The Heterogeneity of Bovine Carboxypeptidase A. I. The Chromatographic Purification of Carboxypeptidase A (Anson)*

Philip H. Pétra and Hans Neurath

ABSTRACT: Bovine carboxypeptidase A (Anson) has been shown to contain at least five components when chromatographed on DEAE-cellulose (DE-52) in the presence of the competitive inhibitor β-phenylpropionate. All five fractions have the same specific activity toward peptide and ester substrates. Partial sequence determination of the amino- and carboxyl-terminal fragments obtained by cleavage with cyanogen bromide of fractions I, II, III, IV, and V reveal that they are carboxypeptidase A_α, carboxypeptidase A_β^{Val}, carboxypeptidaseA_β^{Leu}, carboxypeptidase A_γ^{Val}, and carboxypeptidase A_γ^{Leu}, respectively. All five species differ in their rates of heat inactivation at 50° indicating that both chain length and the amino acid replacement may affect the conformational stability of the molecule as a whole. An additional amino acid replacement genetically linked with the valine-leucine replacement is proposed.

The recent advances in the study of the chemical structure and enzymatic function of bovine carboxypeptidase A (for a review, see Neurath *et al.*, 1969; Lipscomb *et al.*, 1969; Vallee and Riordan, 1969) have placed this enzyme in the forefront of proteins for which a unique relationship between molecular structure and mechanism of action will soon be established. The solution of the structure–function problem presupposes that the protein under investigation is molecularly homogeneous or that such variations in chemical structure as may exist are inconsequential for the experimental analysis. Rigorous proof of the molecular homogeneity of a protein is difficult to establish since common methods of protein separation and analysis are not sufficiently sensitive to detect minor variations in structure. Thus minor impurities or variations in structure resulting from deletion, addition, or replacement of chemically similar amino acid residues may well escape detection. In any case, proof for molecular homogeneity is based on negative evidence and hence inferential.

Although crystalline bovine carboxypeptidase A was first thought to be a pure protein (Neurath, 1960), it was subsequently shown that depending upon the conditions of activation and isolation, the enzyme exists in various chemical forms which differ in their amino-terminal regions as shown in Figure 1 (Sampath Kumar *et al.*, 1964b). The major products contain preponderantly carboxypeptidase A_α (Cox *et al.*, 1964), A_γ (Anson, 1937), or A_δ (Allan *et al.*, 1964). In addition, carboxypeptidase A_β containing amino-terminal serine can be generated upon tryptic hydrolysis of carboxypeptidase A_α (Sampath Kumar *et al.*, 1964b). Pure preparations of the β enzyme have heretofore not been obtained.

In addition to these variations in the amino-terminal region, carboxypeptidase A also shows variations in the carboxyl-terminal region. These arise from amino acid replacements due to allelomorphism (Walsh *et al.*, 1966). The presence of a valine-leucine replacement in the antepenultimate position of the polypeptide chain of carboxypeptidase A_α has been demonstrated (Bargetzi *et al.*, 1964). Experiments with single pancreas glands isolated from 14 individual animals revealed that the 2 allotypic replacements may occur separately or together according to the prediction of Mendelian genetics, indicating that they represent the products of 2 alleles of the same gene and that both alleles are fully expressed in the animal heterozygous with regard to this trait (Walsh *et al.*, 1966).

The preparation of a molecularly homogeneous form of carboxypeptidase A thus appears a formidable task requiring first pancreatic juice from an animal known to be homozygous with regard to the established amino acid replacements, and secondly the development of procedures that would yield a single product of activation. An alternate route to a homogeneous enzyme involves the development of chromatographic procedures that would clearly separate all molecular species from each other. This latter undertaking proved difficult at the outset since in the past, the extreme insolubility of the enzyme had resisted all attempts at ion-exchange chromatography. Eventually, a reproducible system of ion-exchange chromatography has been found which can be applied to relatively large quantities of carboxypeptidase A prepared according to Anson (1937). This chromatographic method, and some of the chemical and enzymatic properties of the purified fractions are reported in this communication.

Experimental Section

Enzyme. Carboxypeptidase A, prepared according to Anson (1937), was purchased from Worthington Biochemical Corp. in lots of 5–10 g (lots CoA-7AE, CoA-7HA, CoA-7KA, CoA-7FB, and CoA-7AD).

Chemicals and Substrates. Z-Gly-Phe and sodium hippuryl-DL-phenyllactate were purchased from Cyclo Chemical Co. and Fox Chemicals, respectively. β-Phenylpropionic acid and LiCl were Baker Analyzed reagents. In some cases the former had to be recrystallized from an ethanol–water mixture. α-Aminobutyric acid and L-serine were recrystallized and both

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received January 20, 1969.* This work was supported by the National Institutes of Health (GM 04617 and GM 15731-01), the American Cancer Society (P-79K), Office of Naval Research (NONR 477-35), and the National Science Foundation (GB 4990).

hydantions were synthesized according to Stark and Smyth (1963). All other materials were of reagent grade quality and were used without further purification.

Activity Measurements. Enzyme stock solutions were prepared by dissolving the crystals in 1 M NaCl–0.05 M Tris-Cl (pH 7.5) at 0°. The absorption at 278 mμ was measured with the Zeiss PM Q II spectrophotometer and the concentration was determined from A_{278} (mg^{-1} ml^{-1}) = 1.88 (Bargetzi *et al.*, 1963). Peptidase activity was determined spectrophotometrically (Perkin-Elmer, Model 350) by measuring the rate of decrease in absorption at 223 mμ, according to Whitaker *et al.* (1966). Esterase activity was measured according to Bargetzi *et al.* (1964). The assay solution contained 3 ml of 10^{-2} M hippuryl-DL-phenyllactic acid, 5×10^{-3} M sodium Veronal, and 4.5×10^{-2} M NaCl (pH 7.5). The rate of hydrolysis was measured with a pH-Stat comprising a TTT-1 Radiometer autotitrator equipped with a scale expander (type PHA 630T) and glass electrode. The rate was linear with time and was treated in terms of zero-order kinetics (Putnam and Neurath, 1946).

Chromatographic Procedure (Preparative Method). The enzyme was separated by equilibrium ion-exchange chromatography in one buffer containing β-phenylpropionate. Recycled DE-52 (microgranular DEAE-cellulose, preswollen wet powder, Reeve Angel Co.) was equilibrated with 0.05 M β-phenylpropionate–0.04 M LiCl–0.05 M Tris (pH 7.5) (the pH was adjusted with 10 N NaOH at room temperature). The adsorbent was packed in a Pharmacia column (2.5 × 100 cm) at room temperature with a flow rate of 300 ml/hr until a height of 90 cm was obtained. A flow adapter was placed in position and the column was transferred to the cold room (4°) and equilibrated overnight with the chromatography buffer described above at a flow rate of 150 ml/hr adjusted with a Technicon peristaltic pump. All subsequent operations were done in the cold (0–4°). A crystal suspension containing 800 mg of carboxypeptidase A was centrifuged and the crystals were dissolved in 4.5 ml of 2 M Tris–0.05 M β-phenylpropionate–0.04 M LiCl (pH 7.5).[1] The solution was centrifuged and the supernatant was decanted, diluted with 37 ml of 0.05 M β-phenylpropionate–0.04 M LiCl (pH 7.5), and dialyzed 4 hr against 2 l. of chromatography buffer.[2] The sample was applied to the column at a flow rate of 48 ml/hr. The elution was started at a flow rate of 93 ml/hr collecting 19-ml fractions. Chromatography was completed in approximately 72 hr. The appropriate peak fractions were pooled, concentrated to 10–20 ml in the Amicon Diaflow pressure cell (Model 400) equipped with a UM-1 membrane, and dialyzed against 0.005 M Tris-Cl (pH 7.5) until crystals were obtained (usually overnight). These were collected by centrifugation, carefully washed several times with cold 0.001 M Tris-Cl (pH 7.5), and stored in the refrigerator under an atmosphere of toluene. The same chromatography column can be reused as long as the brownish material which accumulates on top of the column is removed after two to three runs and replaced with equilibrated adsorbant so as to keep the column height constant. The method can be scaled down to accommodate 100 mg of enzyme by using a 1.5 × 90 cm column, a flow rate of 34 ml/hr, and collecting 12-ml fractions.

