Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Neurath, H., Bradshaw, R. A., Ericsson, L. H., Babin, D. R., Petra, P. H., and Walsh, K. A. (1969a), *Brookhaven Symp. Biol. 21*, 1.
Neurath, H., Bradshaw, R. A., Petra, P. H., and Walsh, K. A. (1969b), *Proc. Roy. Soc. Ser. B (London)* (in press).
Nomoto, M., Srinivasin, N. G., Bradshaw, R. A., Wade, R. D., and Neurath, H. (1969), *Biochemistry 8*, 2755.
Sampath Kumar, K. S. V., Clegg, J. B., and Walsh, K. A. (1964), *Biochemistry 3*, 1728.
Shearer, W. T., Bradshaw, R. A., Gurd, F. R. N., and Peters, T., Jr. (1967), *J. Biol. Chem. 242*, 5451.

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).

† Supported by U. S. Public Health Service Postdoctoral Fellowship 1-F2-GM-23, 968-02. Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo. 63110.

[1] R. A. Bradshaw, K. A. Walsh, and H. Neurath, in preparation.

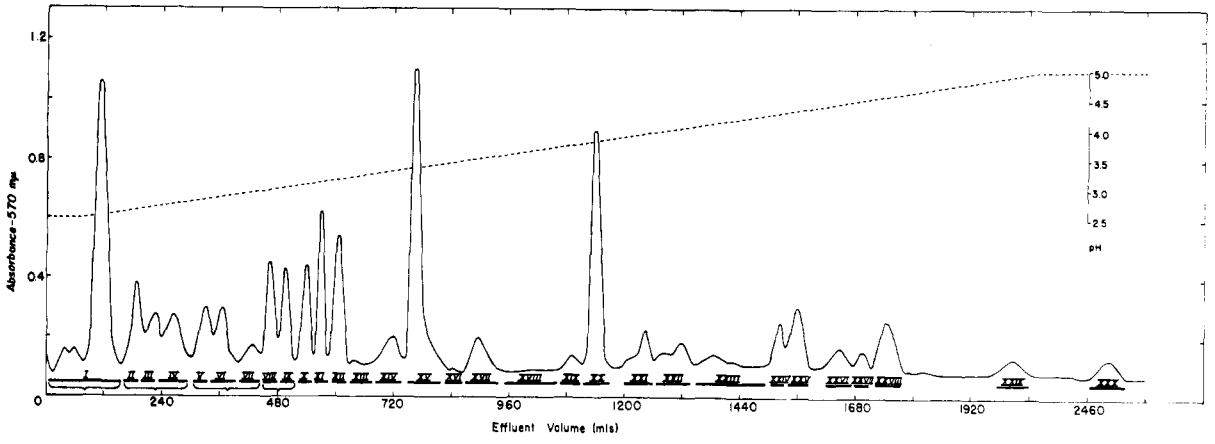

FIGURE 1: Elution profile of the soluble thermolytic peptides of fragment F_{III} of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine acetate buffers as described in the text. Fractions of 6.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis. Fractions were pooled according to the solid bars. Those pools marked with brackets were pooled and subfractionated on Dowex 1-X2. The pH gradient is indicated by the dashed line.

Methods. Thermolytic digestion was performed in a pH-Stat at pH 8.0. Lyophilized F_{III} was suspended in water to a concentration of 1–2% and dissolved by the addition of 1 N NaOH. After all the protein was dissolved, the solution was readjusted to pH 8.0. Thermolysin (0.5 mg/ml in 0.001 M calcium acetate) was added to a final concentration of 0.5% (w/w) and digestion was allowed to proceed for 2 hr at 37°. At this time, a second aliquot of enzyme was added and digestion was continued for 1 more hr. The reaction was terminated by the addition of 6 N HCl to pH 2.0. Only traces of insoluble material were formed, which were removed by centrifugation.

The supernatant was fractionated on a 2.0 × 25 cm column of Dowex 50-X8 using linear gradients of pyridine acetate. Subfractionations were carried out on Dowex 1-X2 and Dowex 50-X2. Details of these procedures have been described previously (Bradshaw *et al.*, 1969b).

All other methods employed have been reported (Bradshaw *et al.*, 1969a).

