}$,Ile,Val,Thr,Asn,Pro,Asn,Gly) |

[a] The same peptide was also isolated from the β-phenylpropionate-protected enzyme. It was sequenced to the same extent as the corresponding peptide shown in the table.

amount of radioactivity represents breakdown products arising from partial hydrolysis of the incorporated methoxamide group. However, two acidic, radioactive peptides were isolated from fraction I′ in very low yields (Table I) by paper electrophoresis at pH 6.5. Although the resulting peptides still contained impurities, the amino acid composition and N-terminal analysis (Tables I and II) indicated that peptide Th-I′-c corresponds to residues 107–115 and peptide Th-I′-d to residues 107–117. Due to scarcity of material, the location of the radioactive label could not be identified directly by Edman degradations. However, on the basis of the amino acid sequence, it may be tentatively associated with $Glu_{108}$.

FRACTIONS II AND II′. Fractions II and II′ were pooled separately and evaporated to dryness. They were each dissolved in 4 ml of 3% pyridine (pH 8.8) and purified on columns of Dowex 1-X2. The elution profiles are shown in Figure 3A,B. In each case, three radioactive peaks were observed. Fractions II-1 and II′-1 contained less than 3% of the total radioactivity applied initially to the SE-Sephadex column (Figure 1A,B) and were not investigated further. Since identical radioactive peptides were isolated from both fractions, only those from fraction II, derived from the inactivated enzyme, are described below.

SCHEME I




FRACTION II-2. Fraction II-2 (Figure 3A) contained one impure radioactive peptide and was further purified by paper electrophoresis at pH 6.5. One major ninhydrin positive band and five minor ones were obtained. The radioactivity was found only in the acidic *peptide Th-II-2f*. Edman degradations gave the following results.

first step:[b] **Phe, 0.1;** Thr, 1.0; Glx, 2.0; Asx, 2.1; Tyr, 0.9; Gly, 1.0; Pro, 0.9; Ser, 1.1
second step: Phe, 0.1; **Thr, 0.1;** Glx, 2.1; Asx, 2.1; Tyr, 0.9; Gly, 1.1; Pro, 0.8; Ser, 1.1
third step: Phe, 0.1; Thr, 0.1; **Glx, 1.4;** Asx, 2.0; Tyr, 1.0; Gly, 0.9; Pro, 1.0; Ser, 0.8

Hence the amino-terminal sequence of this peptide is Phe-Thr-Glx. These data, together with the amino acid composition, established the identity of this peptide with residues 86–95 in the amino acid sequence of carboxypeptidase. By examining the amount of radioactivity released in the ethyl acetate extracts after each turn of the Edman degradation, the label was located on $Glu_{88}$. No label was released in the first two turns; however, 30% of the total radioactivity was extracted in the ethyl acetate layer after the third degradation with a concomitant 24% loss of glutamic acid. The incomplete cleavage, observed with all radioactive peptides containing the label on a glutamic acid residue, is probably due to the acid-catalyzed formation of pyrrolidone as shown in Scheme I. This reaction is probably enhanced by the relative instability of the methoxamide label when compared to the amide group of glutamine.

[b] The amino acid lost at each step is given in boldface type.