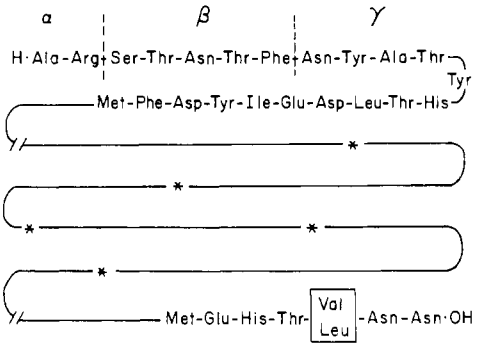

FIGURE 1: Schematic representation of the terminal regions of bovine carboxypeptidase A.

Amino-Terminal Analysis (Cyanate Method). The method of Stark and Smyth (1963) was used with slight modifications; 2.4 g of urea was added to 4 ml of a 50% (v/v) aqueous solution of redistilled *N*-ethylmorpholine adjusted to pH 8 with glacial acetic acid. Carboxypeptidase (50 mg, 1.4 μmoles) was acid denatured and Zn^{2+} was removed as described by Bargetzi *et al.* (1964). The wet precipitate of inactive enzyme was dissolved by adding 20 mg of solid sodium dodecyl sulfate, and the protein solution was added dropwise (using rapid stirring) to the above urea solution followed by 250 mg of potassium cyanate. Carbamylation was allowed to continue for 15 hr at 50° with stirring. Cyclization, chromatography, and base hydrolysis of the hydantoin were carried out as described by Stark and Smyth (1963); 4 μmoles of norleucine and 0.5 μmole of hydantoin from α-aminobutyric acid were added *before* cyclization in order to correct for mechanical losses, and for accurate measurements of protein concentration (Gertler and Hofmann, 1967). An aliquot corresponding to 2 mg of enzyme was removed *before* application to the Dowex 50-X2 column, hydrolyzed in 6 M HCl for 24 hr at 110°, and subjected to amino acid analysis. The total protein concentration of the sample was calculated on the basis of 19 or 20 alanine residues per mole and correcting on the basis of norleucine recovery (Walsh and Brown, 1962). The concentration of amino-terminal amino acid in the sample was corrected on the basis of recovery of α-aminobutyric acid. A control, consisting of uncarbamylated carboxypeptidase, was also carried through the same procedure.

Heat Inactivation. NaCl (1.0 M)–phosphate (0.005 M, pH 7.5, 4 ml) was placed in a thermostated reaction vessel kept at 50.0 ± 0.1° (Haake circulator-thermostat) for 15 min with efficient stirring; 50 μl of enzyme stock solution (15.7 mg/ml, kept at 0°) was added at zero time; 50-μl aliquots were removed at different time intervals and directly assayed for hippuryl-DL-phenyllactic acid activity as described above (Bar-

[1] One particular commercial preparation was extremely insolub'e (lot CoA-7KA). This material (1 g) was suspended in 5 M NaCl–0.005 M Tris-Cl (pH 7.5) at 0°. The pH was raised to 9.5 to dissolve the enzyme and rapidly brought back to 7.5 with HCl. The solution was centrifuged and crystals were obtained after dialyzing the supernatant overnight at 4° against 6 l. of 0.005 M Tris-Cl (pH 7.5). Some enzyme was irreversibly denatured during this process; however, the crystals obtained were 90–100% active and were used directly for the chromatography. The resulting elution pattern was identical with those of other lots.

[2] The removal of Tris by dialysis produces a supersaturated enzyme solution and occasionally the sample will crystallize if dialysis is continued for a longer period of time. If this is the case, the content of the dialysis bag is centrifuged, and the collected crystals are dissolved in 4.5 ml of 2 M Tris, 0.04 M LiCl, and 0.05 M β-phenylpropionate (pH 7.5). The supernatant is added and the solution is redialyzed for 4 hr as described in the text.

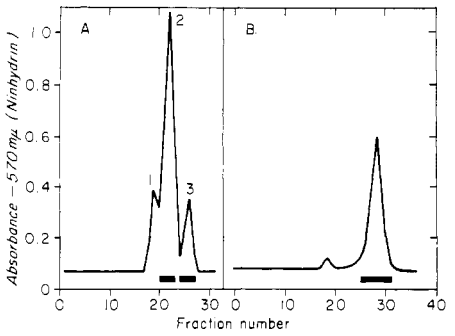

FIGURE 2: Elution pattern for the isolation of amino- and carboxyl-terminal peptides derived from fraction IV (Figure 1). (A) Gel filtration on a column (0.9 × 100 cm) of Sephadex G-25 of 15 mg of 5% trifluoroacetic acid soluble cyanogen bromide fragments. (B) Gel filtration on a column (0.9 × 100 cm) of Sephadex G-50 of the pooled fractions from peak 2 of part A; 1.5-ml fractions were collected for both elutions at a flow rate of 5 ml/hr. Peptides were pooled as indicated by the solid heavy line.

getzi *et al.*, 1963). The specific activity (100% value) was obtained by adding 50 μl of the enzyme stock solution to 4.9 ml of 1 M NaCl–0.005 M sodium phosphate (pH 7.5) at 0° and by removing a 50-μl aliquot for hippuryl-DL-phenyllactic acid activity measurement. The same pipets (50 μl and 4.0 ml) were used for all these transfers. The error in enzyme concentration based on optical density measurements at 278 mμ did not vary more than 2%. Precipitation of the enzyme occurs as denaturation proceeds.

Amino acid analysis was performed according to Spackman *et al.* (1958) with the use of T-Ala[3] and 2-amino-β-guanidopropionic acid as internal standards to correct for any difference in recovery between the long and short columns resulting from pipetting and instrumental errors (Walsh and Brown, 1962); 2 mg of carboxypeptidase was placed into each of three ignition tubes along with 2 ml of constant-boiling HCl. The tubes were evacuated, sealed, put into an oven at 110°, and removed at 24, 48, and 72 hr, respectively. The contents were taken to dryness three times, allowed to remain 4–6 hr at pH 6.5 (Moore and Stein, 1963), taken up in 2.2 ml of 0.2 N sodium citrate buffer (pH 2.2) along with the internal standards, and analyzed on a Spinco Model 120 amino acid analyzer. Peptides were hydrolyzed in 6 N HCl at 110° for 15 hr. Tryptophan was determined according to Edelhoch (1967) assuming 19 residues of tyrosine/mole of protein and correcting for the spectral contribution of 1 residue of cystine/mole of protein (Lipscomb *et al.*, 1969) at 280 and 288 mμ.

Performic Acid Oxidation. Carboxypeptidase (15 mg, 0.41 μmole) was heat denatured as described by Bargetzi *et al.* (1964). The precipitate was collected, washed several times with distilled water, and lyophilized; 0.3 ml of 98% formic acid was added to the lyophilized powder and the solution was allowed to remain at 0° for 0.5 hr; 0.3 ml of performic acid was added and the oxidation was continued overnight at 0° (Hirs, 1956). Cysteic acid was determined by amino acid analysis after acid hydrolysis.

