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The Amino Acid Sequence of Bovine Carboxypeptidase A. II. Tryptic and Chymotryptic Peptides of the Cyanogen Bromide Fragment F_{III}*

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ABSTRACT: An 81 amino acid residue fragment (F_{III}) of bovine carboxypeptidase A, obtained by cleavage with cyanogen bromide and isolated by gel filtration on Sephadex G-75, has been subjected to tryptic and chymotryptic hydrolysis. The resulting peptides have been isolated and characterized by Edman degradations and hydrazinolysis, and by hydrolysis with carboxypeptidase A and B and leucineaminopeptidase. Alignment of these peptides yields a tentative structure for the entire fragment, which is in exact agreement with the amino acid composition deduced from acid hydrolysates.

As the first step in the complete sequence analysis of bovine carboxypeptidase A, the three residues of methionine have served as cleavage sites in the cyanogen bromide reaction to produce four fragments. These fragments account satisfactorily for the molecular weight and amino acid composition of the native enzyme (Nomoto *et al.*, 1969). Two of these fragments, termed F_N and F_C, have been examined in detail and their complete primary structure has been elucidated (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1964). One of the remaining two fragments, F_I, contains 198 amino acids and is attached in the sequence to the carboxyl-terminal peptide F_C, while the other, F_{III}, containing 81 amino acids, has been positioned adjacent to the amino-terminal fragment, F_N (Nomoto *et al.*, 1969). This fragment is devoid of half-cystine and possesses a single amino-terminal residue of aspartic acid.

Sequence analysis of F_{III} has been initiated by isolation and characterization of the tryptic and chymotryptic peptides. These data are sufficient to produce a tentative structure of the fragment. The additional information necessary to provide the complete structure has been obtained from peptides produced by hydrolysis of F_{III} with thermolysin and is reported in the accompanying paper (Bradshaw, 1969).

Experimental Procedure

Materials. Carboxypeptidase A (Anson) was purchased in 10-g lots as twice-crystallized material from Worthington Biochemicals and used without further purification.

Fragment F_{III} was prepared by cyanogen bromide cleavage and purified as previously described (Nomoto *et al.*, 1969)

Trypsin, chymotrypsin, carboxypeptidase B, and leucineaminopeptidase were obtained from Worthington Biochemicals.

Pyridine and *N*-ethylmorpholine were redistilled from solid ninhydrin (1 g/l.) before use.

Phenyl isothiocyanate and trifluoroacetic acid were purchased from Eastman Organic Chemicals and redistilled before use.

Hydrazine was obtained from Matheson Coleman and Bell.

Methods. Tryptic and chymotryptic peptides were prepared in the following manner. Lyophilized F_{III} was suspended in water (10 mg/ml) and dissolved by the addition of 1 N NaOH. When all of the protein had dissolved, the solution was readjusted to pH 8.5–9.0 with 1 N HCl to yield a uniform suspension. Trypsin or chymotrypsin, prepared as a stock solution (10 mg/ml) in 10^{-3} M HCl, was added to a final concentration of 1% (w/w) relative to the protein substrate. The pH of the reaction mixture (37°) was held constant with a Radiometer pH-Stat equipped with an Ole Dich recorder. At the completion of the reaction (3–6 hr), the hydrolysate was adjusted to pH 2.0 with 6 N HCl. The insoluble material formed was removed by centrifugation.

The soluble peptides from both digests were fractionated on a column (2.0 × 25 cm) of Dowex 50-X8 (Spinco amino acid

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received May 14, 1969.* This work was supported by the National Institutes of Health (GM 04617 and GM 15731-01), the American Cancer Society (P-79K), the Office of Naval Research (NONR 477-35), and the National Science Foundation (GB 4990).

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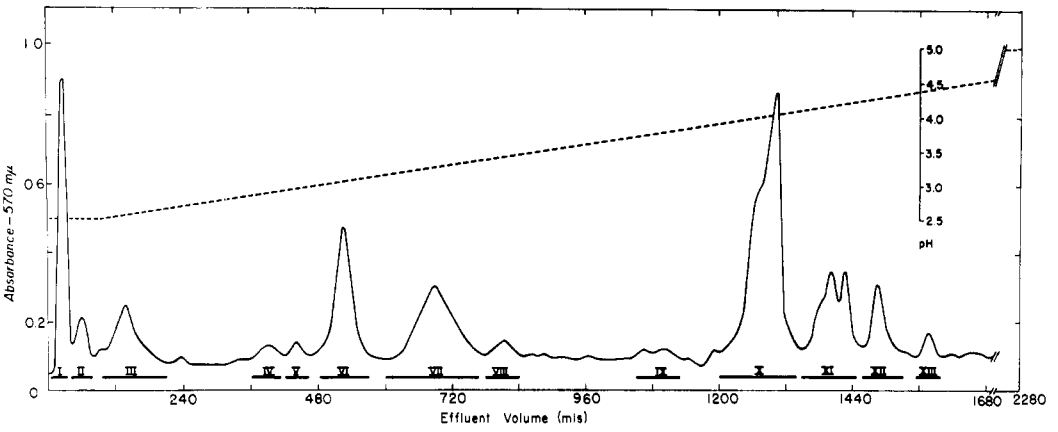

FIGURE 1: Elution profile of the soluble tryptic peptides from fragment F_{III} of carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine acetate as described in the text. Fractions of 6.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis. Fractions were pooled as indicated by the solid bars. The pH gradient is indicated by the dashed line.

TABLE I: Amino Acid Composition of the Tryptic Peptides of Fragment F_{III}.[a]

Amino Acid	T-1	T-2	T-3	T-3a	T-4	T-5	T-6	T-6b	T-6c	T-6d
Lysine	1.02 (1)		1.02 (1)			1.00 (1)	1.05 (1)			
Histidine	0.95 (1)				0.83 (1)					
Arginine		1.00 (1)	0.98 (1)	0.93 (1)	1.88 (2)					
Aspartic acid	1.07 (1)				2.20 (2)		3.32 (3)	3.16 (3)	2.20 (2)	1.15 (1)
Threonine					1.04 (1)	1.82 (2)	2.04 (2)	2.24 (2)	1.16 (1)	0.94 (1)
Serine	0.81 (1)		0.94 (1)	0.99 (1)	2.79 (3)		1.50 (2)	1.56 (2)		1.72 (2)
Glutamic acid	1.97 (2)	1.07 (1)	1.12 (1)	1.07 (1)		1.88 (2)	2.36 (2)	2.00 (2)	2.20 (2)	
Proline	0.86 (1)		0.99 (1)		0.83 (1)		1.10 (1)	1.06 (1)		0.77 (1)
Glycine		0.97 (1)	1.32 (1)	1.14 (1)	3.04 (3)	1.17 (1)	1.18 (1)	1.21 (1)	1.00 (1)	
Alanine	1.10 (1)				1.21 (1)	2.02 (2)	1.02 (1)	1.16 (1)		1.00 (1)
Valine	1.84 (2)		1.04 (1)			1.00 (1)				
Isoleucine		0.84 (1)	0.87 (1)		2.87 (3)	1.00 (1)	1.10 (1)	1.12 (1)		0.98 (1)
Leucine	2.70 (3)	0.81 (1)	0.88(1)		1.21 (1)		0.90 (1)	0.91 (1)		0.96 (1)
Tyrosine			1.52 (2)	0.78 (1)			0.92 (1)	0.94 (1)	0.60 (1)	
Phenylalanine					0.96 (1)	0.92 (1)	1.86 (2)	1.86 (2)	0.65 (1)	0.82 (1)
Tryptophan					0.46 (1)	1.20 (2)				
Homoserine and lactone							0.98 (1)	1.03 (1)		0.94 (1)
Total	13	5	11	5	20	13	19	18	8	10
% yield	82	50	10	50	6	48	13	24	12	6
Purification process[b]			E	E			DX1	DX1	DX1	DX1
Fraction no.	VI	X	XI-E-1	XII	Insol-1	VII	I-III-2	I-III-3	I-III-4	I-III-1