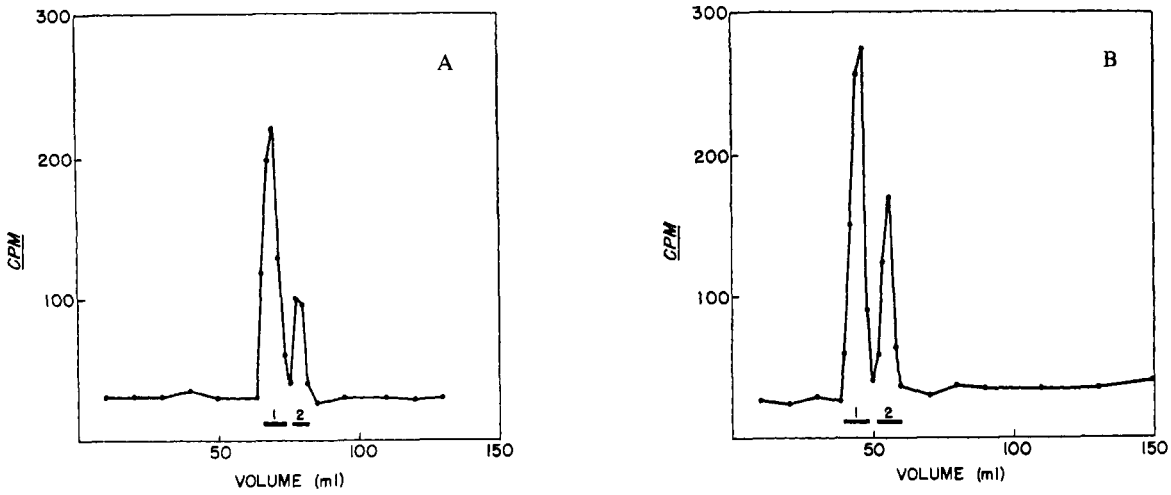



FIGURE 4: Elution profile of fractions IV and IV′ on Dowex 1-X2 at 37°. The peak fractions were pooled as indicated by the solid bar. 95% of the radioactivity applied to the column was recovered. (A) Chromatography of fraction IV on a 0.9 × 100 cm column. The column was eluted at 15 ml/hr using the same four-chamber gradient described in the legend to Figure 2. Fractions of 1.8 ml were collected. (B) Chromatography of fraction IV′ on a 0.9 × 60 cm column using the same conditions as in Figure 4A.

FRACTION II-3. Fraction II-3 (Figure 3A) contained three acidic radioactive peptides which were purified by paper electrophoresis at pH 6.5.

PEPTIDE Th-II-3a. Subtractive Edman degradations gave the following results.

| | | | | | |
|---|---|---|---|---|---|
| first step: | **Leu, 0.1;** | Asx, 2.0; | Glx, 1.2; | Ile, 1.0; | Tyr, 0.8 |
| second step: | Leu, 0.1; | **Asx, 1.0;** | Glx, 1.0; | Ile, 0.9; | Tyr, 0.8 |
| third step: | Leu, 0.1; | Asx, 1.1; | **Glu, 0.3;** | Ile, 1.0; | Tyr, 0.9 |
| fourth step: | Leu, 0.1; | Asx, 1.1; | Glu, 0.3; | **Ile, 0.1;** | Tyr, 0.9 |
| fifth step: | Leu, 0.2; | Asx, 1.0; | Glu, 0.1; | Ile, 0.1; | **Tyr, 0.2** |

According to these data, the peptide was identified with residues 15–20 in the primary sequence (Table II). The radioactivity remained in the aqueous extracts during all successive Edman degradations, providing proof that the label was associated with the last residue, $Asp_{20}$. The low specific radioactivity of this peptide (Table I) probably arises from a loss of label from the C-terminal aspartic acid by the formation of a cyclic imide (Naughton *et al.*, 1960). This reaction occurs under acidic conditions as shown in Scheme II. Peptides corresponding to structures A and B would be expected to have similar electrophoretic mobility and the resulting mixture would exhibit a lower specific radioactivity due to the presence of the nonradioactive peptide B (Scheme II).

PEPTIDE Th-II-3b. Subtractive Edman degradations gave the following results.

first step: **Leu, 0.3;** Asx, 2.0; Glx, 1.1; Ile, 0.8; Tyr, 0.8
second step: Leu, 0.3; **Asx, 1.3;** Glx, 0.9; Ile, 0.9; Tyr, 0.9

This peptide is identical in amino-terminal sequence and amino acid composition (Table I) with peptide Th-II-3a (Table II). However, in contrast to the latter, 75% of the radioactivity was released into the ethyl acetate extract after the second turn of the Edman degradation, indicating that the label was associated with $Asp_{16}$ rather than $Asp_{20}$.