Synthesis of α-Aminobutyric Acid Hydantoin and L-*Serine Hydantoin.* Recrystallized α-aminobutyric acid (1 g) was added to a solution containing 50 ml of distilled water, 15 ml of *N*-ethylmorpholine, 25 g of potassium cyanate, and adjusted to pH 8 with glacial acetic acid. The reaction mixture was kept at 50° for 4 hr and evaporated to dryness. The residue was taken up in 10 ml of water, adjusted to pH 2, and again taken to dryness. The residue was taken up in 40 ml of 6 N HCl and placed in a boiling-water bath for 30 min. After evaporation to dryness the residue was dissolved in 5 ml of hot water (about 70°) and crystallized upon cooling in ice. Two crystallizations yielded a pure product (50% yield, mp 114–117°) as shown by thin-layer chromatography on silica gel in three solvents: chloroform–methanol (3:2, v/v), R_F 0.7; chloroform–dioxane (7:3, v/v), R_F 0.3; and CCl_4–pyridine (7:3, v/v), R_F 0.4. Pure α-aminobutyric acid remained at the origin in all three solvents; 98.6% recovery of α-aminobutyric acid was obtained when the hydantoin was subjected to amino acid analysis after base hydrolysis in 0.2 N NaOH for 24 hr at 110° (Stark and Smyth, 1963).

L-Serine hydantoin was synthesized from L-serine using the same conditions. A twice-crystallized product (45% yield, mp 187–180°) was obtained which was shown to be pure by thin-layer chromatography (95% ethanol–water (62:37, v/v), R_F 0.73; 1-propanol–water (64:36, v/v), R_F 0.55); 25.5% recovery of L-serine was obtained (20%, Stark and Smyth, 1963) after base hydrolysis in 0.2 N NaOH for 24 hr at 110°. The per cent recovery of serine was estimated by including the hydantoin of α-aminobutyric acid as an internal standard since the latter is quantitatively hydrolyzed to α-aminobutyric acid under these conditions (*vide supra*).

Purification of Amino- and Carboxyl-Terminal Peptides. Carboxypeptidase A (100 mg, 2.7 μmoles) was treated with cyanogen bromide as described by Sampath Kumar *et al.* (1964a) and stored as a lyophilized powder. The carboxyl-terminal peptide was separated on a column of Sephadex G-25 (0.9 × 100 cm) by dissolving the lyophilized powder (15 mg) in 1.5 ml of 50% acetic acid, centrifuging, and applying the supernatant with gravity flow. Elution was carried out with 50% acetic acid at a flow rate of 5 ml/hr (LKB peristaltic pump), collecting 1.5-ml fractions in plastic cups; 0.05-ml aliquots were removed from each cup and analyzed with ninhydrin after base hydrolysis (Hirs *et al.*, 1956) with the use of a Technicon Autoanalyzer (Schroeder and Robberson, 1965). The fractions containing the carboxyl-terminal peptide (peak 3, Figure 2A) were pooled, taken to dryness, and further purified by high-voltage paper electrophoresis using a pyridine–formic acid–water buffer (0:3:4.0:95.7, v/v, pH 2.1) at 2000 V for 60 min (Bargetzi *et al.*, 1964). The major ninhydrin-positive area, located with the aid of a guide strip, was eluted from the paper with water and analyzed for amino acid composition after acid hydrolysis. The amino-terminal peptide obtained from peak 2 (Figure 2A) was further purified on a column of Sephadex G-50 (0.5 × 100 cm) equilibrated with 50% acetic acid. The pooled fractions from peak 2 (Figure 2A) were taken to dryness, dissolved in 1.5 ml of 50% acetic acid, centrifuged, and applied with gravity flow. Elution and monitoring of the fractions were carried out as described for the Sephadex G-25 filtration (*vide supra*). The resulting peak fractions (Figure 2B) were pooled, taken to dryness, and stored in the freezer for further analysis.

Peptide Sequence Analysis. The modified subtractive Edman degradation procedure (Konisberg and Hill, 1962) was used as described by Shearer *et al.* (1967). The solution containing the peptide (0.5–1.5 μmoles in 1 ml of 50% acetic acid)

[3] T-Ala, β-thienylalanine.

TABLE I: Comparison of Specific Activities of Various Chromatographic Fractions of Carboxypeptidase A.[a]

	Sp Act. (μmoles of hippuryl-DL-phenyllactic acid/min μg of enzyme)
Pool sample	0.142
Breakthrough peak	0.01
Peak I	0.193
Peak II	0.210
Peak III	0.199
Peak IV	0.212
Peak V	0.207

[a] Under conditions according to Bargetzi *et al.* (1964): 10^{-2} M hippuryl-DL-phenyllactic acid, 5×10^{-3} M sodium Veronal, 4.5×10^{-2} M NaCl, and $1\text{–}9 \times 10^{-8}$ M carboxypeptidase (pH 7.5), 25°. The experimental error is within 10%.

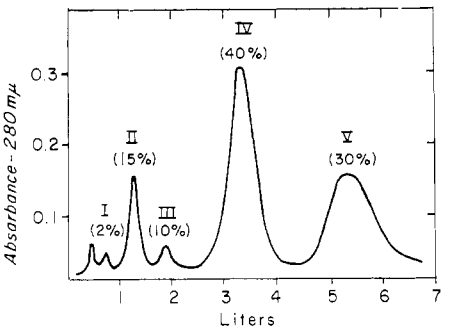

FIGURE 3: Elution pattern of 900 mg of twice-crystallized carboxypeptidase A (Anson) on a column (2.5 × 90 cm) of DE-52 developed at 150 ml/hr at 4° with a buffer containing 0.05 M β-pehnylpropionic acid, 0.04 M LiCl, and 0.05 M Tris, adjusted to pH 7.5 with 1.0 N NaOH. The preparation of the sample to be chromatographed is described in the Experimental Section.

was put into a screw-cap tube. An aliquot was removed and hydrolyzed with 6 N HCl at 110° for 15 hr to establish purity and concentration. The remainder was evaporated to dryness. The Edman degradations were carried out as described.

Results

Chromatography of Carboxypeptidase A. Figure 3 represents the elution profile of the chromatographic separation of 900 mg of carboxypeptidase A (lot CoA-7HA). Other lots tested gave identical patterns except for lot CoA-7AE which lacked the breakthrough peak. Each of the five peak fractions was crystallized by dialyzing against 0.005 M Tris-Cl (pH 7.5). The specific activities (hippuryl-DL-phenyllactic acid) of the five isolated fractions and of the sample applied on the column (pool sample) are listed in Table I. There are no major differences among the fractions but the specific activity of the fractions is higher than that of the pool sample. This latter value, however, varies from lot to lot whereas the specific activities of the chromatographed fractions remain essentially the same within the experimental error of the assay. The kinetic parameters, k_{cat} and K_m, for the enzyme-catalyzed hydrolysis of hippuryl-DL-phenyllactic acid and Z-Gly-Phe by fraction I–V showed no major variations (J. Uren, unpublished experiments). The total amount of protein recovered after crystallization corresponded to 80–85% yield explaining perhaps the increase in specific activity since the breakthrough peak contained only 3% of hippuryl-DL-phenyllactic acid specific activity (based on 0.208 μmole min^{-1} μg^{-1} as the 100% value; Bargetzi *et al.*, 1964) and significant amounts of brown color accumulated on top of the column after each chromatography (*vide infra*). No additional protein fraction could be obtained after stripping the column with a solution containing 3 M LiCl, 0.05 M Tris, and 0.2 M β-phenylpropionic acid adjusted to pH 7.5 with NaOH. The four major fractions (II, III, IV, and V) were distributed in the ratio 15:10:40:30 and there was little variation in this ratio among the lots tested. Rechromatography of each peak of fractions IV and V under the same conditions gave a single component which separated at its appropriate position in the elution pattern of the original chromatography.

