Chemical Relationships Among Various Forms of Bovine Pancreatic Carboxypeptidase A*

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Carboxypeptidase A_α and carboxypeptidase A_γ, which differ in their N-terminal amino acid residues and in their amino acid composition, were compared by analyzing the chymotryptic digests of the oxidized protein. The peptide patterns revealed the presence of an additional peptide in carboxypeptidase A_α. This peptide, which is derived from the N-terminal region of the parent protein, has the amino acid sequence H.Ala.Arg.Ser.Thr.AspN.OH. and accounts for the difference in amino acid composition between these two proteins. A scheme depicting the origin of the various chemical species of carboxypeptidase A from a common precursor is presented.

Carboxypeptidase A of bovine pancreas has been shown to occur in more than one chemical form which differ in their N-terminal groups and in their amino acid compositions (Bargetzi *et al.*, 1963, 1964). The original method of preparing the enzyme (Anson, 1937) from autolyzing tissues of the gland yields a protein, designated as carboxypeptidase A_γ[1], with the sequence AspN.Tyr.Ala.... at the N-terminal region of this molecule (Thompson, 1953; Coombs and Omote, 1962; Vallee *et al.*, 1963). A second method of preparation has been described in which the enzyme was isolated from extracts of acetone powders of the pancreas after activation (Allan *et al.*, 1964). This enzyme (carboxypeptidase A_δ) also has an N-terminal asparagine (Vallee *et al.*, 1963). A third form of carboxypeptidase A (A_α) has been obtained in higher yields by a third method of preparation (Cox *et al.*, 1964) in which the activation was carried out after initial purification of procarboxypeptidase A. This enzyme has been shown to contain N-terminal alanine (Bargetzi *et al.*, 1964) and to contain five or more amino acid residues in addition to those found in the other two preparations (Bargetzi *et al.*, 1963). In order to clarify the relations among these preparations of carboxypeptidase A and their formation from a common precursor, an attempt was made in the present work to define the exact chemical differences among these preparations in the amino terminal regions of the proteins. The present investigation has revealed that differences in amino acid composition between carboxypeptidase A_α and A_γ could be explained by the existence of an additional pentapeptide sequence at the N-terminus of carboxypeptidase A_α.

Materials and Methods

Enzymes.—Carboxypeptidase A_γ, prepared according to the method of Anson (1937), was purchased from Worthington Biochemical Corporation. Dinitrophenylation of the thrice-crystallized protein yielded DNP-aspartic acid on acid hydrolysis.[2] Carboxypeptidase A_α, prepared according to the method of Cox *et al.* (1964), was crystallized three times and its N-terminal group was found to be alanine. In both the preparations a small but variable amount of DNP-serine was always present (Bargetzi *et al.*, 1964).

Bovine trypsin and α-chymotrypsin were crystalline products obtained from the Worthington Biochemical Corporation.

Performic Acid Oxidation.—The protein was dried and treated with performic acid according to Hirs (1956). After 9 hours at 0°, the protein solution was treated with ether to remove the reagents and to precipitate the oxidized protein (Bettelheim, 1955). The protein was washed with ether, dissolved in formic acid, and reprecipitated with ether. This procedure was repeated twice. The protein precipitate was then washed with water, washed twice with 0.25 M ammonium acetate buffer, *p*H 8.0, and finally suspended in a suitable volume of the same buffer.

Chymotryptic Digestion.—The oxidized protein, finely suspended in ammonium acetate buffer (2 mg/ml) was treated with α-chymotrypsin (enzyme-substrate weight ratio of 1:50) at 37° for 24 hours. The digest was lyophilized three times to eliminate the volatile buffer, dried *in vacuo* over H_2SO_4, and dissolved in a minimal amount of water.

Peptide Mapping.—Digests corresponding to 4 mg of the original protein were spotted on Whatman No. 3 paper. Electrophoresis was first carried out in pyridine-acetate buffer, *p*H 6.5, with a potential difference of 35 v/cm for 60 minutes. An amino acid mixture was run at the same time as a marker which could be separately stained with ninhydrin to indicate the position of the neutral components. The area on the paper containing the neutral peptides was cut off and sewn onto a new sheet of paper, and electrophoresis was repeated at *p*H 2.1 in pyridine-formate buffer. The paper area comprising these neutral peptides was then sewn onto another Whatman No. 3 sheet and subjected to descending chromatography in the second dimension for 20 hours using *n*-butanol–acetic acid–water (3:1:1) as the solvent. The basic and the acidic peptides from electrophoresis at *p*H 6.5 were also separated in the second dimension by chromatography in the latter solvent. After the papers were dried the chromatograms were developed with ninhydrin reagent.