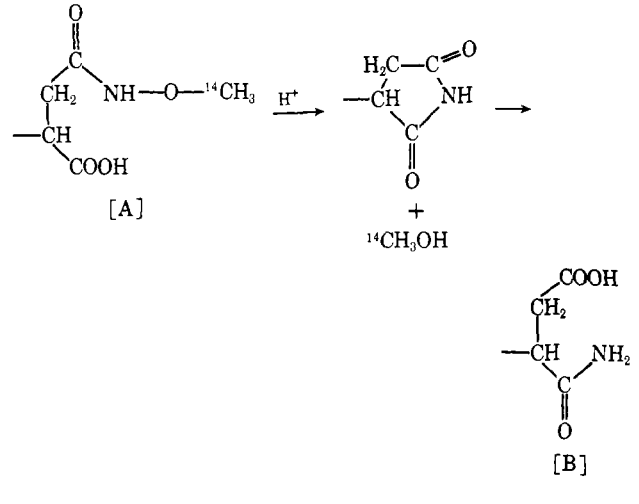
PEPTIDE Th-II-3c. Subtractive Edman degradations gave the following results.

first step: **Phe, 0.1;** Thr, 0.9; Glx, 2.0; Asx, 2.0; Tyr, 1.0
second step: Phe, 0.1; **Thr, 0.3;** Glx, 2.0; Asx, 2.1; Tyr, 1.0
third step: Phe, 0.1; Thr, 0.3; **Glx, 1.5;** Asx, 2.1; Tyr, 0.9
fourth step: Phe, 0.9; Thr, 0.2; Glx, 1.5; Asx, 1.7; Tyr, 0.7

The amino-terminal sequence is therefore Phe-Thr-Glx, identical with that of peptide Th-II-2f. The peptide lacks, however, proline and serine (Table I) and thus includes only residues 86–93. Thermolytic cleavage of the $Asn_{93}$–$Pro_{94}$ bond has been previously observed by Bradshaw (1969) in the cyanogen bromide fragment III of carboxypeptidase and probably is responsible for the formation of peptide Th-II-3c in addition to Th-II-2f. As in the case of the nonapeptide Th-II-2f, the radioactive peptide Th-II-3c was associated with $Glu_{88}$ since 40% of the radioactivity was released after the third Edman degradation, together with 50% of the glutamic acid. The incomplete degradation of glutamic acid was probably due to cyclization to the pyrrolidone since attempts to further degrade the peptide failed after step 3.

FRACTIONS IV AND IV′. These fractions were isolated by SE-Sephadex chromatography (Figure 1A,B) and each pool was subfractionated on Dowex 1-X2 as shown in Figure 4. Each fraction contained two basic radioactive peptides. Those of fraction IV-1 were separated by electrophoresis at pH 3.75. Two major ninhydrin positive bands and one minor one were obtained. The radioactivity was associated with the two major bands.

SCHEME II



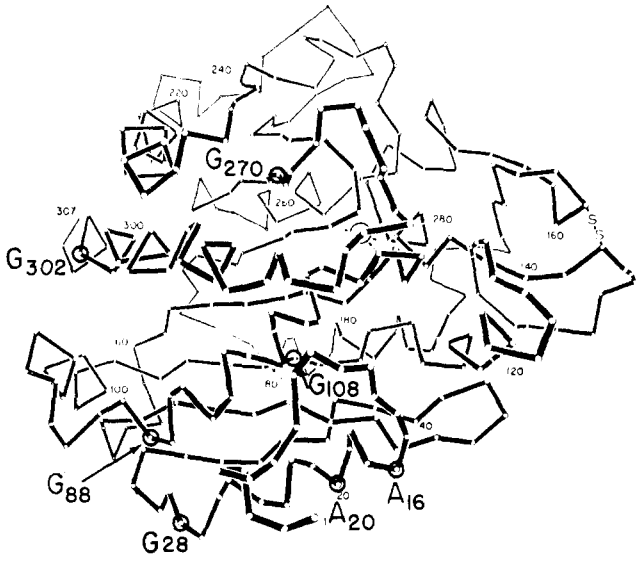



FIGURE 5: Structure of bovine carboxypeptidase A showing the α carbons of the modified glutamic and aspartic acid residues. Taken from Lipscomb *et al.* (1969a).