Amino Acid Composition. Table II summarizes the amino acid composition obtained for each of the five fractions including the breakthrough peak. The data represent an average of at least three determinations for each fraction and have been calculated on the basis of 19, 20, or 21 residues of alanine per molecular weight of 34,600 (Bargetzi *et al.*, 1963). The selection of the proper value of alanine was chosen so as to approach the maximum number of integral values for all other amino acids (Walsh *et al.*, 1966). This value was 21 residues of alanine in the case of fractions I, 20 for II and IV, and 19 for fractions III and V. Recoveries of the unstable and more slowly released amino acids during acid hydrolysis were estimated by an average of two sets of determinations at three different times of hydrolysis: 24, 48, and 72 hr (not done for breakthrough fraction). Serine and threonine were obtained by linear extrapolation to zero-time hydrolysis (Smith and Stockell, 1954); the maximum standard deviation (calculated from the least squares) for both amino acids was +0.6 of a residue. Valine and isoleucine represent maximum values obtained after 72 hr (Bargetzi *et al.*, 1964). The determination of cysteic acid (Hirs, 1956) was carried out on pooled carboxypeptidase A (lots CoA-7AE, CoA-7HA, and CoA-7FB) and on the chromatographically separated fractions of lot CoA-7HA. The results are shown in Table III. Performic acid oxidation done according to Moore (1963) gave identical results.

Amino-Terminal Analysis. The amino-terminal residues of each fraction are shown in Table IV. The values for serine were corrected for 75% destruction as described in the Experimental Section. A correlation exists between fractions II, III, and carboxypeptidase A_β, containing amino-terminal serine, and between fractions IV, V, and carboxypeptidase A_γ which contains asparagine in this position.

Isolation of the Carboxyl-Terminal Peptides Derived from Fractions I–V. The lyophilized powders obtained from each of the cyanogen bromide treated fractions were chromatographed on Sephadex G-25. A typical gel filtration is shown in Figure 2A, which represents the elution pattern of peak fraction IV. The fractions corresponding to the third peak containing the carboxyl-terminal peptide were pooled and

TABLE II: Comparison of Amino Acid Composition of Chromatographically Separated Fractions of Carboxypeptidase A (Anson) (residues per mole).

Amino Acid	A (Cox)[a]	A (Anson)[a]	A_β[b] (cald)	Peak Fractions BP[h]	I	I	III	IV	V
Aspartic acid	27.9	26.5	27.5	23.6	27.9	28.2	27.6	26.8	27.0
Threonine	27.8	23.4	25.4	15.1	24.5[c]	25.3[c]	25.0[c]	22.6[c]	23.8[c]
Serine	33.0	30.0	31.0	20.8	31.4[c]	30.9[c]	30.5[c]	31.2[c]	31.2[c]
Glutamic acid	25.0	24.9	24.9	20.9	25.1	25.0	26.0	25.7	26.0
Proline	9.7	9.8	9.8	12.5	9.8	9.5	10.1	10.3	9.5
Glycine	22.5	22.5	22.5	27.9	22.8	22.6	22.2	22.8	22.7
Alanine[d]	20.0	19.0	19.0	(20.0)	(21.0)	(20.0)	(19.0)	(20.0)	(19.0)
Valine	15.8	15.6	15.6	15.0	16.0[c]	16.0[c]	16.0[c]	16.2[c]	15.5[c]
Methionine	3.0	2.7	2.7	3.1	2.8	2.8	2.6	2.9	2.7
Isoleucine	20.3	19.8	19.8	14.8	19.0[c]	19.8[c]	19.8[c]	22.0[c]	19.0[c]
Leucine	23.2	22.9	22.9	18.7	22.0	22.7	23.0	22.8	23.5
Tyrosine	19.2	18.8	18.8	13.7	18.2	18.6	17.9	18.1	18.8
Phenylalanine	16.0	14.6	15.6	10.6	16.7	16.2	16.2	14.8	15.5
Lysine	15.0	15.0	15.0	15.1	15.4	14.5	14.7	14.7	14.8
Histidine	8.2	7.7	7.7	5.0	8.0	8.0	7.7	7.8	7.8
Arginine	11.1	9.9	9.9	8.6	10.8	9.6	9.6	10.0	9.8
Half-cystine	2	2	2		2.0[e]	2.0[e]	2.0[e]	1.9[e]	1.9[e]
Tryptophan	8[g]	8[g]	8		8.5[f]	8.0[f]	8.6[f]	8.3[f]	8.3[f]

[a] Bargetzi *et al.* (1963). [b] Calculated by adding the amino acids comprising the amino-terminal difference peptide between carboxypeptidase A_α and carboxypeptidase A_β to the published values of carboxypeptidase A (Cox) (Sampath Kumar *et al.*, 1964b). [c] Corrected (see text). [d] Reference amino acid (see text). [e] Calculated as cysteic acid (Hirs, 1956). [f] Edelhoch (1967). [g] Bencze and Schmid (1957). [h] Breakthrough peak (Figure 3).

further purified by high-voltage paper electrophoresis. Table V shows the amino acid composition of all five carboxyl-terminal peptides obtained from the chromatographically separated fractions shown in Figure 2. The pooled enzyme used for the chromatography contained approximately a 60–40 distribution of valine and leucine in the antepenultimate position of the peptide. However, in the case of fractions II and IV leucine is absent from the peptides, whereas the opposite is true for fractions III and V where valine is absent. Peak I contains both valine and leucine enzymes (Table V). However, their distribution in this particular fraction is 80:20 (valine to leucine, respectively) instead of the predicted 60:40 ratio (see Discussion).

Isolation of the Amino-Terminal Peptides Derived from Fractions I–V. The fractions corresponding to peak 2 (from the Sephadex G-25 system illustrated in Figure 2A) were pooled, taken to dryness, and rechromatographed on Sephadex G-50.

TABLE III: Determination of Cysteic Acid.[a]

Sample	Residues of $CySO_3H$/Mole of Protein
Lot CoA-7AE	1.86
Lot CoA-7FB	2.41
Lot CoA-7HA	3.22
Peak I (lot CoA-7HA)	1.99[b]
Peak II (lot CoA-7HA)	1.99[c]
Peak III (lot CoA-7HA)	1.96[d]
Peak IV (lot CoA-7HA)	1.88[c]
Peak V (lot CoA-7HA)	1.88[d]

[a] Methods of Hirs (1956) and Moore (1963). [b] Based on 21 alanines (see text and Table I). [c] Based on 20 alanines (see text and Table I). [d] Based on 19 alanines (see text and Table I).

TABLE IV: Amino-Terminal Residues of Chromatographically Separated Fractions (residues per mole).