[a] Values are given in residues per mole. The assumed integral values are given in parentheses. [b] Abbreviations used are: DX1, Dowex 1-X2; E, preparative high-voltage electrophoresis.

analyzer resin, AA15). The column was equilibrated with 0.05 N pyridine acetate (pH 2.4) prior to the addition of the sample. Elution was carried out at a flow rate of 80 ml/hr with a double linear gradient composed of 500 ml of 0.05 N pyridine acetate (pH 2.4) and 500 ml of 0.5 N pyridine acetate (pH 3.75) followed by 500 ml of 0.5 N pyridine acetate (pH 3.75) and 500 ml of 2.0 N pyridine acetate (pH 5.0) (Bradshaw *et al.*, 1969). The column was stripped with 2.5 M trimethylamine. The eluent was collected in 6.0-ml fractions. The separation was monitored by ninhydrin color at 570-mμ after alkaline hydrolysis and the fractions were pooled accordingly. Peptides were recovered after removal of the solvent by rotary evaporation or lyophilization.

Subfractionations were carried out on columns of Dowex 1-X2 and Dowex 50-X2 (Bio-Rad). Dowex 1-X2 columns (0.9 × 100–150 cm) were equilibrated in 3% pyridine and

TABLE II: Characterization of Peptides from Fractions Tp I–III.

Fraction Tp I-III-1:
  Peptide T-6d:
    Sequence: Pro-Ser-Phe-Thr-Ala-(Ile, Leu, Asx, Ser, HSer)

Fraction Tp I-III-2:
  Peptide T-6:
    Sequence: Lys-Phe-Thr-Glx-Asx-Tyr-Gly-Glx(Asx, Pro, Ser, Phe, Thr, Ala, Ile, Leu, Asx, Ser, HSer)

Fraction Tp I-III-3:
  Peptide T-6b:
    Sequence: (Phe, Thr, Glx, Asx, Tyr, Gly, Glx, Asx, Pro, Ser, Phe, Thr, Ala, Ile, Leu, Asx, Ser, HSer)
    Chymotryptic subdigest:
      Peptide T-6b C-1:
        Sequence: Thr-Glu-Asn-Tyr
        LAP: 3 hr; Thr, 1.00; Glu, 0.32; Asn, 0.20
      Peptide T-6b C-2:
        Sequence: Gly-Glx-Asx-Pro(Ser, Phe)
      Peptide T-6b C-3:
        Sequence: (Thr, Ala, Ile, Leu)
      Peptide T-6b C-4:
        Sequence: Asx(Ser, HSer)

Fraction Tp I-III-4:
  Peptide T-6c:
    Sequence: Phe-Thr-Glx-Asx-Tyr-Gly-(Glx, Asx)

developed at a rate of 30 ml/hr with a continuous gradient of 3% pyridine, 0.5 N pyridine acetate (pH 6.0), 1.0 N pyridine acetate (pH 6.0), and 2.0 N pyridine acetate (pH 5.0) (Bradshaw *et al.*, 1969). Dowex 50-X2 columns (0.9 × 50 cm) were developed at 30 ml/hr utilizing various gradients of pyridine acetate buffers as dictated by the elution of the fraction on the initial Dowex 50-X8 column.

The isolated fractions were checked for purity by either high-voltage electrophoresis at pH 3.75 and 6.5 or by paper chromatography using a pyridine–1-butanol–acetic acid–H_2O (10:15:3:12, v/v) solvent. Preparative separations on paper were carried out in the same systems.

Subtractive Edman degradations were carried out by a modified version (Shearer *et al.*, 1967) of the method of Konigsberg and Hill (1962). Digestion of the peptides with carboxypeptidase A or B and leucineaminopeptidase was carried out in 0.01 M Tris-Cl buffer (pH 8.5) at room temperature. Aliquots (0.1 ml) for analysis were withdrawn at appropriate time intervals and diluted with 0.9 ml of sodium citrate buffer (pH 2.2) to stop the reaction.

Hydrazinolysis was performed as described by Fraenkel-Conrat and Tsung (1967). The carboxyl-terminal residue was detected on the automatic amino acid analyzer without prior removal of the contaminating hydrazides.

Results

Isolation of Tryptic Peptides. The mixture of tryptic peptides obtained after acidification of the digestion mixture was separated on a column of Dowex 50-X8 with a double linear gradient of pyridine acetate; the pattern obtained is shown in Figure 1. Each fraction was pooled according to the solid bars and the peptides were recovered after rotary evaporation. The treatment of each pool is described in detail below. The ten pure tryptic peptides obtained are listed in Table I. In addition to the composition, the total number of residues, the per cent yield, and the pool number are given for each peptide. Peptides that were found to be impure after initial separation are designated by the subsequent procedure used to purify them. For convenience, pure peptides have been designated (in

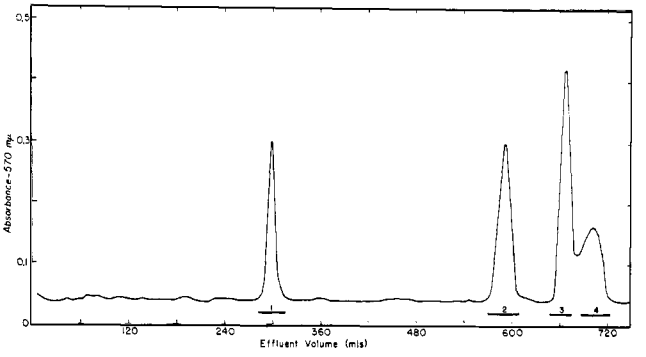

FIGURE 2: Elution profile of fractions Tp I–III on a 0.9 × 150 cm column of Dowex 1-X2 at 35°. The column was developed at 30 ml/hr with a gradient of pyridine acetate as described in the text. Fractions of 2.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis. Fractions were pooled as indicated by the solid bars.

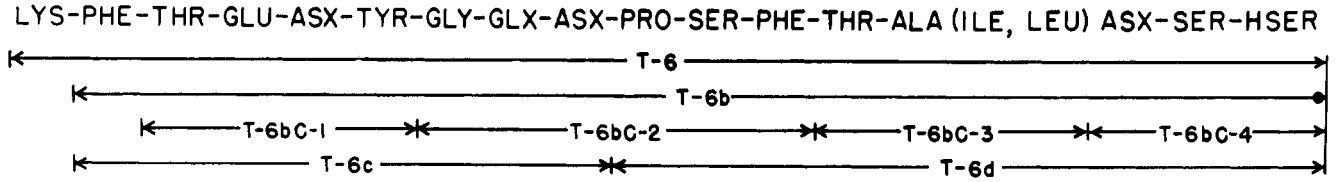

FIGURE 3: Alignment of the tryptic peptides of fractions Tp I–III.

TABLE III: Characterization of Peptides from Fraction Tp VI.

Fraction Tp VI:
Peptide T-1:
Sequence: Asx-Leu-Leu-Val(Ala, Glx, His, Pro, Glx, Leu, Val, Ser)-Lys
Carboxypeptidase B: 30 min; Lys, 1.00
Chymotryptic subdigest:
Peptide T-1 C-1:
Sequence: (Asx, Leu, Leu)
HVE, pH 6.5: Acidic
Peptide T-1 C-2:
Sequence: Val-Ala-Glx-His-Pro(Glx, Leu, Val, Ser, Lys)
HVE,[a] pH 6.5: Basic

[a] In all tables HVE is high-voltage electrophoresis.