PEPTIDE Th-IV-1c. Subtractive Edman degradations gave the following results.

first step: **Val, 0.1;** Ala, 1.0; Glx, 2.1; His, not determined; Pro, 0.9
second step: Val, 0.1; **Ala, 0.1;** Glx, 2.1; His, not determined; Pro, 0.9
third step: Val, 0.1; Ala, 0.1; **Glx, 1.5;** His, not determined; Pro, 0.9
fourth step: Val, 0.2; Ala, 0.1; Glx, 1.2; His, 0.7; Pro, 1.0

Hence the amino-terminal sequence is Val-Ala-Glx. The peptide corresponds to residues 26–31 in the sequence of carboxypeptidase A (Tables I and II). After the third Edman degradation, 40% of the radioactivity and 50% of the glutamic acid were removed, indicating that the label was associated with $Glu_{28}$. Residues 28 and 31 had previously been identified as glutamine and glutamic acid, respectively (Bradshaw *et al.*, 1969). However, reexamination of the amino-terminal sequence of bovine carboxypeptidase A using the sequenator (P. H. Pétra, M. A. Hermodson, K. A. Walsh, and H. Neurath, submitted to *Biochemistry*) revealed these positions to be occupied by $Glu_{28}$ and $Gln_{31}$, which is in accord with the present labeling experiments.

PEPTIDE Th-IV-1b. Amino acid composition and Edman degradations (Tables I and II) revealed that this peptide was similar to peptide Th-IV-1c but that it contained an additional leucine as the amino-terminal residue. In analogy to the structural analysis of peptide Th-IV-1c, the label must be at $Glu_{28}$.

FRACTION IV-2. This fraction (Figure 4A) contained one basic, impure, radioactive peptide which was purified by paper electrophoresis at pH 2.1. Five major ninhydrin-positive bands were obtained.

PEPTIDE Th-IV-2d contained all of the radioactivity. Subtractive Edman degradations gave the following results.

first step: **Ile, 0.1;** Met, 1.0; Glx, 1.1; His, not determined; Thr, 0.8
second step: Ile, 0.1; **Met, 0.1;** Glx, 1.0; His, not determined; Thr, 0.9
third step: Ile, 0.1; Met, 0.1; **Glx, 0.6;** His, 0.8; Thr, 1.0

The amino-terminal sequence is therefore Ile-Met-Glx. From the data and the amino acid composition (Table I), it was concluded that the peptide corresponds to residues 300–304 in the C-terminal region of the enzyme (Table II). After the third Edman degradation, 30% of the radioactivity and 40% of the glutamic acid were released, indicating that the label was associated with $Glu_{302}$.

## Discussion

The reaction of bovine carboxypeptidase A with Woodward's reagent K results in the modification of several carboxyl groups, but only one of these is responsible for loss of peptidase and esterase activities. Since the chemical modification and enzymatic inactivation are simultaneously prevented by β-phenylpropionate, it follows that the active site contains a functional carboxyl group. Because β-phenylpropionate (Simpson *et al.*, 1963; Bethune, 1965) and its analog β-(iodophenyl)propionate (Steitz *et al.*, 1967) are bound at other loci as well, it is important to demonstrate that the "difference" peptide(s) derived from the enzyme labeled in the presence and absence of β-phenylpropionate, respectively, contain only the modified carboxyl group of the active site. It was therefore essential to identify most if not all of the radioactive peptides from enzymatic digests of the labeled enzyme. As shown in Table I, 85% of the label incorporated in the inactivated enzyme could be accounted for by peptides of proven structure. It was evident that the only "difference" peptide which could be correlated with loss of activity is Phe-$Glu_{270}$ and that $Glu_{270}$ is in fact the group whose modification is responsible for enzyme inactivation.[6]