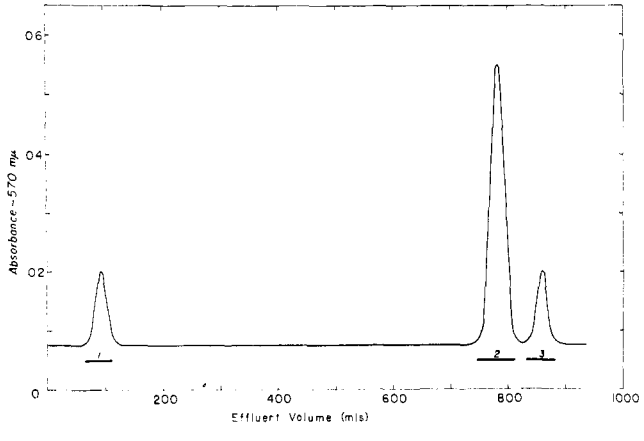

FIGURE 2: Elution profile of fraction Th I on a 0.9 × 150 cm column of Dowex 1-X2 at 35°. The column was developed at 30 ml/hr with a gradient of pyridine acetate buffers as described in the text. Fractions of 2.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis. Fractions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

Results and Discussion

The fractionation of the soluble thermolytic peptides on Dowex 50-X8 is shown in Figure 1. A cursory examination of this elution profile indicates that thermolysin produces a more complex mixture of peptides than the tryptic digest but of the same degree of fragmentation caused by chymotrypsin acting on the same material. The initial nine fractions were combined to form four pools (indicated by brackets), and each was fractionated on Dowex 1-X2. The elution profiles of fractions I, II-III-IV, and VIII-IX are shown in Figures 2–4. The subfractionation of fraction VIII-IX-1 on Dowex 50-X2 is shown in Figure 5. The purification of fraction V-VI-VII yielded a single peptide containing carboxyl-terminal homoserine which may account for the apparent heterogeneity on the Dowex 50-X8 column. Fraction X was inexplicably found to be devoid of material when examined by electrophoresis and amino acid analysis. The remaining fractions were found to contain a single peptide or were devoid of sufficient material to warrant further purification.

In accord with previous procedures (Bradshaw *et al.*, 1969a), the column pools have been designated with Roman numerals

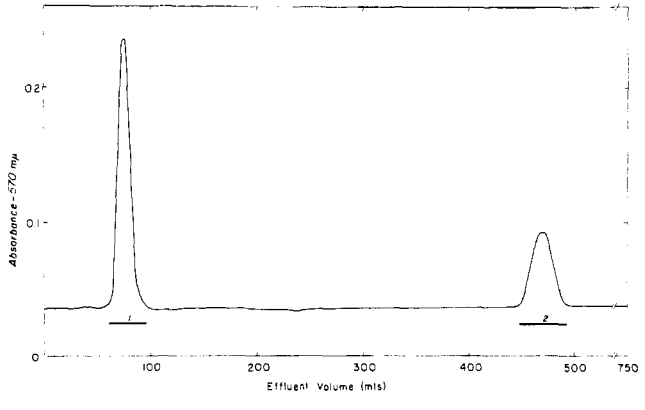

FIGURE 3: Elution profile of fractions Th II, III, and IV on a 0.9 × 150 cm column of Dowex 1-X2. Details as described in Figure 2.

TABLE I: Characterization of the Peptides in Fractions Th XV, Th XXV, and Th XXX.

Fraction Th XV:
Peptide Th 8:
Sequence: Phe-Thr-Ser-Gly-Gly-Ser-Asx-Arg-Pro-Ala

Fraction Th XXV:
Peptide Th 7a:
Sequence: Leu-Lys-Phe
Carboxypeptidase A: 30 min; Phe, 0.54

Fraction Th XXX:
Peptide Th 13:
Sequence: Phe-Ala-Lys-Lys
Carboxypeptidase B: 30 min; Lys, 2.02

and the pure peptides are numbered (Arabic) in the order that they occur in the sequence. The treatment of the three peptides required to complete the sequence, along with the identification by composition of the remaining peptides, is given below.

Analysis of Fractions Th XV, Th XXV, and Th XXX.[2] Three fractions, each containing a single peptide, were examined in detail in order to obtain further sequence data on F_{III}. The description of these data is given in Table I. Fraction Th XV contained a single peptide, Th 8, of ten residues. Nine rounds of Edman degradation[3] were completed, yielding the complete structure. Fraction Th XXV contained a tripeptide with the structure Leu-Lys-Phe. The structure was ascertained by a single Edman degradation and by digestion with carboxypeptidase A. The final fraction, Th XXX, contained a tetrapeptide whose structure was deduced by a single Edman degradation and by digestion with carboxypeptidase B.