Arabic numbers) in the order that they occur in the fragment, starting at the amino terminus, while column pools from the original Dowex 50-X8 fractionation have been indicated by Roman numerals.

Fractions Tp I–III.[1] Fractions Tp I–III from the initial separation were pooled together and subfractionated on Dowex 1-X2. Four major pools were obtained, as shown in Figure 2, and were treated as summarized in Table II. Coincidentally, each of the four peptides isolated from fractions Tp I–III can be readily identified as being derived from the carboxyl-terminal portion of the fragment. Peptides T-6d, T-6, and T-6b each contained a single residue of homoserine (and its lactone), whereas peptide T-6c was readily identified as being identical with a portion of T-6.

The sequence data obtained for each peptide are indicated by subscript arrows in the following manner: Edman degradation ($\rightarrow$), hydrazinolysis ($\leftarrow$), carboxypeptidase A or B ($\leftarrow$), and LAP ($\rightarrow$). The analytical data are listed below each peptide except for the Edman degradations.[2] The results of high-voltage electrophoresis at pH 6.5 are recorded in terms of the character of the peptide, basic peptides being those which move toward the negative pole.

Since each of the four peptides of fractions Tp I–III is derived from the same portion of the molecule, they can be assembled to produce nearly a complete sequence of the last 19 residues of F_{III}. These data are summarized in Figure 3. The sequence of the first 8 residues is supplied by Edman degradations of T-6 and T-6c. By difference in composition, this places an Asx residue in the ninth position. The tenth through fourteenth residues are positioned from T-6d. The last three residues are supplied by T-6b C-3 with the assumption that homoserine is the carboxyl-terminal residue of this peptide. Only the order of Ile and Leu in positions 15 and 16 and three amide assignments are undetermined by these data.

Fraction Tp VI. This fraction contained a single pure peptide, T-1, described in Table III. The first four residues were obtained by Edman degradations and the carboxyl-terminal Lys by carboxypeptidase B digestion. A subdigest of this peptide by chymotrypsin gave two peptides, accounting for the composition of the whole peptide. The first was readily identifiable as the amino-terminal portion from the presence of the single residue of aspartic acid and the absence of lysine. High-voltage electrophoresis indicated the aspartic acid residue to be in the acid form. Five rounds of Edman degradations of the second peptide left only four residues of T-1 not in sequence.

Fraction Tp VII. This fraction also contained a single peptide (Table IV) comprising 13 residues. Only a single turn of Edman degradation was accomplished before the remainder of the peptide was inexplicably lost. Carboxypeptidase B digestion of a separate sample showed a single carboxyl-terminal lysine residue. The assignment of two residues of tryptophan to this peptide was made on the basis of an extraordinarily high recovery of tryptophan after acid hydrolysis (1.2 resi-

[1] Abbreviations used are: Tp-, tryptic pool; Cp-, chymotryptic pool; T-, tryptic peptide; C-, chymotryptic peptide; CPA, carboxypeptidase A; CPB, carboxypeptidase B; LAP, leucineaminopeptidase.

[2] Edman degradations were analyzed differentially by use of the automatic amino acid analyzer. No degradation was considered successful unless the decrease was at least 50% greater than the loss in any other amino acid over that seen in the previous analysis, under conditions in which *only one* residue was 20% lower than that previously observed. All data were discarded in which the recovery of peptides was less than 5%. When the residue to be removed was penultimate to the carboxyl-terminal residue, a recovery of at least 10% of the remaining amino acid was required. In view of the large number of amino acid analyses required to obtain the requisite amount of sequence data to complete the primary structure of carboxypeptidase A, the analytical data have been omitted.

TABLE IV: Characterization of Peptides from Fractions Tp VII and Tp X.

Fraction Tp VII:
  Peptide T-5:
    Sequence: Glx(Trp, Ile, Thr, Glx, Ala, Thr, Gly, Val, Trp, Phe, Ala)Lys
    Carboxypeptidase B: 30 min; Lys, 0.89

Fraction Tp X:
  Peptide T-2:
    Sequence: Leu-Glx-Ile-Gly-Arg
    Carboxypeptidase B: 30 min; Arg, 1.00
    HVE, pH 6.5: Basic

dues) which was subsequently confirmed by isolation of the chymotryptic peptides from this region (*vide infra*).

Fraction Tp X. This fraction contained a single pentapeptide as shown in Table IV. Three rounds of Edman degradations and digestion by carboxypeptidase B were sufficient to give the complete sequence of peptide T-2. The peptide was basic on high-voltage electrophoresis at pH 6.5, indicating the Glx residue to be glutamine.

Fraction Tp XI. This fraction was found to be impure and was subsequently fractionated by preparative high-voltage electrophoresis at pH 3.75. Two peptides were obtained from this separation as indicated in Table V. The first, T-3, contained 11 residues. Three rounds of Edman degradations indicated a sequence of Ser-Tyr-Glx and carboxypeptidase digestion showed a carboxyl-terminal Lys residue. Subdigestion by chymotrypsin yielded two peptides after fractionation on Dowex 50-X8. The first, T-3 C-1, was assigned to the initial portion of the main peptide from composition while the second, T-3 C-2, gave Val as amino-terminal residue on Edman degradation. Since this tripeptide contained the only Lys residue, already ascertained to be the carboxyl-terminal residue of T-3, the sequence of this peptide can be taken as Val-Leu-Lys.

The second peptide present in fraction Tp XI, T-6a, was shown to be free Lys.

Fraction Tp XII. This fraction contained a single peptide, T-3a, as shown in Table VI. Only compositional data were obtained, but it has been subsequently found that this peptide must have been derived from an Arg-Pro cleavage (*vide infra*).

Fraction Tp Insol. The insoluble core of the tryptic digest contained essentially one pure peptide after repeated acid (0.05 N pyridine acetate) washings (Table VI). It is noteworthy that this peptide had partial acid solubility since fractionation of the initial tryptic digest of F_{III} on Dowex 50-X2 rather than on Dowex 50-X8 showed this peptide to be eluted last.[3] The low yield of this peptide was attributed to the partial acid solubility with the assumption that the soluble portion was not eluted from the more highly cross-linked resin (Dowex 50-X8).

Three rounds of Edman degradations and consecutive carboxypeptidase B and A digestion gave the three terminal and two carboxyl-terminal residues as indicated in Table VI.

It is significant to note that the peptides summarized in Table I account for the amino acid composition of F_{III} exactly (Nomoto *et al.*, 1969), with the following qualifications. Peptide T-6, as shown already (see Figure 3), accounts for the whole carboxyl-terminal sequence and, thus, peptides T-6a, T-6b, T-6c, and T-6d were not included in the total. Peptide T-3a which was deduced to be a portion of T-3 was also excluded. The remaining peptides, T-1, T-2, T-3, T-4, T-5, and T-6, give a total composition in exact agreement with that of whole F_{III}. The alignment of these pieces and the elucidation of the unsequenced regions were obtained from the chymotryptic peptides.

Isolation of Chymotryptic Peptides. The digestion of F_{III} by chymotrypsin resulted in the solubilization of virtually all of the fragment. The small amount of acid-insoluble material, removed by centrifugation, was not examined further.