Residue	Peak II	Peak III	Peak IV	Peak V
Asp	0.04	0.08	0.87	0.88
Ala	0.03	0.10	0.03	0.01
Ser	0.51	0.64	0.03	0.01

H · Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn-Tyr---

α β γ

Figure 2B shows the elution profile for the amino-terminal peptide obtained after cyanogen bromide cleavage of fraction IV. The peptides derived from fractions I, II, III, and V gave identical elution patterns and their amino acid compositions along with fraction IV are given in Table VI. The peptide derived from fraction I contains a total of 22 amino acids and is identical with the amino acid composition of the amino-terminal peptide derived from carboxypeptidase $A_\alpha(N_\alpha)$ (Figure 1, and Sampath Kumar *et al.*, 1964b). The two amino-terminal peptides derived from fractions II and III each contain a total of 20 amino acids and have identical compositions corresponding to the amino acid composition of the amino-terminal peptide obtained from carboxypeptidase $A_\beta(N_\beta)$. Those derived from fractions IV and V which contain 15 amino acids are also identical in composition and correspond to the amino acid composition of the amino-terminal peptide derived from carboxypeptidase $A_\gamma(N_\gamma)$.

Sequence of the Amino-Terminal Peptide Derived from Fraction I (N_I). The composition of the peptide (N_I) is given in Table VI. The substractive Edman procedure was used for the determination of the sequence. The method involves the cleavage of the amino-terminal amino acid leaving the residual peptide with the following compositions at each step.

FIRST DEGRADATION. Asp 4.0, Thr 3.9, Ser 1.1, Glu 1.4, *Ala 1.2*, Ile 1.2, Leu 1.2, Tyr 2.8, Phe 1.9, His 1.0, Arg 0.8, and Met[4] 1.3.

SECOND DEGRADATION. Asp 4.0, Thr 3.7, Ser 1.1, Glu 1.3, Ala 1.2, Ile 1.1, Leu 1.2, Tyr 2.8, Phe 2.0, His 1.0, *Arg 0.2*, and Met 1.2.

The results suggest the following partial sequence of the peptide H·Ala-Arg, which is consistent with part of the sequence of the amino-terminal region of carboxypeptidase A (Cox) (Sampath Kumar *et al.*, 1964b). These data in addition to those shown in Tables IV and VI demonstrate that fraction I (Figure 3) is carboxypeptidase A_α.

Sequence of the Amino-Terminal Peptide Derived from Fraction II (N_{II}). The composition of the peptide (N_{II}) is given in Table VI. The subtractive Edman procedure was used for the determination of the sequence. The method involves the cleavage of the amino-terminal amino acid leaving the residual peptide with the following compositions at each step.

FIRST DEGRADATION. Asp 4.0, Thr 3.6, *Ser 0.4*, Glu 1.2, Ala 1.2, Ile 1.1, Leu 1.2, Tyr 2.8, Phe 2.1, and Homoser 0.3.

SECOND DEGRADATION. Asp 4.0, *Thr 3.1*, Ser 0.3, Glu 1.3, Ala 1.2, Ile 1.1, Leu 1.2, Tyr 2.8, Phe 1.8, and Homoser 0.3.

THIRD DEGRADATION. *Asp 3.0*, Thr 2.7, Ser 0.2, Glu 1.1, Ala 1.0, Ile 1.2, Leu 1.1, Tyr 2.5, Phe 1.6, and Homoser 0.3.

FOURTH DEGRADATION. Asp 3.0, *Thr 2.1*, Ser 0.3, Glu 1.2, Ala 1.0, Ile 1.0, Leu 1.0, Tyr 2.4, Phe 1.7, and Homoser 0.3.

FIFTH DEGRADATION. Asp 3.0, Thr 2.1, Ser 0.5, Glu 1.4, Ala 1.1, Ile 1.1, Leu 1.1, Tyr 2.3, *Phe 1.1*, and Homoser 0.3.

SIXTH DEGRADATION. *Asp 2.3*, Thr 1.8, Ser 0.3, Glu 1.1, Ala 0.9, Ile 0.9, Leu 1.0, Tyr 2.1, Phe 0.9, and Homoser 0.2.

SEVENTH DEGRADATION. Asp 2.0, Thr 1.7, Ser 0.3, Glu 1.0, Ala 0.8, Ile 0.9, Leu 0.9, *Tyr 1.5*, Phe 0.8, and Homoser 0.2.

EIGHTH DEGRADATION. Asp 2.3, Thr 1.7, Ser 0.4, Glu 1.1, *Ala 0.3*, Ile 0.9, Leu 1.0, Tyr 1.4, Phe 0.9, and Homoser 0.2.

The results suggest the following sequence: H-Ser-Thr-Asx-Thr-Phe-Asx-Tyr-Ala. These data in addition to those shown in Tables IV–VI demonstrate that fraction II (Figure 3) is carboxypeptidase A_β^{Val} (see Discussion for nomenclature).

[4] Sum of homoserine and homoserine lactone.

TABLE V: Amino Acid Composition (Residues per Mole) of the Carboxyl-Terminal Cyanogen Bromide Fragments Derived from Chromatographically Isolated Fractions I–V.[a]

	Glu	His	Thr	Val	Leu	Asp
CPA (Anson)	1.03	0.95	0.95	0.64	0.42	2.00
Peak I	1.06	1.30	0.91	0.79	0.25	2.00
Peak II	0.99	1.04	0.90	*0.93*	0.10	2.00
Peak III	1.00	1.09	0.90	0.12	*1.02*	1.90
Peak IV	1.03	0.90	0.94	*1.08*	0.07	2.00
Peak V	1.04	1.14	0.98	0.05	*1.01*	2.00

[a] Calculated on the basis of the aspartic acid content of the peptides (Bargetzi *et al.*, 1964).

Sequence of the Amino-Terminal Peptide Derived from Fraction III (N_{III}). The composition of the peptide (N_{III}) is given in Table VI. The subtractive Edman was used for the determination of the sequence.

FIRST DEGRADATION. Asp 4.0, Thr 3.5, *Ser 0.6*, Glu 1.3, Ala 1.3, Ile 1.2, Leu 1.3, Tyr 2.8, Phe 1.9, and Homoser 0.3.

SECOND DEGRADATION. Asp 4.0, *Thr 2.8*, Ser 0.5, Glu 1.4, Ala 1.3, Ile 1.7, Leu 1.3, Tyr 2.7, Phe 1.8, and Homoser 0.3.

THIRD DEGRADATION. *Asp 3.2*, Thr 2.9, Ser 0.5, Glu 1.3, Ala 1.1, Ile 1.1, Leu 1.2, Tyr 2.3, Phe 1.7, and Homoser 0.3.

FOURTH DEGRADATION. Asp 3.0, *Thr 2.1*, Ser 0.5, Glu 1.5, Ala 1.1, Ile 1.1, Leu 1.1, Tyr 2.3, Phe 1.7, and Homoser 0.3.

FIFTH DEGRADATION. Asp 3.0, Thr 2.1, Ser 0.6, Glu 1.1, Ala 1.1, Ile 1.1, Leu 1.2, Tyr 2.3, *Phe 1.1*, and Homoser 0.3.

SIXTH DEGRADATION. *Asp 2.3*, Thr 1.9, Ser 0.5, Glu 1.1, Ala 1.0, Ile 1.0, Leu 1.1, Tyr 2.4, Phe 0.9, and Homoser 0.3.

SEVENTH DEGRADATION. Asp 1.8, Thr 1.5, Ser 0.4, Glu 0.9, Ala 0.8, Ile 1.2, Leu 1.0, *Tyr 1.2*, Phe 0.8, and Homoser 0.2.

EIGHTH DEGRADATION. Asp 1.8, Thr 1.6, Ser 0.6, Glu 1.1, *Ala 0.4*, Ile 0.9, Leu 0.9, Tyr 1.1, Phe 0.7, and Homoser 0.2.