Analytical Methods.—Amino acid compositions were determined in acid hydrolysates prepared in 6 N HCl *in vacuo* at 105° overnight. Amino acids were separated by the method of Richmond and Hartley (1959) and quantitated by the method of Tigane *et al.* (1961). Conventional methods of end-group analysis and selective hydrolysis by enzymes were used for the determination of amino acid sequences. N-terminal amino acids were identified with FDNB reagent. The DNP-amino acids in the ether extracts of acid hydrolysates

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[1] The nomenclature of the various chemical species of carboxypeptidase A has been described by Bargetzi *et al.* (1963).

[2] The following abbreviations are used: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, dinitrophenyl-.

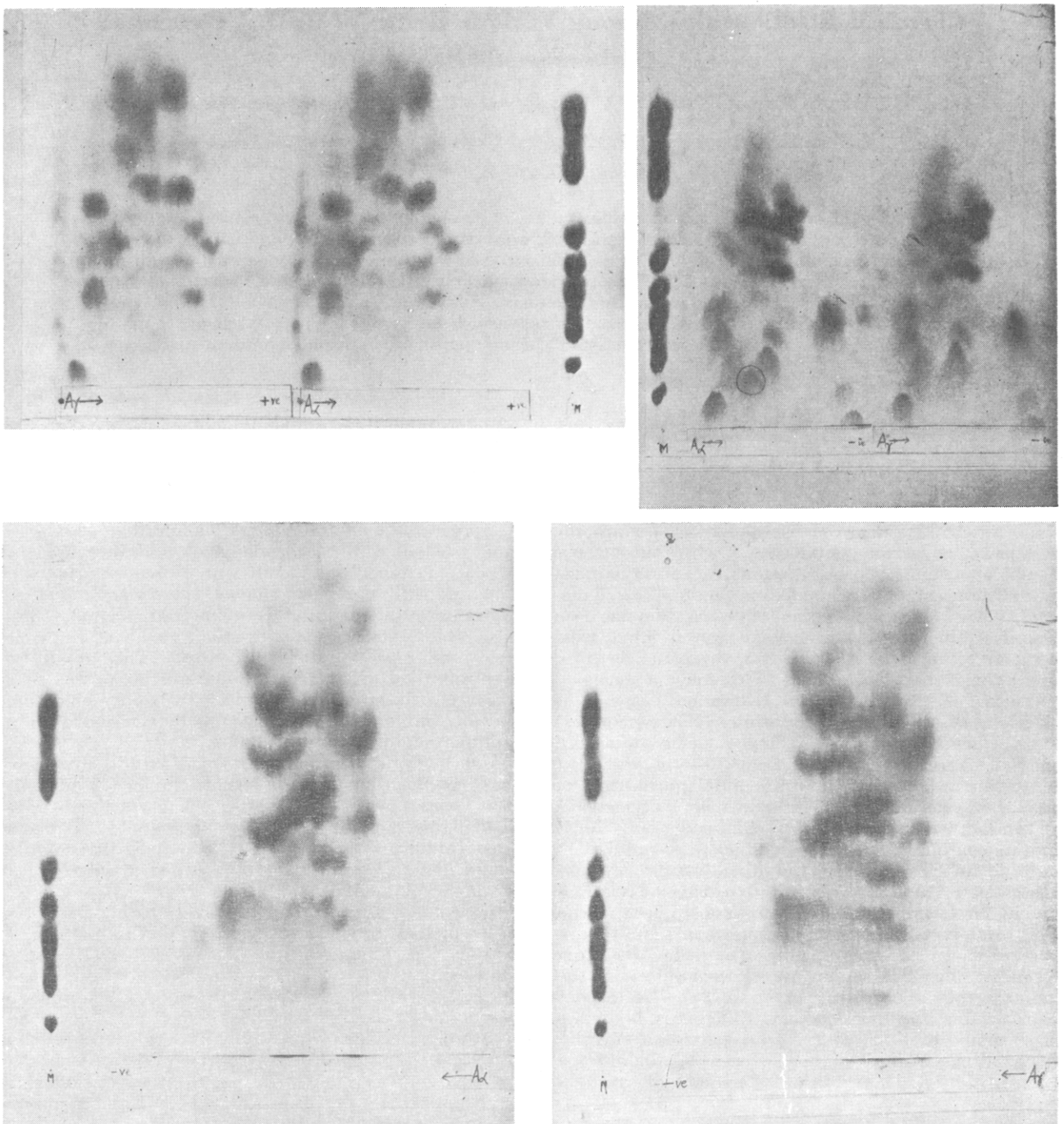

FIG. 1.—Comparison of chymotryptic digests of oxidized carboxypeptidase A_α and A_γ as described in the text. Top left: the acidic peptides. Top right: the basic peptides. Bottom left and right: the neutral peptides. The circled spot in top right is the "difference peptide." M-Amino acid mixture.