The incorporation of each of these peptides into the tentative sequence of F_{III} (Figure 6), proposed from the alignment of the tryptic and chymotryptic peptides, removes the final areas of ambiguity. Peptide Th 8 can be positioned at residues 30–39. The sequence data obtained order the residues from 36 to 39, thus completing this segment of the fragment.

Peptide Th 7a supplies a more definitive overlap at residues 29–30 for aligning T-3 and T-4 (Bradshaw *et al.*, 1969a). The extension of C-6 by one additional residue allows for a more absolute assignment for the relative positions of T-3 and T-4 not possible with the dipeptide C-6 alone.

Finally, peptide Th 13 confirms the asignment of residue 60 as phenylalanine by extending peptide C-11 one additional residue, thus completing the void created by the fact that C-10 and C-11 did not form a continuum in this portion of the molecule.

Identification of the Thermolytic Peptides of F_{III}. The amino acid composition of each of the thermolytic peptides of fragment F_{III} is given in Table II. The total number of residues, the per cent yield, the method of purification, and the fraction number are given at the foot of each column. Although only compositional data—with the three exceptions described above—were employed to incorporate these peptides into the known structure, no major ambiguities were encountered. This observation serves to support the proposed structure of the fragment. The complete array of thermolytic peptides in the order they occur in the F_{III} sequence is shown in Figure 6. Only two residues at positions 44 and 45 were not accounted for by the isolated peptides. Interestingly, only a small amount of heterogeneity of cleavage, resulting in more than one peptide from the same region, was encountered. This observation alone suggests that thermolysin is a very useful enzyme for sequence analyses.

Analysis of the Specificity of Thermolysin. It has been established, using both protein and synthetic substrates (Matsubara *et al.*, 1966; Matsubara, 1966; Ambler and Meadway, 1968; Ohta and Ogura, 1965; Morihara and Tsuzuki, 1966),

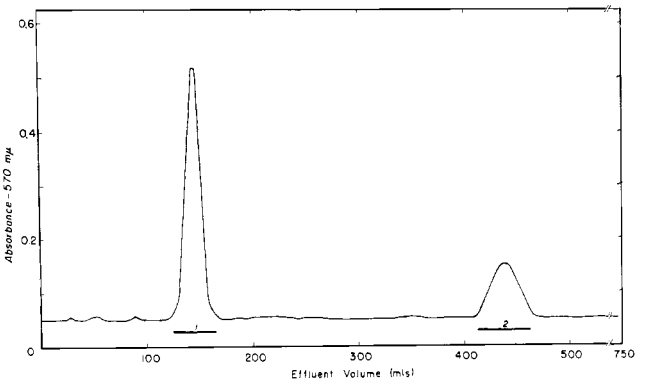

FIGURE 4: Elution profile of fraction Th VIII-IX on a 0.9 × 150 cm column of Doxex 1-X2. Details as described in Figure 2.

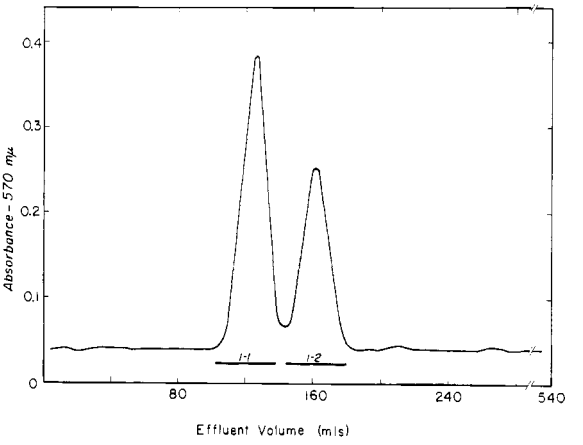

FIGURE 5: Elution profile of fraction Th VII-IX-1 on a 0.9 × 61 cm column of Dowex 50-X2 at 55°. The column was developed at 30 ml/hr with a linear gradient composed of 250 ml of 0.05 M pyridine acetate (pH 2.5) and 250 ml of 0.5 M pyridine acetate (pH 3.75). Fractions of 2.0 ml were collected and monitored by ninhydrin analysis after alkaline hydrolysis. Fractions were pooled according to the solid bars,

[2] Abbreviation used is: Th-, thermolytic fraction or peptide.