The data are consistent with the following sequence: H·Ser-Thr-Asx-Thr-Phe-Asx-Tyr-Ala. These results in addition to those presented in Tables IV–VI indicate that fraction III (Figure 3) is carboxypeptidase A_β^{Leu}.

Sequence of the Amino-Terminal Peptide Derived from Fraction IV (N_{IV}). The composition of the peptide (N_{IV}) given in Table VI correlates well with that published for the amino-terminal peptide derived from carboxypeptidase A (Anson) (Sampath Kumar *et al.*, 1964b). Three turns of the Edman degradation were carried out to complete the identification.

FIRST DEGRADATION. *Asp 2.1*, Thr 1.8, Glu 1.1, Ala 1.0, Ile 1.0, Leu 1.0, Tyr 2.6, Phe 1.0, and Homoser 0.3.

SECOND DEGRADATION. Asp 2.1, Thr 1.8, Glu 1.1, Ala 1.0, Ile 1.1, Leu 1.1, *Tyr 1.8*, Phe 1.0, and Homoser 0.3.

THIRD DEGRADATION. Asp 2.0, Thr 1.8, Glu 1.0, *Ala 0.3*, Ile 1.0, Leu 1.0, Tyr 1.6, Phe 0.9, and Homoser 0.3.

The data are consistent with the following amino-terminal sequence: H·Asx-Tyr-Ala. These results with the ones presented in Tables IV–VI indicate that fraction IV (Figure 3) is carboxypeptidase A_γ^{Val}.

Sequence of the Amino-Terminal Peptide Derived from Frac-

TABLE VI: Amino Acid Composition (residues per mole) of the Amino-Terminal Peptides Derived by Cyanogen Bromide Cleavage from Chromatographically Isolated Fractions I–V.

Amino Acid	N_γ[a]	N_α[a]	N_β[b]	N_I	N_{II}	N_{III}	N_{IV}	N_V
Aspartic acid	3.0	4.0	4.0	4.0	4.0	4.0	2.8	2.9
Threonine	1.9	3.8	4.0	3.7	3.6	3.4	1.8	1.8
Serine		1.2	1.0	1.3	1.0	1.0		
Glutamic acid	1.1	1.4	1.0	1.4	1.2	1.3	1.1	1.1
Alanine	1.0	1.8	1.0	2.0	1.2	1.3	1.0	1.0
Isoleucine	1.0	0.9	1.0	1.0	1.1	1.2	1.0	0.9
Leucine	1.0	1.2	1.0	1.3	1.3	1.3	1.0	1.0
Tyrosine	2.7	2.6	3.0	2.8	2.8	2.8	2.6	2.6
Phenylalanine	1.0	2.0	2.0	1.9	2.0	1.9	1.0	1.0
Histidine	1.0	1.0	1.0	1.1	1.1	1.0	1.1	1.0
Arginine		0.9		0.8				
Methionine[c]	1.0	1.1	1.0	1.1	1.0	0.8	0.9	0.9

[a] Published values (Sampath Kumar *et al.*, 1964b). [b] Theoretical values for the amino-terminal peptide derived from carboxypeptidase A_β based on the amino-terminal region of carboxypeptidase A_α (Sampath Kumar *et al.*, 1964b). [c] Sum of homoserine and homoserine lactone.

tion V (N_V). The composition of the peptide (N_V) shown in Table VI is similar to the one of N_γ and N_{IV}. Three turns of the subtractive Edman procedure completed the identification.

FIRST DEGRADATION. *Asp 2.1*, Thr 1.8, Glu 1.1, Ala 1.0, Ile 1.0, Leu 1.0, Tyr 2.4, Phe 1.0, and Homoser 0.3.

SECOND DEGRADATION. Asp 2.0, Thr 1.7, Glu 1.1, Ala 1.0, Ile 1.0, Leu 1.1, *Tyr 1.7*, Phe 0.9, and Homoser 0.3.

THIRD DEGRADATION. Asp 2.0, Thr 1.8, Glu 1.0, *Ala 0.3*, Ile 1.0, Leu 1.0, Tyr 1.6, Phe 0.9, and Homoser 0.3.

These data suggest the following amino-terminal sequence: H·Asx-Tyr-Ala. These results with the ones presented in Tables IV–VI indicate that fraction V (Figure 3) is carboxypeptidase A_γ^{Leu}. The sequences of all four amino-terminal peptides are summarized in Table VII; N_{IV} and N_V were isolated in 60% yield in contrast to a 30% recovery of N_I, N_{II}, and N_{III}. These results support the earlier evidence that N_α is insoluble in water (Sampath Kumar *et al.*, 1964b) and large amounts are probably lost during the 5% trifluoroacetic acid precipitation after cyanogen bromide cleavage. N_β would be expected to behave similarly. However, N_γ is soluble in water thereby explaining the higher recoveries of N_{IV} and N_V.[5]

Heat Inactivation Studies. The loss of enzymatic activity as a function of time at 50° was measured for the separated fractions, *i.e.*, for carboxypeptidase A_β^{Val}, A_β^{Leu}, A_γ^{Val}, and A_γ^{Leu} (see Discussion for nomenclature). The results are shown in Figure 4. The data for carboxypeptidase A^{Val} and A^{Leu} obtained from the Cox *et al.* (1964) preparation done on pancreatic juices of two individual cows homozygous for either the valine or leucine trait have already been published (Walsh *et al.*, 1966) and were included to illustrate the relationship between all the different forms of carboxypeptidase A. The rate of inactivation appears to depend upon two factors: first, chain length; the longer the N-terminal region of the enzyme, the slower the rate of inactivation. Carboxypeptidase A (Cox) is more heat stable than carboxypeptidase A_β, which in turn is more heat stable than carboxypeptidase A_γ. Second, all the valine enzymes are more heat stable than the corresponding leucine enzymes, indicating that this amino acid replacement or other linked amino acid replacements (Pétra *et al.*, 1969) or both may affect the conformational stability of the molecule as a whole.

Discussion

A method has been developed to chromatograph bovine carboxypeptidase A (Anson).[6] The success of this separation method is due to the presence of β-phenylpropionate which serves not only to stabilize the enzyme but also to render it soluble at ionic strengths suitable for chromatography. It is clear from the present analysis that carboxypeptidase A (Anson) is not a pure protein but actually is composed of at least five active forms having nearly the same specific activity toward small ester substrates and differing from each other mainly in their amino and carboxyl-terminal regions. Commercial preparations of the enzyme contain 70% carboxypep-

[5] Since some of these peptides were obtained in low yield, proof of homogeneity of the isolated fractions by an independent method is essential. Disc gel electrophoresis of all five fractions was made possible at the time this paper went to press. The results confirmed homogenity by this method (P. H. Pétra and H. Neurath, in preparation).

[6] In view of the present results, it appears necessary to modify the nomenclature previously proposed for bovine carboxypeptidase A (Bargetzi *et al.*, 1963). Thus carboxypeptidase A (Anson), A (Cox), and A (Allan) denote the procedures used for the isolation, regardless of the composition and purity of the product. Carboxypeptidase A_α, A_β, and A_γ denote the species containing alanine[1], serine[3], or asparagine[8] as the amino-terminal residue, using the amino acid sequence of carboxypeptidase A_α for numbering the residues (Neurath *et al.*, 1969). The species arising from amino acid replacements are denoted by superscripts as carboxypeptidase A^{Leu} or A^{Val}. If more than one amino acid replacement is to be symbolized, the superscript residues will be numbered according to their position in the amino acid sequence of the protein.

TABLE VII: Amino-Terminal Region of the Amino-Terminal Peptides Derived from Chromatographically Isolated Fractions I–V.