The separation of the soluble chymotryptic peptides is shown in Figure 4. Each fraction was pooled and examined for purity by high-voltage electrophoresis. Impure pools were purified on columns of Dowex 1-X2 or by preparative high-voltage electrophoresis or chromatography. The amino acid composition of each of the pure peptides obtained is summarized in Table VII. As with the tryptic peptides, the chymotryptic peptides have been numbered in the order that they

TABLE V: Characterization of Peptides from Fraction Tp XI.

Fraction Tp XI-E-1:
  Peptide T-3:
    Sequence: Ser-Tyr-Glx(Gly, Arg, Pro, Ile, Tyr, Val, Leu)Lys
    Carboxypeptidase B: 30 min; Lys, 0.79
    Chymotryptic subdigest:
      Peptide T-3 C-1:
        Sequence: (Ser, Tyr, Glx, Gly, Arg, Pro, Ile, Tyr)
        HVE, pH 6.5: Neutral
      Peptide T-3 C-2:
        Sequence: Val(Leu, Lys)

Fraction Tp XI-E-2:
  Peptide T-6a:
    Sequence: Lys

[3] D. R. Babin, unpublished observations.

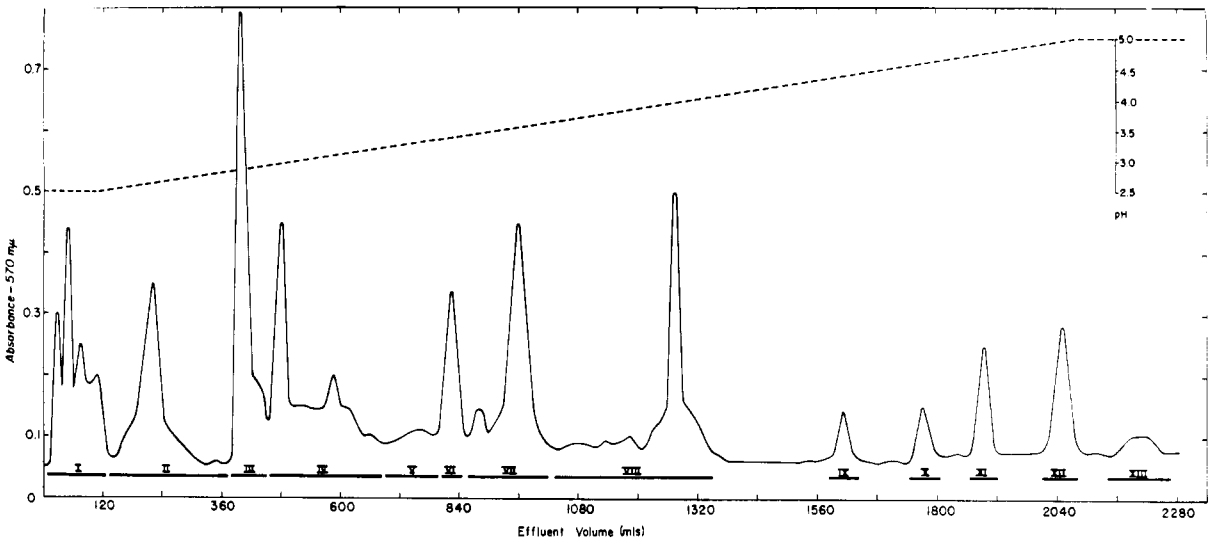

FIGURE 4: Elution profile of the soluble chymotryptic peptides from fragment F_{III} of carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8. Details as in Figure 1.

TABLE VI: Characterization of Peptides from Fraction Tp XII and Tp Insol.

Fraction Tp XII:
Peptide T-3a:
Sequence: (Ser, Tyr, Glx, Gly, Arg)

Fraction Tp Insol-1:
Peptide T-5:
Sequence: Phe-Ser-Thr(Gly, Gly, Ser, Asx, Arg, Pro, Ala, Ile, Trp, Ile, Asx, Leu, Gly, Ile, His)Ser-Arg
Carboxypeptidase B: 30 min; Arg, 0.70
Carboxypeptidase A: 1 hr; Ser, 0.57

occur in the fragment. The treatment of each pool is described below.

Fraction Cp I. This fraction contained three major peptides, C-12, C-12b, and C-12c, which were purified on Dowex 1-X2 as shown in Figure 5. The characterization of these peptides is summarized in Table VIII. Peptide C-12c was subjected to five Edman degradations. Assuming that homoserine is the carboxyl-terminal residue, this information gives the complete structure. High-voltage electrophoresis at pH 6.5 showed two spots, one neutral and one acidic. The origin of this heterogeneity is readily assigned to the homoserine residue which occurs in both the open-chain and lactone forms. This observation allows for the assignment of aspartic acid to the single Asx residue.

Peptides C-12 and C-12b have been assigned on the basis of composition. Because of the extensive information concerning this region that was obtained from other peptides (see Figure 3), no further studies were carried out with these fractions.

Fraction Cp II. Subfractionation of this fraction was carried out on Dowex 1-X2 and the separation obtained is shown in Figure 6. Five peptides were obtained, three in major yield. Each was treated as described in Table IX. By inspection of the compositions, peptides C-10, C-10a, and C-10b appear to be derived from the same portion of the molecule. This conclusion is supported by the single Edman degradation of C-10 and C-10a. Peptide C-10, the longest segment from this region, was successfully treated for five rounds of the Edman reaction. The carboxyl-terminal residue was established as tryptophan by carboxypeptidase A digestion. Both C-10b and C-10 were found to be neutral on electrophoresis, indicating the single Glx residue to be glutamine.

Peptide C-12a was subjected to six Edman degradations which yielded the complete structure. This peptide can be positioned in the carboxyl-terminal portion of the molecule as part of T-6 (*vide supra*).

Fraction Cp III. This fraction contained only a single major yield peptide after chromatography on Dowex 1-X2. The treatment of this peptide is summarized in Table X. The peptide was inert to the Edman reaction but yielded a single residue of tyrosine by CPA digestion. Tryptic subdigestion, followed by isolation of the resulting peptides on a 0.9 × 25 cm column of Dowex 50-X8 (Shearer *et al.*, 1967), yielded two new peptides. Peptide C-3 T-1 possessed carboxyl-terminal Arg whereas peptide C-3 T-2, a dipeptide, was shown to have the structure Ser-Tyr. The whole peptide was deduced to be blocked by cyclization of the amino-terminal glutaminyl residue to pyrrolidonecarboxylic acid.

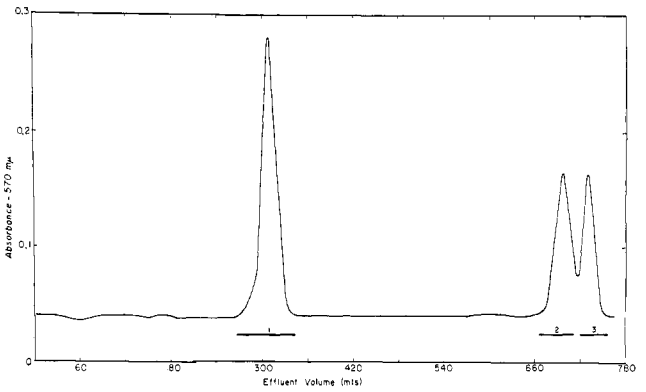

FIGURE 5: Elution profile of fraction Cp I on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

Fraction Cp IV. This fraction was repurified on Dowex 1-X2 and yielded three major pools as shown in Figure 7. The data obtained from the peptides present in fraction Cp IV are summarized in Table XI. Fraction Cp IV-1 was subsequently found to contain two peptides which were isolated by preparative electrophoresis. Peptide C-2a was subjected to five Edman degradations and the structure obtained indicated this peptide to be a portion of T-1. Peptide C-6a, which was separated from C-2a by high-voltage electrophoresis, gave two successful Edman degradations.