were first identified by two-dimensional chromatography in *tert*-amyl alcohol–ammonia and phosphate buffer (Fraenkel-Conrat *et al.*, 1955) and confirmed by quantitative analysis of the amino acids in the aqueous phase. The C-terminal amino acid was determined by digestion with carboxypeptidase A_γ in 0.1 M ammonium acetate buffer, *p*H 8.5. The liberated amino acid was identified on a Spinco amino acid analyzer.

RESULTS

Isolation of Difference Peptide.—A typical peptide map of the two forms of carboxypeptidase is shown in Figure 1. The peptide distribution is similar in both cases except for the presence of an additional spot in A_α which is moving towards the cathode at *p*H 6.5. This spot, designated as the "difference peptide," gave a positive Sakaguchi reaction (Jepson and Smith, 1953), indicating the presence of arginine in the peptide.

The difference peptide was isolated in large amounts for sequence determination by the following procedure: A chymotryptic digest of 200 mg of oxidized carboxypeptidase A_α was applied as 12-cm bands on eight sheets of Whatman No. 3 paper and subjected to electrophoresis at *p*H 6.5 for 60 minutes with a potential difference of 35 v/cm. Digests of 4 mg of oxidized A_α and A_γ were spotted alongside on each sheet as markers. The markers were chromatographed in the

TABLE I

AMINO ACID COMPOSITION OF DIFFERENCE PEPTIDE AND OF ITS TRYPTIC PEPTIDES

Amino Acid	Difference Peptide	Tryptic Peptides DPT_1	DPT_2
Alanine	0.9	1.0	
Arginine	1.1	1.0	
Serine	0.9		1.2
Threonine	0.8		0.9
Aspartic acid	1.1		0.9

butanol–acetic acid–water (3:1:1) system and the position of the difference peptide was located. The areas corresponding to the mobility of the difference peptide in electrophoresis were cut out, sewn onto Whatman No. 3 sheets, and subjected to descending chromatography as before. Since the mobility of the difference peptide is very low in this system, the chromatograms were irrigated with the same solvent for a second time after drying. The area corresponding to the difference peptide was cut out, and electrophoresis was once again carried out at *p*H 6.5 for 100 minutes to eliminate any contaminating peptides. The peptide was eluted with water and dried.

Structure of Difference Peptide.—The amino acid composition of the difference peptide was examined on a hydrolysate of a portion of the eluate; the results are given in Table I. The composition corresponded in integral numbers to one residue each of alanine, arginine, serine, threonine, and aspartic acid. From the cationic mobility of the peptide at *p*H 6.5 it was inferred that the difference peptide contained asparagine rather than aspartic acid. Dinitrophenylation of the peptide yielded DNP-alanine only. Digestion of 0.4 μmole of the pentapeptide with 200 μg of trypsin in 0.2 M ammonium acetate buffer, *p*H 8.0, for 1 hour at room temperature, yielded two peptides, DPT_1 and DPT_2, which were separated by high-voltage electrophoresis at *p*H 2.1. The compositions of the two peptides were for DPT_1 alanine and arginine, and for DPT_2 serine, threonine, and asparagine (Table I). Dinitrophenylation of these two peptides yielded DNP-alanine and DNP-serine, respectively. The presence of serine as the N-terminal residue of DPT_2 was also confirmed by the absence of serine in the aqueous phase of the acid hydrolysate after ether extraction. Digestion of DPT_2 with carboxypeptidase A_γ for 60 minutes at room temperature yielded only asparagine when examined in the Spinco amino acid analyzer. From these results the sequence of the difference peptide was established as H.Ala.Arg.Ser.Thr.AspN.OH. Table II summarizes the evidence for the sequence of the peptide.