CPA_α	H · Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn-Tyr-Ala-
Peak I	H · Ala-Arg-(Ser,Thr,Asx,Thr,Phe,Asx,Tyr,Ala)
Peak II	H · Ser-Thr-Asx-Thr-Phe-Asx-Tyr-Ala-
Peak III	H · Ser-Thr-Asx-Thr-Phe-Asx-Tyr-Ala-
Peak IV	H · Asx-Tyr-Ala-
Peak V	H · Asx-Tyr-Ala-

tidase A_γ, 25% A_β, and approximately 2% A_α. The species, along with their corresponding allotypic variants, A^{Val} and A^{Leu}, can account for all the known and postulated forms of carboxypeptidase A.

Fraction I (Figure 3), identified as carboxypeptidase A_α, contains both valine and leucine enzymes. However, the distribution ratio for the two allotypic variants in this fraction is not the same as the one for the pooled enzyme applied on the column (Table V). This observation would indicate that fraction I contains carboxypeptidase $A_\alpha{}^{Val}$ along with small amounts of the leucine variant of a new species of carboxypeptidase A, and that carboxypeptidase $A_\alpha{}^{Leu}$ probably separates with carboxypeptidase $A_\beta{}^{Val}$ in fraction II (Figure 3). This hypothesis is difficult to test because carboxypeptidase A_α occurs in too small amounts in the commercial preparation. Recently, however, a chromatographic method for the separation of carboxypeptidase A_α into both allotypic variants was developed (P. H. Pétra and H. Neurath, in preparation) thereby providing an opportunity to answer this question. Considering the method of isolation of carboxypeptidase A (Anson), the molecular heterogeneity of the enzyme is not too surprising; in fact, Putnam and Neurath (1946) have already reported that this enzyme, under certain conditions, shows more than one component in moving-boundary electrophoresis. Since the starting material used for chromatography is a crystalline preparation, the demonstration of heterogeneity documents further the well accepted fact that crystallinity is an inadequate criterion for the purity of a protein.

The chromatographic separation of carboxypeptidase A_β and A_γ can be accounted for by the difference in amino acid composition in their amino-terminal region. The same argument cannot be applied to the separation of the valine and leucine variants of carboxypeptidase A_β and A_γ, respectively, since both have the same amino-terminal sequence. The difference in one methylene group alone would hardly account for a chromatographic separation of this kind. Three possible explanations, however, can be offered. First, a slight difference in conformation may exist as a result of the valine-leucine replacement causing unmasking of either charged or aromatic groups or both which may be responsible for alterations of the surface of the enzyme. The results obtained in the heat inactivation studies tend to support this hypothesis. The most recent model of carboxypeptidase A (Lipscomb *et al.*, 1969), although not specified as to either the valine or leucine variant, permits either of these two residues to be accommodated in the penultimate position without requiring any significant conformational changes in this locus. However, induced conformational changes in other regions of the molecule cannot be precluded by the static nature of such a model. *Second*, β-phenylpropionate is known to bind at several loci in carboxypeptidase A including the active center (Bethune, 1965). If one or more of these loci in one of the variant enzymes are modified as a result of the amino acid replacement, the binding properties of the inhibitor will be different, thereby promoting separation in equilibrium chromatography. *Third*, the valine-leucine replacement in the C-terminal region may be linked to one or more amino acid replacements elsewhere in the molecule as indicated by the asterisks in Figure 1. Such replacements should explain the small but significant differences in the amino acid composition of the five forms (Table II). In fact, meaningful amino acid compositions could only be obtained when 20 alanine residues were used as a basis for peaks 2 and 4 (valine enzymes) and 19 alanine residues for peaks 3 and 5 (leucine enzymes) as if there were an additional alanine replacement linked with valine (Table II). The positions of the leucine enzymes in the chromatographic patterns indicate that they are probably more negatively charged than the valine enzymes and hence the other amino acid linked with leucine could be an aspartic or glutamic acid residue, a prediction which has been recently confirmed (Pétra *et al.*, 1969).

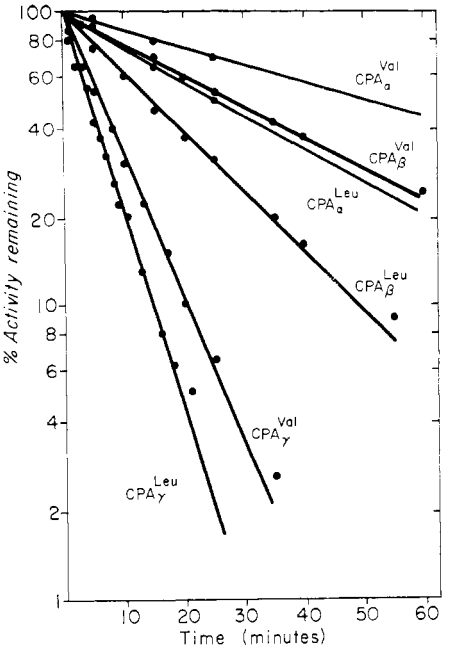

FIGURE 4: Heat inactivation of various forms of carboxypeptidase A at 50 ± 0.2°. Protein concentration is 0.2 mg/ml in 1 M NaCl–0.005 M phosphate (pH 7.5). Enzymatic acitivity was measured with hippuryl-DL-phenyllactic acid. See text for more details.

The differences in chemical composition, in structure, or in both between the four fractions of carboxypeptidase A have no significant effects on the catalytic activity of the enzyme towards ester and peptide substrates. This is in accord with the three-dimensional model of Lipscomb *et al.* (1969) which indicates that the extremities of the polypeptide chain in which these chemical variations are located, occupy distant positions relative to the active center.

The chromatographic separation of commercial carboxypeptidase A (Anson) and more recently of carboxypeptidase A (Cox) (P. H. Pétra and H. Neurath, in preparation) pro-

vides an opportunity for a reexamination of certain structural and functional features of the enzyme which previously had been determined on impure preparations. Although 95–97% of the protein recovered after chromatography of the commercially available enzyme is carboxypeptidase A, some lots contain a brownish impurity which is retained on top of the chromatographic column and another colorless impurity which separates as a small breakthrough peak (Table II). Samples which contain this latter fraction yield a higher cysteic content after oxidation than the corresponding chromatographic fractions (Table III). The presence of these impurities which cannot be removed by dialysis or repeated crystallization will affect the interpretation of the results in the determination of total sulfur content and may introduce difficulties in the sequence analysis of the whole molecule.

Although most of the chemical modifications have been done on commercially available enzymes which contain approximately a 60:40 distribution of valine to leucine variants, there is no *a priori* reason to expect that the sites or consequences of chemical modification are different in the four chromatographic species that have been identified in this investigation. There is no evidence that any of the residues that differentiate one of the forms from the remainder is affected by the specific reagents that have been described. However, in view of the possible effects of amino replacements on conformation, it remains to be determined whether the allotypic variants react with and respond to group specific reagents in the same manner and whether the chemically modified species have the same or different kinetic properties.

Since measurements of the rates of heat inactivation indicate differences among the four species in conformational stabilities, it will be of interest to examine in a like manner the effects of urea and other denaturing agents and to correlate enzyme inactivation with certain physical properties such as optical rotatory dispersion and circular dichroism. The findings will be of particular importance when compared with X-ray structure analysis of crystalline preparations of all three forms of carboxypeptidase A along with their corresponding allotypic variants.