Peptide C-2b was identified as corresponding to peptide C-2a, with one less residue. One round of Edman degradation gave a single residue of valine. Treatment with leucineaminopeptidase released valine, followed by equal amounts of alanine and glutamine. Although these data do not allow for determining the order of the alanyl and glutaminyl residues, they do provide the information concerning the side-chain nature of the first Glx residue. Carboxypeptidase A treatment released equal amounts of valine and leucine. The order of these two residues was established by hydrazinolysis of the whole peptide, which indicated valine to be the carboxyl-terminal residue. High-voltage electrophoresis showed that the peptide was neutral, indicating the second Glx residue to be glutamic acid.

Peptide C-1, found in the last pool of fraction Cp IV, possessed an identical composition and electrophoretic mobility as peptide T-1 C-1, released from T-1 by chymotrypsin.

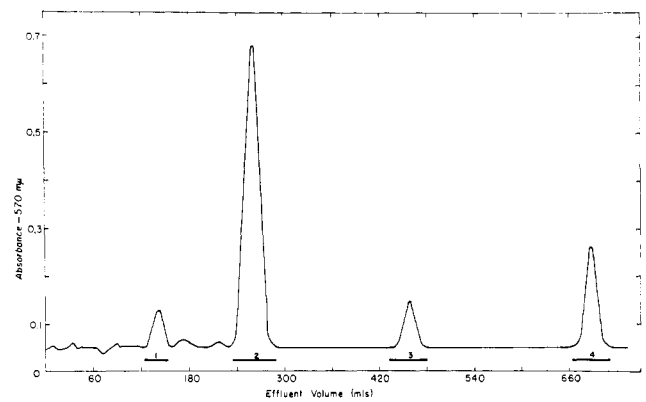

FIGURE 6: Elution profile of fraction Cp II on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

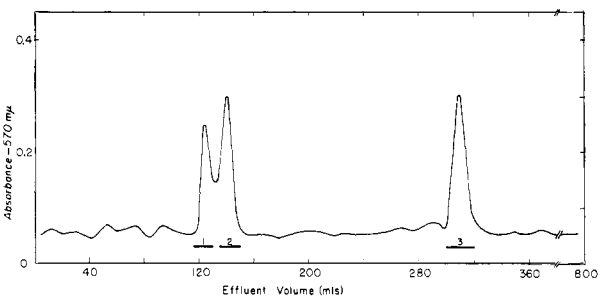

FIGURE 7: Elution profile of fraction Cp IV on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

Fraction Cp V. Three pure peptides were obtained after Dowex 1-X2 purifications, shown in Figure 8. The characterization of these peptides is detailed in Table XII. Fraction Cp V-1 contained a single dipeptide, C-5. Fraction Cp V-2 contained the same peptide found in fraction Cp IV-2, peptide C-2b. The third pool contained a tetrapeptide, C-10c, that was completely structured after three Edman degradations.

Fraction Cp VI. This fraction contained a single peptide, C-8, whose characterization is shown in Table XII. Five rounds of Edman degradation gave the complete structure. Hydrolysis by leucineaminopeptidase released the amino acids consistent with this structure and, in addition, indicated the single Asx residue to be aspartic acid. High-voltage electrophoresis confirmed this assignment.

Fraction Cp VII. Fractionation of this pool on Dowex 1-X2 resulted in a single peak that was heterogeneous on electrophoresis. Consequently this sample was purified by preparative high-voltage electrophoresis to yield two peptides described in Table XIII. Peptide C-2 possessed amino-terminal valine as judged by Edman degradation. Digestion by carboxypeptidase A for 1 hr yielded a full residue of leucine. Addition of carboxypeptidase B followed by digestion for a second hour yielded a full residue of lysine and serine, and partial residues of valine and leucine.

Peptide C-7 was subjected to four Edman degradations and to digestion by carboxypeptidase A for 30 min. The latter method yielded a carboxyl-terminal sequence of Ile-Trp.

Fraction Cp VIII. This fraction contained a single major peptide, C-4, which is described in Table XIV. Five rounds of

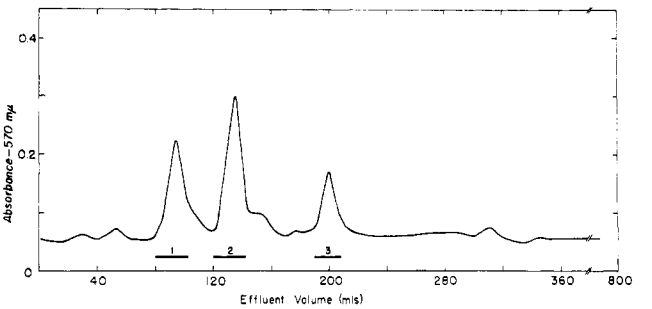

FIGURE 8: Elution profile of fraction Cp V on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

TABLE VII: Amino Acid Composition of the Chymotryptic Peptides of Fragment F_{III}.[a]

Amino Acid	C-1	C-2	C-2a	C-2b	C-2c	C-3	C-3a	C-4	C-5
Lysine		0.89 (1)			1.10 (1)				
Histidine		0.78 (1)	0.83 (1)	1.01 (1)					
Arginine						0.96 (1)	1.19 (1)	1.12 (1)	
Aspartic acid	1.12 (1)								
Threonine									
Serine		1.11 (1)	1.30 (1)			0.88 (1)	0.98 (1)		
Glutamic acid		1.92 (1)	1.83 (2)	2.03 (2)		1.10 (1)		1.05 (1)	
Proline		1.06 (1)	0.90 (1)	1.00 (1)				0.87 (1)	
Glycine						1.09 (1)	1.02 (1)	1.02 (1)	
Alanine		1.02 (1)	1.07 (1)	0.97 (1)					
Valine		1.61 (1)	1.86 (2)	1.97 (2)					1.02 (1)
Isoleucine						0.98 (1)	0.98 (1)	1.01 (1)	
Leucine	1.88 (2)	1.80 (2)	0.99 (1)	1.13 (1)	0.96 (1)				1.00 (1)
Tyrosine						0.70 (1)	0.83 (1)	0.94 (1)	
Phenylalanine									
Tryptophan									
Homoserine and lactone									
Total	3	11	9	8	2	6	5	6	2
% yield	70	7	5	18	95	48	21	90	25
Purification process[b]	DX1	E	DX1, E	DX1		DX1			DX1
Fraction no.	IV-3	VII-E-1	IV-1	IV-2	X	III-1	IX	VIII	V-1

	C-6	C-7	C7-a	C-8	C-9	C-10	C-10a	C-10b	C-10c
Lysine	1.02 (1)								
Histidine				1.01 (1)					
Arginine		1.00 (1)			1.18 (1)				
Aspartic acid		1.15 (1)		0.99 (1)					
Threonine		0.98 (1)	1.00 (1)			1.78 (2)	1.71 (2)	1.15 (1)	0.99 (1)
Serine		1.83 (2)	1.79 (2)		0.90 (1)				
Glutamic acid					1.05 (1)	1.12 (1)	1.11 (1)	1.17 (1)	
Proline		1.18 (1)							
Glycine		2.26 (2)	1.82 (2)	1.08 (1)		1.00 (1)	1.08 (1)		0.93 (1)
Alanine		1.13 (1)				1.06 (1)	1.01 (1)	0.91 (1)	1.00 (1)
Valine						1.12 (1)	1.00 (1)		1.13 (1)
Isoleucine		0.96 (1)		1.98 (2)		0.95 (1)	0.98 (1)	0.85 (1)	
Leucine				1.00 (1)					
Tyrosine									
Phenylalanine	0.97 (1)								
Tryptophan		0.42 (1)			0.51 (1)	0.50 (1)			
Homoserine and lactone									
Total	2	11	5	6	4	8	7	4	4
% yield	95	24	7	85	40	47	12	6	7
Purification process[b]		E	DX1, E			DX1	DX1	DX1	DX1
Fraction no.	XI	VII-E-1	IV-1	VI	XII	II-2	II-3	II-1	V-3