Determination of the Penultimate Residues at the N-Terminal Region of the Protein.—The N-terminal amino acid residue was selectively removed by Edman degradation essentially according to the method of Konigsberg and Hill (1962), and the protein was subsequently dinitrophenylated to determine the second residue. Twenty mg of the crystals of the protein was suspended in 4.0 ml of *N*-ethylmorpholine buffer. One hundred μl of freshly distilled phenylisothiocyanate was added to the suspension and the contents were incubated at 37° with occasional shaking. At the end of 4 hours the suspension was extracted with ether twice; the protein was removed by centrifugation, washed with alcohol, then twice with acetone, and finally with ether before drying the sample *in vacuo*. The protein was dissolved in 4.0 ml of anhydrous trifluoroacetic acid and incubated at room temperature for 2 hours. It was then precipitated with ether, centrifuged, washed with acetone twice, and finally washed with water. The protein was suspended in 1% sodium bicarbonate and the dinitrophenylation was carried out in the usual way.

TABLE II

SUMMARY OF EVIDENCE FOR SEQUENCE OF DIFFERENCE PEPTIDE

Composition	Ala,Arg,Ser,Thr,AspN	
Dinitrophenylation	Ala.(Arg,Ser,Thr,AspN)	
Tryptic digestion	(Ala,Arg) and	(Ser,Thr,AspN)
Dinitrophenylation of the two tryptic peptides	Ala.Arg	Ser.(Thr,AspN)
Carboxypeptidase A_γ digestion of the tripeptide		(Ser,Thr).AspN.
Sequence	Ala.Arg.Ser.Thr.AspN	

TABLE III

AMINO ACIDS AT THE N-TERMINAL PORTIONS OF CARBOXYPEPTIDASES A_α AND A_γ

Protein	Treatment	Amino Acid Found
Carboxypeptidase A_α	Dinitrophenylation of the native protein	DNP-alanine
	Dinitrophenylation after alanine is cleaved by Edman degradation	DNP-arginine
Carboxypeptidase A_γ	Dinitrophenylation of the native protein	DNP-aspartic acid
	Dinitrophenylation after asparagine is cleaved by Edman degradation	di-DNP-tyrosine

The ether-soluble DNP-amino acids were identified by chromatography as described. For the determination of DNP-arginine in the aqueous phase, the following procedure was adopted. The hydrolysate, after extraction with ether, was dried *in vacuo* over NaOH. It was then dissolved in a minimum amount of water, and the free amino groups were acetylated. The acetylation was carried out at *p*H 8.0 in a *p*H-stat by adding freshly distilled acetic anhydride in amounts of 5–10 μl and titrating with 1 N NaOH until the base uptake decreased to the level caused by the slow hydrolysis of the anhydride. The acetylated sample was then acidified to 1 N in HCl and extracted with ether four times to remove the acylated derivatives. DNP-arginine was extracted from the aqueous phase with *n*-butanol, and this extract was dried. It was then spotted on Whatman No. 1 paper, and electrophoresis was carried out in a Spinco Model R paper electrophoresis apparatus in 0.02 M sodium borate containing 2% formaldehyde[3], at 400 v for 7 hours. Markers of synthetic α-DNP-arginine and ε-DNP-lysine were run at the same time. Under the conditions employed, DNP-arginine moved 2 cm from the origin towards the cathode, and ε-DNP-lysine moved 5 cm from the origin towards the anode.

The results obtained with carboxypeptidase A_α and A_γ are shown in Table III. The data indicate that arginine follows alanine at the N-terminal region of carboxypeptidase A_α, while tyrosine is the penultimate amino acid at the N-terminal region of carboxypeptidase A_γ.

[3] We are indebted to Dr. E. O. P. Thompson for suggesting the buffer system for separating DNP-arginine.

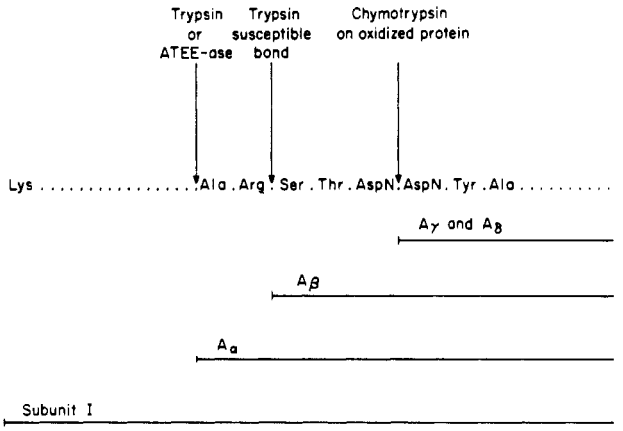

FIG. 2.—A schematic representation of the origin of various forms of carboxypeptidase A from their common precursor. ATEEase = acetyltyrosine-ethylesterase.