[3] Edman degradations are indicated by arrows (→) without analytical data. A description of the criteria for such data has been summarized previously (Bradshaw *et al.*, 1969a).

TABLE II: Amino Acid Composition of the Thermolytic Peptides of Fragment F_{III} of Bovine Carboxypeptidase A.[a]

Amino Acid	Th-1	Th-2	Th-3	Th-3a	Th-4	Th-5	Th-6	Th-7	Th-7a
Lysine			1.00 (1)	0.94 (1)				0.94 (1)	1.00 (1)
Histidine		0.95 (1)							
Arginine						1.99 (2)			
Aspartic acid	1.03 (1)								
Threonine									
Serine			1.05 (1)	1.06 (1)		0.95 (1)			
Glutamic acid		2.18 (2)			1.01 (1)	1.06 (1)			
Proline		0.95 (1)				0.98 (1)			
Glycine						2.06 (2)			
Alanine		1.07 (1)							
Valine		1.03 (1)	1.00 (1)	0.74 (1)			1.14 (1)		
Isoleucine						1.05 (1)	1.01 (1)		
Leucine	0.98 (1)	1.25 (1)	0.95 (1)		0.99 (1)			1.06 (1)	1.06 (1)
Tyrosine						0.96 (1)	0.85 (1)		
Phenylalanine									0.98 (1)
Tryptophan									
Homoserine and lactone									
Total	2	7	4	3	2	9	3	2	3
% yield	54	59	80	15	72	79	51	39	25
Purification process[b]	DX1				DX1, DX50X2				
Fraction no.	VIII-IX-2	XII	XX	XXI	VIII-IX-1-2	XXVIII	XIV	XXIV	XXV

	Th-8	Th-9	Th-10	Th-10a	Th-11	Th-11a	Th-12	Th-13	Th-14
Lysine								2.15 (2)	
Histidine			0.81 (1)	0.90 (1)					
Arginine	0.91 (1)		1.00 (1)	1.10 (1)					
Aspartic acid	1.19 (1)	0.98 (1)							2.01 (2)
Threonine	1.08 (1)				2.16 (2)	1.00 (1)			0.96 (1)
Serine	2.00 (2)		0.98 (1)	0.91 (1)					0.94 (1)
Glutamic acid			1.05 (1)	1.00 (1)	0.97 (1)				1.79 (2)
Proline	1.01 (1)								1.11 (1)
Glycine	2.36 (2)				1.09 (1)	0.97 (1)			1.01 (1)
Alanine	1.26 (1)				1.01 (1)	1.02 (1)		0.91 (1)	
Valine							1.00 (1)		
Isoleucine		2.00 (2)	0.91 (1)	0.96 (1)	0.92 (1)				
Leucine									
Tyrosine									0.77 (1)
Phenylalanine	0.83 (1)							1.09 (1)	0.98 (1)
Tryptophan		0.56 (1)	0.61 (1)				0.92 (1)		
Homoserine and lactone									
Total	10	4	6	5	6	3	2	4	10
% yield	66	7	12	20	13	36	60	53	33
Purification process[b]						DX1, DX50X2			DX1
Fraction no.	XV	XVII	XXIX	XXVI	II, III, IV-1	VIII-IX-1-1	XXIII	XXX	I-2

	Th-14a	Th-14b	Th-15	Th-16
Lysine				
Histidine				
Arginine				
Aspartic acid	1.97 (2)			1.20 (1)
Threonine	0.97 (1)		0.98 (1)	

TABLE II (*Continued*)

	Th-14a	Th-14b	Th-15	Th-16
Serine		1.05 (1)		1.05 (1)
Glutamic acid	1.81 (2)			
Proline		0.95 (1)		
Glycine	1.02 (1)			
Alanine			1.08 (1)	
Valine				
Isoleucine				0.94 (1)
Leucine				1.00 (1)
Tyrosine	0.68 (1)		1.00 (1)	
Phenylalanine	0.71 (1)			
Tryptophan				
Homoserine and lactone				0.99 (1)
Total	8	2	3	5
% yield	12	25	75	76
Purification process[b]	DX1	DX1	DX1	DX1
Fraction no.	I-3	I-1	XI	II, III, IV-2 (V, VI, VII-1)

[a] Values are given in residues per mole of peptide. The assumed integral values are given in parentheses. [b] Abbreviations used are: DX1, Dowex 1-X2; DX50X2, Dowex 50-X2.