	C-11	C-12	C-12a	C-12b	C-12c
Lysine	2.04 (2)				
Histidine					
Arginine					
Aspartic acid		3.20 (3)	1.87 (2)	1.98 (2)	1.17 (1)
Threonine		1.89 (2)	0.72 (1)	0.96 (1)	0.94 (1)
Serine		1.91 (2)		0.92 (1)	0.92 (1)
Glutamic acid		2.01 (2)	2.20 (2)	2.06 (2)	
Proline		0.91 (1)		0.91 (1)	
Glycine		1.15 (1)	1.18 (1)	1.15 (1)	
Alanine	1.00 (1)	1.02 (1)			1.05 (1)
Valine					

TABLE VII (*Continued*)

	C-11	C-12	C-12a	C-12b	C-12c
Isoleucine		0.98 (1)			1.00 (1)
Leucine		1.00 (1)			1.11 (1)
Tyrosine		0.86 (1)	0.80 (1)	0.95 (1)	
Phenylalanine	0.96 (1)	0.97 (1)		0.99 (1)	
Tryptophan					
Homoserine and lactone		0.84 (1)			0.75 (1)
Total	4	17	7	10	7
% yield	20	20	11	25	40
Purification process[b]		DX1	DX1	DX1	DX1
Fraction no.	XIII	I-2	II-4	I-3	I-1

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Abbreviations used are: DX1, Dowex 1-X2; E, preparative high-voltage electrophoresis.

TABLE VIII: Characterization of Peptides from Fraction Cp I.

Fraction Cp I-1:
Peptide C-12c:
Sequence: Thr-Ala-Ile-Leu-Asx-(Ser, HSer)
HVE, pH 6.5: Neutral and acidic (two spots)

Fraction Cp I-2:
Peptide C-12:
Sequence: (Thr, Glx, Asx, Tyr, Gly, Glx, Asx, Pro, Ser, Phe, Thr, Ala, Ile, Leu, Asx, Ser, HSer)

Fraction Cp I-3:
Peptide C-12b:
Sequence: (Thr, Glx, Asx, Tyr, Gly, Glx, Asx, Pro, Ser, Phe)

Edman degradation were sufficient to structure this peptide. High-voltage electrophoresis at pH 6.5 indicated that this peptide was neutral, *i.e.*, the Glx residue was present as glutamic acid.

Fraction Cp IX. This fraction also contained a single pure peptide, C-3a, which was completely structured by Edman degradation. These data are summarized in Table XIV.

Fractions Cp X and Cp XI. Each of these fractions contained a single dipeptide, as described in Table XIV, which was sequenced by a single Edman degradation.

Fraction Cp XII. This fraction contained a single peptide, C-9, as described in Table XV. The initial two residues were identified by Edman degradation while the last two residues were released by carboxypeptidase A. The assignment of glutamic acid to the third residue from the CPA data was confirmed by high-voltage electrophoresis.

Fraction Cp XIII. A single peptide, C-11, was obtained from the last column fraction. Amino-terminal alanine was identified from Edman degradation and carboxyl-terminal phenylalanine from carboxypeptidase A digestion.

In view of the greater heterogeneity accompanying proteolysis by chymotrypsin, it was not possible to sum the chymotryptic compositions to produce the exact composition of F_{III}. However, as described below, when these peptides are aligned in the correct sequence, virtually all of the fragment is accounted for, in keeping with the observation that there was very little acid-insoluble material removed from the whole digest.

Alignment of Tryptic and Chymotryptic Peptides. The tentative alignment of the tryptic and chymotryptic peptides is shown in Figure 9. The residues have been numbered relative to this fragment and it should be noted that these numbers do not correspond to the residue numbers of native carboxypeptidase, since F_{III} occupies an internal position in the order of the cyanogen bromide fragments (Nomoto *et al.*, 1969). In each line, the tryptic peptides are positioned above and the chymotryptic peptides below the sequence.

Peptide T-1 was tentatively assigned to the amino-terminal position since it was the only tryptic peptide isolated which contained amino-terminal aspartic acid (or asparagine). The first seven residues were aligned by Edman data of this peptide and chymotryptic peptides derived from it. Peptide C-1 was positioned at the amino terminus on the basis of its composition, which was identical with that derived from T-1 by

TABLE IX: Characterization of Peptides from Fraction Cp II.

Fraction Cp II-1:
Peptide C-10b:
Sequence: Ile(Thr, Glx, Ala)
HVE, pH 6.5: Neutral

Fraction Cp II-2:
Peptide C-10:
Sequence: Ile-Thr-Glx-Ala-Thr(Gly, Val)-Trp
Carboxypeptidase A: 60 min; Trp, 0.71
HVE, pH 6.5: Neutral

Fraction Cp II-3:
Peptide C-10a:
Sequence: Ile(Thr, Glx, Ala, Thr, Gly, Val)

Fraction Cp II-4:
Peptide C-12a:
Sequence: Thr-Glx-Asx-Tyr-Gly-Glx-Asx

TABLE XI: Characterization of Peptides from Fraction Cp IV.

Fraction Cp IV-1:
Preparative HVE:
Peptide C-2a:
Sequence: Val-Ala-Glx-His-Pro(Glx, Leu, Val, Ser)
Peptide C-7a:
Sequence: Ser-Thr(Gly, Gly, Ser)

Fraction Cp IV-2:
Peptide C-2b:
Sequence: Val(Ala, Gln)(His, Pro, Glx)Leu-Val
Hydrazinolysis: Val, 0.73
Carboxypeptidase A: 30 min; Val, 0.80; Leu, 0.80
Leucineaminopeptidase A: 1 hr; Val, 1.00; Ala, 0.33; Gln, 0.33
HVE, pH 6.5: Neutral

Fraction Cp IV-3:
Peptide C-1:
Sequence: (Asp, Leu, Leu)
HVE, pH 6.5: Acidic

chymotryptic digestion. Chymotryptic peptides C-2, C-2a, and C-2b were readily identified as containing the same sequence as T-1 C-2 and were thus used to position the remaining residues in T-1. The sequence of residues 9-12 was deduced in the following manner. Carboxypeptidase A digestion of C-2 indicated that Leu, Val, and Ser occupied positions 10–12, but the data were insufficient to distinguish the order. By difference from peptide C-2a, the Ser was assigned to position 12. In addition, carboxypeptidase A digestion of C-2b confirmed that Leu and Val were in positions 10 and 11, but again these data could not distinguish the order. Hydrazinolysis identified Val as the carboxyl-terminal residue of C-2b, thus identifying Val as occupying position 11, and, by difference, Leu occupying position 10. By subtracting the assigned residues of peptide C-2a from both ends, glutamic acid was assigned to position 9. The side-chain identification was by electrophoresis.

Peptide C-2 extends T-1 by one residue, placing Leu in position 14. Only peptide T-2 contained amino-terminal Leu and was, consequently, aligned adjacent to T-1. Since one residue represents a minimal overlap, this assignment must be considered tentative. T-2 was completely structured and allowed a firm basis for aligning peptides C-3, its tryptic subpeptides, and C-3a. These peptides supply a clear overlap of Ser-Tyr for the next tryptic peptide, T-3.