DISCUSSION

Carboxypeptidase A has been isolated by a variety of procedures, and the enzymes so obtained have been shown to vary in their amino acid composition and N-terminal residues (Bargetzi *et al.*, 1963, 1964). The present investigation indicates that two of these proteins, viz., carboxypeptidases A_α and A_γ, having the same enzymatic activity and isolated from the same source by different procedures, differ by the existence of an additional pentapeptide in carboxypeptidase A_α. The following observations indicate that the pentapeptide must have been derived from the N-terminal region of the molecule: (1) Amino acid analysis indicated that carboxypeptidase A_α was at least five residues larger than A_γ (Bargetzi *et al.*, 1963), and the additional amino acids were those found in the difference peptide. (2) The two proteins have different N-terminal sequences, Ala.Arg.. in the case of A_α and AspN.Tyr.... in the case of A_γ; and therefore differences in peptides deriving from the N-terminal region of the proteins should be anticipated. (3) Only one difference was seen on comparing peptide maps of the two proteins. This "difference peptide" occurred only in digests of carboxypeptidase A_α and had the structure Ala.Arg.Ser.Thr.AspN. The N-terminal Ala.Arg sequence is identical to that of the protein (A_α) whence it was derived. (4) The similarity of the C-terminal amino acids of the two proteins (Bargetzi *et al.*, 1964) precludes the possibility of the difference peptide originating from the C-terminal region of the molecule. (5) Recent experiments (Sampath Kumar *et al.*, 1964) have indicated that carboxypeptidase A_α can be converted to other active forms, and the conversions are reflected in the new forms having predictably different N-terminal residues.

The existence of more than one chemical species of carboxypeptidase A raises the question as to the mechanism of their formation. While carboxypeptidase A_γ is prepared from autolyzing tissues of the pancreas and as such might represent a limit product of an extensive degradation of the proenzyme by the various proteolytic enzymes existing in such crude extracts, carboxypeptidase A_α has been isolated only after the initial purification of procarboxypeptidase and its subsequent activation with trypsin. In the latter case, the enzyme must result from cleavage either by trypsin or by the transient endopeptidase (acetyltyrosine ethylesterase) that is also formed on activation, or from the action of both trypsin and endopeptidase (Neurath, 1964). These observations suggest that it might be possible to convert carboxypeptidase A_α to A_γ by an enzymic mechanism, and raise the question whether other as yet unidentified forms of carboxypeptidase A exist or can be produced by altering the conditions of activation either in crude extracts or in the purified state of the precursor. It may be of interest to mention here that Folk and Schirmer (1963) have also pointed out the existence of three forms of carboxypeptidase A in porcine pancreas.

Brown *et al.* (1963) have recently established that bovine pancreatic procarboxypeptidase A is composed of three subunits, and that one of the subunits (I), with a molecular weight of about 35,000, contains lysine as the N-terminal amino acid residue and is the immediate precursor of carboxypeptidase A. The formation and relationship among various forms of carboxypeptidase A from this common precursor are schematically represented in Figure 2 based on the observations of Brown *et al.* (1963) and on known N-terminal sequences in carboxypeptidase A_α and A_γ.

The scheme, in its present form, envisages not only the origin of the various forms of carboxypeptidase A, but significantly points out that the formation of any one species might involve the rupture of only one bond to form the active enzyme in close analogy with the mechanism of activation of trypsinogen or chymotrypsinogen (Neurath, 1957, 1964). The scheme predicts that yet another species, carboxypeptidase A_β with N-terminal serine, might be formed by the tryptic hydrolysis of the arginyl-serine bond. In this regard, it is significant that dinitrophenylation of some preparations of the various forms of carboxypeptidase A has yielded variable amounts of DNP-serine. It is not immediately apparent why the bond between arginine and serine is not readily split by trypsin during activation of procarboxypeptidase A. That the bond between the two asparagines might be susceptible to chymotrypsin is suggested by the finding that the difference peptide was isolated from chymotryptic digestion of the oxidized protein.

Carboxypeptidase A_γ and A_δ differ in their solubility and in the ability of the apoenzymes to reactivate; but they possess the same N-terminal amino acid, asparagine. The identity of the N-terminal asparagines in the amino acid sequence of these two proteins has yet to be established.

The chemical relationships among various forms of carboxypeptidase A as depicted in the scheme can be unequivocally confirmed by transformation of carboxypeptidase A_α into other enzyme species. Studies on such interconversions are in progress and will be the subject matter of a future communication.

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