TABLE III: Sites of Cleavage in F_{III} by Thermolysin in Comparison with the Action of Trypsin and Chymotrypsin against the Same Bond.[a]

		Thermolysin		Trypsin		Chymotrypsin	
Bond Type	Total	Major	Minor[b]	Major	Minor[b]	Major	Minor[b]
X–Leu	7	4	1	1	0	0	0
X–Ile	7	5	1	0	0	1	1
X–Phe	4	4	0	2	0	1	0
X–Val	4	1	1	0	0	0	0
X–Tyr	3	0	0	0	0	0	0
X–Trp	3	1	0	0	0	0	0
X–Ala	5	1	0	0	0	0	1
Other	47						
Th susceptible		2	0	0	1	1	1
Tp + Cp susceptible		0	0	3	1	12	3
Total	80	18	3	6	2	15	6
		21		8		21	

[a] The major and minor cleavages by trypsin and chymotrypsin of each bond type are restricted to those bonds also cleaved by thermolysin. Bonds of the X–Leu, X–Ile, X–Phe, X–Val, X–Tyr, X–Trp, and X–Ala type that were hydrolyzed by trypsin or chymotrypsin that were not cleaved by thermolysin are included in the last line of the table. [b] Minor cleavages are arbitrarily defined as being less than 20%, based on the recovery of the contributing peptides.

that thermolysin has the general specificity of hydrolyzing peptide bonds on the amino side of hydrophobic residues. In order to extend these observations to other protein substrates, an analysis of the sites of cleavage with the cyanogen bromide fragment F_{III} of bovine carboxypeptidase A has been made. The isolated thermolytic peptides are shown in Figure 6 along with the sites of cleavage of the same substrate by trypsin and chymotrypsin (Bradshaw *et al.*, 1969a). The downward arrows indicate the bonds susceptible to trypsin and the upward arrows those susceptible to chymotrypsin. The dashed arrows indi-