The carboxyl group of $Glu_{270}$ was first implicated by Lipscomb *et al.* (1968) as being the particular nucleophile (Vallee, 1964) in the mechanism of action of carboxypeptidase A. This identification was confirmed by the sequence analysis by Bradshaw *et al.* (1969). The data presented in this and the preceding communication (Pétra, 1971) provide direct chemical proof of the involvement of $Glu_{270}$ in the enzymatic process. First, enzymatic activity is abolished when $Glu_{270}$ is modified. Second, the effectiveness of competitive inhibitors in protecting the enzyme against chemical inactivation is directly related to their efficiency ($1/K_i$) in inhibiting enzymatic hydrolysis of peptide substrates. Third, the pH dependence of inhibition by reagent K (Pétra, 1971) is almost identical with that of enzyme-catalyzed hydrolyses of peptide and ester substrates in the acid region (Neurath and Schwert, 1950; Carson and Kaiser, 1966; Riordan and Vallee, 1963; Auld and Vallee, 1970) and indicates that the unprotonated form of a group with a p$K$ of 7 is the reactive species. While this may be an unusually high value for the ionization constant of a γ-carboxyl group, the highly nonpolar environment of $Glu_{270}$ may strongly affect its ionization (Lipscomb *et al.*, 1969b). Recent experiments with *N*-bromoacetyl-*N*-methyl-L-phenylalanine, an affinity label for carboxypeptidase A, have provided analogous results which led to the same conclusion (Hass and Neurath, 1971a,b).

In addition to $Glu_{270}$, the carboxyl groups of $Asp_{16}$, $Asp_{20}$, $Glu_{28}$, $Glu_{88}$, $Glu_{302}$, and $Glu_{108}$ are also modified by reagent K

[6] While this manuscript was in preparation, Riordan and Hayashida (1970) described the inactivation of bovine carboxypeptidase A by a carbodiimide and the protection against inactivation by β-phenylpropionate. The data were interpreted by implicating $Glu_{270}$ as the site of modification. While this conclusion is in agreement with the present analytical data, it is not compelling by itself since the labeled peptide(s) was not isolated and identified, and inactivation due to modification at other sites was not excluded.

but without loss of enzymatic activity. These "nonfunctional" carboxylic acid side chains were identified in the primary structure of the enzyme by analyzing the radioactive peptides isolated from fully active enzyme, chemically modified in the presence of β-phenylpropionate. These residues are located on the surface of the protein as shown in Figure 5, except for $Glu_{108}$ which is situated at the center of the molecule in the region of the twisted, pleated sheet of the β structure, an unusual position for a charged amino acid residue (Lipscomb *et al.*, 1969a; Bradshaw *et al.*, 1969). Peptides containing this residue were not found in the inactivated enzyme, suggesting that modification at $Glu_{270}$ prevented incorporation of reagent K at $Glu_{108}$, perhaps by blocking the site of interaction. Examination of the X-ray model reveals that the carboxylic acid side chains of $Asp_{16}$-$Asp_{20}$ and $Glu_{28}$-$Glu_{88}$, respectively, are paired. In the case of the first pair, $Asp_{16}$-$Asp_{20}$, modifications of the two carboxyl groups are indeed mutually exclusive. Mutual exclusion could not be demonstrated for reaction with $Glu_{28}$ and $Glu_{88}$, as this would have required the isolation of a peptide containing 61 or more amino acid residues. However, in view of the proximity of the side chains of $Glu_{28}$ and $Glu_{88}$, it is reasonable to assume that reaction with one would also exclude reaction of the other. The pairing of these carboxyl groups may also be responsible for the conversion of reagent K into the active ketoketenimine. In fact, it has been suggested that such a reaction, *i.e.*, the abstraction of the proton at position 3 of reagent K with subsequent opening of the ring (Pétra, 1971, Figure 9), may be promoted by a protein carboxyl group different from that actually modified (Feinstein *et al.*, 1969); both pairs of carboxylates could act in a concerted mechanism.

Finally, it should be noted that all carboxyl groups reactive toward reagent K appear to be located on one side of the surface of the enzyme molecule and that none of the "back side" has been modified (Figure 5). Such a polarized response may be fortuitous and may simply reflect localized affinity regions on the protein surface. It is also possible that the unreactive surfaces are inaccessible to the reagent as a result of protein dimerization, even though there is no evidence that under the particular conditions of modification, carboxypeptidase A is associated in such a fashion. At any rate, the lack of absolute specificity of reaction of Woodward's reagent K with the active site of carboxypeptidase A has enabled a differentiation between functional and nonfunctional carboxyl groups of the enzyme, and within the latter class, has differentiated further between those that are reactive toward the reagent and others that remain free. In this sense, the present investigation has provided a functional and topographical analysis of a class of side chains in this enzyme.

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