Peptide T-3, which covers residues 19–29, was structured only through the first three residues. However, subdigestion with chymotrypsin gave two peptides, one of which, T-3 C-1, could be used to align peptide C-4. The complete structure of this peptide extended the sequence through residue 26. Residues 27–29 were deduced from the subpeptide, T-3 C-2, which possessed amino-terminal Val, and from carboxypeptidase B data on T-3.

The only overlap peptide available from the chymotryptic digest to align T-3 and T-4 was the dipeptide C-6. This overlap is insufficient since a second Lys-Phe sequence occurs at resi-

TABLE X: Characterization of Peptides from Fraction Cp III.

Fraction Cp III-1:
Peptide C-3:
Sequence: (Glx, Ile, Gly, Arg, Ser)Tyr
Carboxypeptidase A: 6 hr; Tyr, 0.6
Tryptic Subdigest:
Peptide C-3 T-1:
Sequence: (Glx, Ile, Gly)Arg
Carboxypeptidase B: 30 min; Arg, 0.81
Peptide C-3 T-2:
Sequence: Ser-Tyr

TABLE XII: Characterization of Peptides from Fractions Cp V and Cp VI.

Fraction Cp V-1:
Peptide C-5:
Sequence: (Val, Leu)

Fraction Cp V-3:
Peptide C-10c:
Sequence: Ala-Thr-Gly-Val

Fraction Cp VI:
Peptide C-8:
Sequence: Ile-Asp-Leu-Gly-Ile-His
Leucineaminopeptidase: 3 hr; Ile, 0.48; Asp, 0.21; Leu, 0.21; Gly, 0.19
HVE, pH 6.5: Neutral

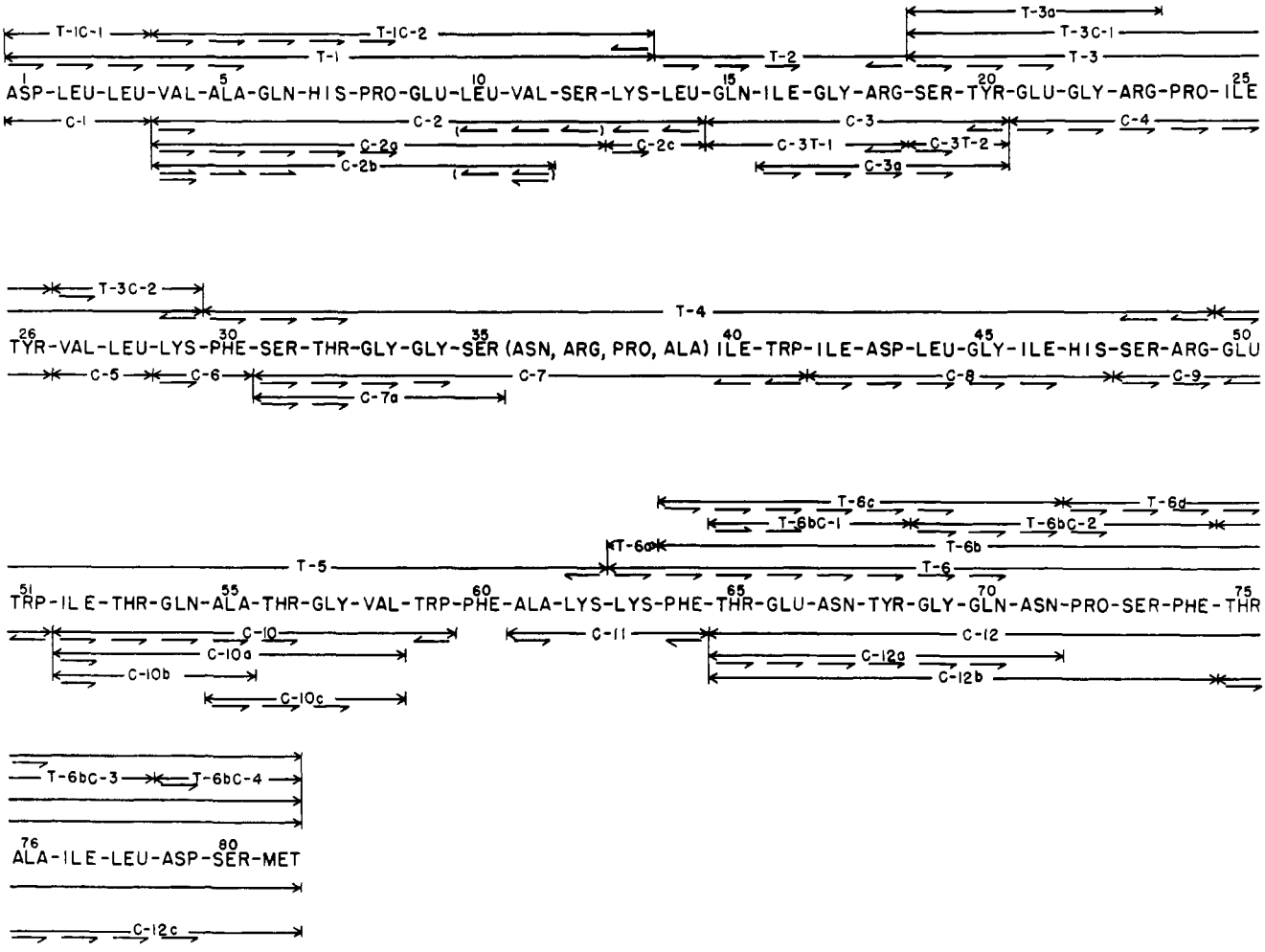

FIGURE 9: Tentative alignment of the tryptic and chymotryptic peptides of fragment F_{III} of bovine carboxypeptidase A. The tryptic peptides are indicated by double-headed arrows above the residues, while the chymotryptic peptides are listed in the same manner below the residues. The small single-headed arrows refer to assignments by Edman degradation (→), hydrazinolysis (←), carboxypeptiase A and B hydrolysis (←), and leucineaminopeptidase hydrolysis (→). Residue numbers are for this fragment and do not correspond to residue numbers in the whole protein.

TABLE XIII: Characterization of Peptides from Fraction Cp VII.

Fraction Cp VII-E-1:	
Peptide C-2:	
Sequence: Val(Ala, Glx, His, Pro, Glx)(Leu, Val, Ser)Lys-Leu	
Carboxypeptidase A: 1 hr; Leu, 1.00	
Carboxypeptidase A and B: 1 hr; Leu, 1.42; Lys, 0.96; Ser, 0.94; Val, 0.81	
Fraction Cp VII-E-2:	
Peptide C-7:	
Sequence: Ser-Thr-Gly-Gly(Ser, Asx, Arg, Pro, Ala)Ile-Trp	
Carboxypeptidase A: 30 min; Trp, 1.02; Ile, 0.54	

dues 63–64 and, hence, absolute assignment of C-6 is impossible. It can be argued that the peptide T-6 (T-6a) can be aligned in this position since it has amino-terminal phenylalanine also. However, since this peptide has been completely structured (see Figure 3) and can be aligned at the carboxy terminus, the assignment of T-6 (T-6a) to residue 30 and following is untenable. Furthermore, all the remaining overlaps are rigorous and therefore, by an inductive process, it is only

TABLE XIV: Characterization of Peptides from Fractions Cp VIII, IX, X, and XI.