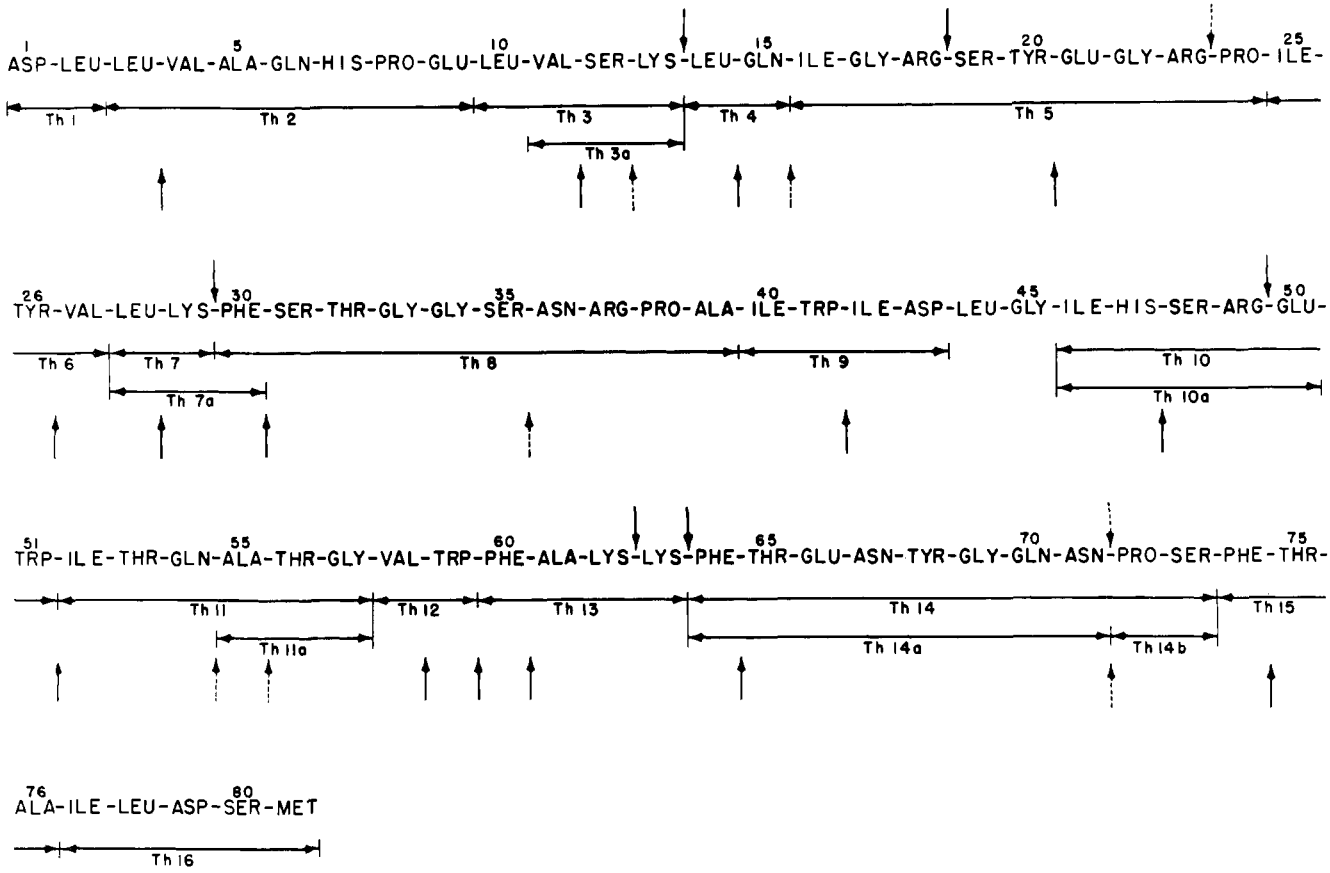

FIGURE 6: Alignment of the thermolytic peptides and the complete sequence of fragment F_{III} of bovine carboxypeptidase A. The thermolytic peptides are indicated by double-headed arrows. The sites of tyrptic (↓) and chrymotryptic (↑) cleavages are indicated by vertical arrows. Minor cleavages are indicated in the same fashion by dashed arrows.

cate the minor cleavages. Clearly, there is only a partial overlap, with the majority of the thermolytic sites being unique to this agent. A summary of these data is given in Table III. In each case, a major unique bond, *e.g.*, X–Leu, has been considered in terms of the number of such examples cleaved by thermolysin *vs.* the number present. The cleavage sites have been considered in terms of major and minor (less than 20%) yields. As can be seen, 3 of the 8 bonds hydrolyzed by trypsin were also susceptible to thermolysin, as were 6 of 21 bonds cleaved by chymotrypsin. In all, 9 of 21 bonds (43%) hydrolyzed by thermolysin were also cleaved by trypsin or chymotrypsin. It should be noted that this comparison has only limited general significance since substrates which contain large numbers of Lys–Y and Arg–Y bonds, where Y is Leu, Ile, or Phe, would be expected to be cleaved at these sites by both trypsin and thermolysin. A similar consideration of bonds of the Phe–Y, Tyr–Y, etc., type holds for chymotrypsin. Nonetheless, if F_{III} can be taken to be generally indicative of the distribution of hydrophobic and basic residues, a considerable number of bonds should exist which are cleaved by thermolysin that are not susceptible to trypsin or chymotrypsin.

Table III also reveals a very decided profile of the specificity of thermolysin. Among the first three types of bonds listed, *i.e.*, X–Leu, X–Ile, and X–Phe, 15 of the 18 present were hydrolyzed. One of the X–Leu bonds is adjacent to the amino terminus and as such is inert to attack (Matsubara, 1966), thus making the total available bonds only 17. In addition, two of the four X–Val bonds were cleaved. If these are added to the total, 17 of 22 (77%) of this type were cleaved. Interestingly only four other bonds were hydrolyzed.[4] Clearly, the specificity of thermolysin is overwhelmingly directed toward bonds of the type X–Leu, X–Ile, X–Phe, and X–Val.

One of the other bonds cleaved, Asn-Pro, is interesting in that both trypsin and chymotrypsin were observed to act at the same site. It is apparent that this bond is highly susceptible in whole F_{III} and its tendency for hydrolysis is not related to the specificity of the attacking enzyme.