Another area of interest which may be clarified by the use of these pure carboxypeptidases is the complex problem of zymogen activation. Since all preparations of carboxypeptidase A invariably contain small amounts of carboxypeptidase A_β (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1964b) which can now be separated chromatographically, it becomes possible to carry out a systematic investigation of the activation process using pure procarboxypeptidase A. Furthermore, the enzymatically catalyzed interconversion between carboxypeptidase A_α, A_β, and A_γ previously investigated on impure preparations (Sampath Kumar *et al.* 1964b) can now be studied quantitatively.

The chromatographic separation of carboxypeptidase A^{Val} from A^{Leu} in all three forms of the enzyme (A_α, A_β, and A_γ) provides an opportunity to determine quantitatively their occurrence and distribution in a single animal. Preliminary experiments, carried out on carboxypeptidase A (Cox) isolated from the pancreatic juice of a cow, containing both variants, indicate a 50:50 distribution between carboxypeptidase $A_\alpha{}^{Val}$ and carboxypeptidase $A_\alpha{}^{Leu}$ (P. H. Pétra, R. W. Tye, R. Sande, and H. Neurath, in preparation). This finding is in agreement with previous data obtained from the valine:leucine distribution in the C-terminal hexapeptide obtained by degradation with cyanogen bromide (Sampath Kumar *et al.*, 1964a). The question presents itself whether such polymorphism is a unique property of pancreatic carboxpeptidase A or a general attribute of bovine pancreatic enzymes in general. The early discovery of the two variants of carboxpeptidase A was facilitated by the ease of isolation in high yields of the souble hexapeptides containing the amino acid replacement, as well as by the equal distribution of the two proteins. A more disproportioned amino acid replacement or an operationally less favorable site in the protein might have escaped detection by current methods of enzyme degradation and peptide isolation. However, if the replacement involves two amino acid residues of different charge, it may be possible to separate both proteins on ion-exchange adsorbents, thereby making the problem of detection relatively easy. The linked second replacement in carboxypeptidase A may very well illustrate this point (Pétra *et al.*, 1969). The question of the generality of this phenomenon or its phylogenetic significance remains to be clarified. It is doubtful that bovine carboxypeptidase A is a unique case among the proteolytic enzymes; however, extension of these studies to other carboxypeptidases (Neurath *et al.*, 1967) and to other enzymes can provide an answer to this question.

Acknowledgments

The authors wish to thank Dr. Kenneth A. Walsh for his interest and helpful suggestions in this research. The authors are also indebted to Mr. Richard R. Granberg and Mr. Mark S. Kupperberg for their invaluable technical assistance, and to Mrs. Brita Moddy and Miss Mila Cabildo for the preparation of reagents for sequence analysis.

Reference

Allan, B. J., Keller, P. J., and Neurath, H. (1964), *Biochemistry 3*, 40.

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 777.

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Bargetzi, J. P., Thompson, E. O. P., Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964), *J. Biol. Chem. 239*, 3767.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Bethune, J. L. (1965), *Biochemistry 4*, 2698.

Cox, D. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Gertler, A., and Hofmann, T. (1967), *J. Biol. Chem. 242*, 2522.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.

Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Ludwig, M. L., Steitz, T. A., and Bethge, P. (1969), *Brookhaven Symp. Biol. 21*, 24.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 7*, 819.

Neurath, H. (1960), *Enzymes 4*, 11.

Neurath, H., Bradshaw, R. A., Ericsson, L. H., Babin, D. R.,

Pétra, P. H., and Walsh, K. A. (1969), *Brookhaven Sym. Biol. 21*, 1.
Neurath, H., Walsh, K. A., Winter, W. P. (1967), *Science 158*, 1638.
Pétra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry* (in press).
Putnam, F. W., and Neurath, H. (1946), *J. Biol. Chem. 166*, 603.
Sampath Kumar, K. S. V., Clegg, J. B., and Walsh, K. A. (1964b), *Biochemistry 3*, 1728.
Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964a), *Biochemistry 3*, 1726.
Schroeder, W. A., and Robberson, B. (1965), *Anal. Chem. 37*, 1583.
Shearer, W. T., Bradshaw, R. A., Gurd, F. R. N., Peters, T., Jr. (1967), *J. Biol. Chem. 243*, 5451.
Smith, E. L., and Stockell, A. (1954), *J. Biol. Chem. 207*, 501.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Vallee, B. L., and Riordan, J. F. (1969), *Brookhaven Symp. Biol. 21*, 91.
Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.
Walsh, K. A., Ericsson, L. H., and Neurath, H. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1339.
Whitaker, T. R., Menger, F., and Bender, M. L. (1966), *Biochemistry 5*, 386.

The Stoichiometry and Site Specificity of the Uncoupling of Mitochondrial Oxidative Phosphorylation by Salicylanilide Derivatives*

David F. Wilson

ABSTRACT: The compound 5-chloro-3-*t*-butyl-2′-nitrosalicylanilide is effective at concentrations lower than any other known compound which can uncouple mitochondrial electron transport from the energy conservation reactions. In rat liver mitochondria complete loss of respiratory control and maximal stimulation of adenosine triphosphatase activity requires 0.6 and 1.0 molecule of uncoupler per respiratory chain, respectively. The release of the inhibition of respiration by azide (which is specific for electron transport at cytochrome oxidase) requires 1.35 molecules of uncoupler/respiratory chain.

This titer is independent of the rate of electron flux and the rate of generation of high-energy intermediates. This salicylanilide derivative therefore binds stoichiometrically to a site which is approximately one per respiratory chain. The site is inferred (from the specificity of the azide inhibition) to be associated with the cytochrome oxidase portion of the respiratory chain.

A very large number of compounds have been discovered which uncouple mitochondrial oxidation from phosphorylation of ADP (Loomis and Lipman, 1948), and other energy-requiring reactions including divalent cation transport (Saris, 1963; Chappell *et al.*, 1963), antibiotic induced monovalent cation transport (Moore and Pressman, 1964), the energy-linked reduction of NAD^+ (Chance and Hollunger, 1957, 1960), and the energy-linked transhydrogenase (Klingenberg and Schollmeyer, 1961; Estabrook *et al.*, 1962). There is very little known about their actual mechanism(s) of action and several hypotheses have been proposed (Slater, 1953; Lardy and Wellman, 1953; Chance and Hollunger, 1957; Mitchell, 1961, 1966; Harris *et al.*, 1967; Slater and Colpa-Boonstra, 1961). These hypotheses include such diverse mechanisms as that the uncouplers increase the permeability of the mitochondrial membrane to protons (Mitchell, 1961, 1966), that they are substituting for substrate anions in an energy-requiring anion pump (van Dam and Slater, 1967), and they catalyze the hydrolysis of a chemical high-energy intermediate (Lardy and Wellman, 1953; Slater, 1953).

A new and very powerful class of uncouplers has recently been reported by Williamson and Metcalfe (1967). They reported that some of these salicylanilide dervatives (including 5-Cl-3-*t*-butyl-2′-Cl-4′-NO_2-salicylanilide) are effective at concentrations lower than the carbonyl cyanide derivatives prepared by Heytler (1963).

In the present communication the observations of Williamson and Metcalf (1957) are confirmed. It is also shown that the 5-chloro-3-*t*-butyl-2′chloro-4′-nitrosalicylanilide (S-13) is a stoichiometric uncoupler of mitochondrial oxidative phosphorylation and that it appears to be specific for the energy conservation site at the cytochrome oxidase of the electron transport chain. A preliminary report of this work has been published (Wilson and Azzi, 1968).

* From the Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received January 17, 1969.* Supported by U. S. Public Health Grant 12202. The author is a fellow of the Pennsylvania Plan to Develop Scientists in Medical Research.