Fraction Cp VIII:	
Peptide C-4:	
Sequence:	Glx-Gly-Arg-Pro-Ile-Tyr
HVE, pH 6.5:	Neutral
Fraction Cp IX:	
Peptide C-3a:	
Sequence:	Ile-Gly-Arg-Ser-Tyr
Fraction Cp X:	
Peptide C-2c:	
Sequence:	Lys-Leu
Fraction Cp XI:	
Peptide C-6:	
Sequence:	Lys-Phe

TABLE XV: Characterization of Peptides from Fractions Cp XII and XIII.

Fraction Cp XII:	
Peptide C-9:	
Sequence:	Ser-Arg-Glu-Trp
Carboxypeptidase A:	6 hr; Trp, 1.00; Glu, 0.37
HVE, pH 6.5:	Neutral
Fraction Cp XIII:	
Peptide C-11:	
Sequence:	Ala-Lys-Lys-Phe
Carboxypeptidase A:	30 min; Phe, 0.85

possible to align the peptides in this manner. Definitive proof that this assignment is indeed correct has been obtained (Bradshaw, 1969).

Only limited data on the structure of T-4 were obtained, but considerable additional evidence was supplied by the chymotryptic peptides. However, a four-residue segment (residues 36–39) was not ordered from these peptides. This segment has been deduced from data on the thermolytic peptides of F_{III} (Bradshaw, 1969).

The alignment of T-4 and T-5 is supplied by C-9. The remainder of the structure of T-5 was deduced from peptides C-10, C-10a, C-10b, and C-10c. The assignment of phenylalanine to residue 60 is by difference only and is somewhat tentative. Further proof of structure for this segment has been obtained (Bradshaw, 1969).

The alignment of T-5 with T-6, by use of C-11, is facilitated by the knowledge that the other two lysyl residues, at positions 13 and 29, cannot be fit to C-11. The structure of T-6 has been amply documented by the large number of peptides obtained from this region. The Ile-Leu sequence, not obtained from the tryptic peptides (Figure 3), was readily deduced from C-12c.

Discussion

The tentative structure of fragment F_{III}, shown in Figure 9, accounts exactly for the amino acid composition of F_{III} obtained from acid hydrolysates. Those areas of uncertainty which have already been indicated (*vide supra*) have been resolved and are reported in the accompanying communication (Bradshaw, 1969). However, two other areas of the sequence deserve comment. First, position 21 is now considered to be glutamic acid. Earlier reports on the structure listed residue 21 as glutamine (Neurath *et al.*, 1969a,b) based on preliminary observations that indicated peptide C-4 possessed a pyrrolidinecarboxylic acid end group.[4] Subsequent isolations have consistently indicated that this residue was present as glutamic acid. Consequently this residue has been assigned to the acid form with the appropriate qualifications. The earlier reports also indicated that the side-chain nature of residues 66 and 67 was unknown. This uncertainty has now been resolved by leucineaminopeptidase digestion, and residue 66 has been assigned as glutamic acid and residue 67 as asparagine.

The pattern of cleavage of trypsin and chymotrypsin is much along the lines expected for these enzymes (Hill, 1965). However, some anomalies should be noted. Trypsin, in addition to the hydrolysis of lysyl and arginyl bonds, showed a limited tendency to hydrolyze bonds 23–24 and 71–72. The first site represents an Arg-Pro cleavage, classically considered inert to attack by trypsin. Some evidence, not reported here,[3] for hydrolysis of the Arg-Pro bond at residues 37–38 (the structure of which is documented elsewhere (Bradshaw, 1969) has also been obtained. The other abnormal cleavage, at residues 71–72, involves an Asn-Pro linkage. This bond was also found to be susceptible to chymotrypsin. Significantly, the bond appears to be labile only in intact F_{III} as chymotryptic hydrolysis of T-6b did not show this cleavage. It may be presumed that some structural feature present in F_{III} even after apparent denaturation is responsible for the facile hydrolysis of this bond.

Acknowledgment

The authors are indebted to Mrs. Brita Moody, Mrs. Milagros Santarin, and Mrs. Rosa Mae MacDonald for their assistance with the sequence analyses and to Mr. C. V. Shih for his assistance in preparing fragment F_{III}. Mr. Richard R. Granberg and Mr. John Richardson performed the amino acid analyses.

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The Amino Acid Sequence of Bovine Carboxypeptidase A. III. Specificity of Peptide-Bond Cleavage by Thermolysin and the Complete Sequence of the Cyanogen Bromide Fragment F_{III}*

Ralph A. Bradshaw†

ABSTRACT: The 81-residue fragment (F_{III}) obtained from bovine carboxypeptidase A after cleavage with cyanogen bromide has been digested by thermolysin and the resulting peptides were isolated. Three of the peptides have been completely structured by Edman degradations and by digestion with carboxypeptidase A and B. These peptides supply the remaining information to produce a rigorous proof for the primary structure of the fragment. The remaining peptides from the thermolytic digest have been assigned in the structure, on the basis of composition, to produce a profile for thermolytic hydrolysis of this substrate; 17 of the 21 bonds hydrolyzed were of the X–Leu, X–Ile, X–Phe, and X–Val type, confirming previous reports of the specificity of this enzyme against other protein substrates. Secondary cleavages of the type X–Trp and X–Ala were also noted. Only a small percentage of the bonds hydrolyzed by thermolysin were also cleaved by trypsin and chymotrypsin. These results suggest that thermolysin is an excellent enzyme for use in sequence analyses.

In the preceding communication (Bradshaw *et al.*, 1969a) a tentative primary structure for fragment F_{III} of bovine carboxypeptidase A was reported. This structure, which was deduced from the tryptic and chymotryptic peptides, contained a few areas of uncertainty which could not be resolved with the peptides derived from these two digests.

Consequently, a third digest of F_{III}, utilizing the thermolytic protease, thermolysin (Endo, 1962), has been prepared. The choice of this enzyme was dictated by two reasons. First, limited reports on the specificity of this enzyme (Matsubara *et al.*, 1966; Matsubara, 1966; Ohta and Ogura, 1965; Ambler and Meadway, 1968) suggested that a rather different set of peptides than those obtained by either tryptic or chrymotryptic cleavage would be obtained. These observations have been corroborated by digestion of the other large cyanogen bromide fragment of carboxypeptidase A, F_I. In fact, the singular success obtained with this enzyme in digesting F_I was of key importance in elucidating this structure.[1] Second, the rapidly accumulating evidence (Matsubara and Sasaki, 1968; Ambler and Meadway, 1969; Blow *et al.*, 1969) that this enzyme is of marked value in sequence analysis prompted a detailed examination of the sensitive bonds in F_{III} in order to characterize the specificity of this enzyme toward large peptide substrates.

Since only a limited amount of information was necessary to complete the sequence of F_{III}, only those peptides actually employed to resolve the ambiguities in the tentative structure (Bradshaw *et al.*, 1969a) have been characterized. The remaining peptides have been placed by composition to yield the nature of the cleavage sites.

Experimental Procedure

Materials. Carboxypeptidase A (Anson) was obtained from Worthington Biochemicals as a twice-crystallized suspension and was used without further purification. Fragment F_{III} was prepared as described previously (Nomoto *et al.*, 1969).

Thermolysin was obtained as a crystalline preparation from Daiwa Kasei K. K., Osaka, Japan.

All other materials were the same as described previously (Bradshaw *et al.*, 1969a).

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received May 14, 1969.* This work was supported by the National Institutes of Health (GM 04617 and GM 15731-01), the American Cancer Society (P-79K), the Office of Naval Research (NONR 477-35), and the National Science Foundation (GB 4990).

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