These observations are supported by the data of other investigators. Digestion of *Pseudomonas fluorescens* azurin (Ambler and Meadway, 1968) with thermolysin produced 26 major and moderate cleavages at sites involving X–Leu, X–Ile, C–Phe, X–Val, X–Tyr, and X–Ala bonds as compared with 38 such bonds that were available; 24 out of 30 of these sites were of the X–Leu, X–Ile, X–Phe, and X–Val variety. Matsubara *et al.* (1969) have examined the action of thermolysin on

[4] In view of the fact that several minor yield peptides were not purified from the F_{III} digest, there may well exist examples of many minor cleavages of other bond types. However, for the most part, it may be concluded that these bonds represent a secondary specificity of the enzyme which can be greatly minimized by controlled digest conditions. Ambler and Meadway (1968) have demonstrated the effect of limited digestion on azurin with results that indicate that digestion can be virtually confined to bonds of the X–Leu, X–Ile, X–Phe, and X–Val type.

tobacco mosaic virus protein and the oxidized A and B chains of insulin. In each case, the profile of cleaved bonds was very similar although the extent of hydrolysis seemed to be somewhat lower. With tobacco mosaic virus protein, 15 of the 17 cleavages reported were of the X–Leu, X–Ile, X–Phe, and X–Val type. However, 43 such sites existed in the protein, indicating that only 35% of these bonds was hydrolyzed, compared with 80% cleavage in azurin and 77% cleavage in F_{III}. These results probably reflect more limited hydrolysis conditions rather than a greatly decreased number of susceptible bonds in tobacco mosaic virus protein. The results of these workers with the insulin chains again support these conclusions, although the greatly restricted number of cleavage sites present in these smaller substrates makes interpretation more difficult.

The present results also indicate that the nature of residue contributing the carboxyl group to the peptide bond in question is relatively unimportant. Acidic, basic, and hydrophobic amino acids, including proline, were found in this position in the hydrolyzed bonds.

It should be noted that no information concerning X–Met bonds was obtained in these studies because of the absence of this amino acid in F_{III}. The results of Ambler and Meadway (1968) do indicate a relatively high percentage of cleavages for bonds of this type in the hydrolysis of azurin.

Thus the observations described in this communication as well as those of other investigators suggest that thermolysin possesses a high degree of specificity of the order of magnitude exhibited by chymotrypsin against protein substrates (Hill, 1965). In addition, its somewhat novel specificity clearly indicates that this enzyme should be of great value in sequence studies. It has been suggested (Ambler and Meadway, 1968) that thermolysin digests of large proteins are likely to be too complex to be worth fractionating. However, in view of the fact that its specificity is clearly of the same degree as chymotrypsin, this conclusion would seem to be unfounded. Data indicating that thermolysin digests of large proteins are, in fact, amenable to separation have been obtained from digests of the 198-residue fragment, F_1, of carboxypeptidase A. In this case, the mixture has been readily fractionated to produce a virtually complete complement of peptides. In contrast, digests of the same fragment by trypsin, chymotrypsin, and pepsin yielded only partial sets of peptides. These data will be reported in an ensuing communication.[1]

Acknowledgment

The author thanks Dr. Hans Neurath for his advice and encouragement during this work and for supplying the facilities in which it was done. Dr. Kenneth A. Walsh provided many helpful discussions,

The author would also like to thank Mrs. Lynne Maston and Mrs. Brita Moody for their help with the peptide isolations and Mr. C. V. Shih for his assistance in preparing the fragment F_{III}. Mr. Richard R. Granberg and Mr. Thomas A. Oas provided invaluable assistance in carrying out the amino acid analyses.

References

Ambler, R. P., and Meadway, R. J. (1968), *Biochem. J. 108*, 893.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature 221*, 337.

Bradshaw, R. A., Babin, D. R., Nomoto, M., Srinivasin, N. G., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969a), *Biochemistry 8*, 3859.

Bradshaw, R. A., Garner, W. H., and Gurd, F. R. N. (1969b), *J. Biol. Chem. 244*, 2149.

Endo, S. (1962), *J. Fermentation Tech. 40*, 346.

Hill, R. L. (1965), *Advan. Protein Chem. 20*, 37.

Matsubara, H. (1966), *Biochem. Biophys. Res. Commun. 24*, 427.

Matsubara, H., and Sasaki, R. (1968), *J. Biol. Chem. 243*, 1732.

Matsubara, H., Sasaki, R., Singer, A., and Jukes, T. H. (1966), *Arch. Biochem. Biophys. 115*, 324.

Morihara, K., and Tsuzuki, H. (1966), *Biochim. Biophys. Acta 118*, 215.

Nomoto, M., Srinivasin, N. G., Bradshaw, R. A., Wade, R. D., and Neurath, H. (1969), *Biochemistry 8*, 2755.

Ohta, Y., and Ogura, Y. (1965), *J. Biochem. (Tokyo) 58*, 607.