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Bovine Pancreatic Procarboxypeptidase B. II. Mechanism of Activation*

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Received August 20, 1962

The present report extends a preceding investigation of the isolation, characterization, and activation of bovine pancreatic procarboxypeptidase B (Wintersberger *et al.*, 1962). The amino acid composition of the zymogen and of crystalline carboxypeptidase B derived from it is given in detail. The amino terminal residue of carboxypeptidase B has been identified as threonine. The initial stage of activation of procarboxypeptidase B has been examined, and evidence has been obtained that the appearance of activity coincides with the hydrolysis of a single arginyl-threonine linkage in the zymogen. Preliminary data indicate that crystalline bovine carboxypeptidase B, like carboxypeptidase A and porcine carboxypeptidase B, contains approximately one gram atom of zinc per mole (molecular weight 34,000).

In the accompanying paper (Wintersberger *et al.*, 1962), we have reported the isolation of procarboxypeptidase B from acetone powder of bovine pancreas. The protein was found to be homogeneous according to chromatography, sedimentation analysis, electrophoresis, and potential enzymatic activity, and upon activation gave rise to carboxypeptidase B which has been isolated in crystalline form. The active enzyme hydrolyzed substrates containing both aromatic and basic carboxyl-terminal residues, and evidence was presented to suggest that this dual specificity is mediated by the same or overlapping active sites.

The present investigation extends the characterization of bovine pancreatic carboxypeptidase B and its zymogen in terms of amino acid composition and amino-terminal groups. In addition, further details are given of the initial stage of the trypsin-catalyzed reaction which results in the conversion of the zymogen to the active enzyme.

MATERIALS AND METHODS[1]

Bovine procarboxypeptidase B was isolated from acetone powders of beef pancreas and *carboxypeptidase B* was prepared from the purified proenzyme by the methods described in the accompanying paper (Wintersberger *et al.*, 1962).

Carboxypeptidase B activity was determined by the assay for HPLA outlined in the accompanying paper (Wintersberger *et al.*, 1962). Procarboxypeptidase B was estimated from the maximum activity of carboxypeptidase B reached during tryptic activation.

Protein concentrations were measured spectrophotometrically at 280 mμ. Extinction coefficients for procarboxypeptidase B and for carboxypeptidase B were determined by the method of Walsh and Brown (1962). The values obtained for $E^{1\%}_{280}$ were 16 for procarboxypeptidase B and 21 for carboxypeptidase B. These values are considered accurate to within ±0.5.

Amino acid analyses were performed with a Beckman/Spinco Model 120 automatic amino acid analyzer by the method of Spackman *et al.* (1958). Hydrolysis of 5-mg samples was carried out in evacuated, sealed ignition tubes at 105°, in constant-boiling HCl. Each hydrolyzed sample was dried and dissolved in 4.8 ml of 0.2 M citrate buffer. Following the suggestion of Walsh and Brown (1962), 0.2 ml of a standard solution containing thienylalanine and aminoguanidopropionic acid, each at a concentration of 0.01 M, was added to allow correction for unequal recoveries from the long and short columns of the analyzer. Aliquots of 2 ml were taken for analysis on each column. Half-cystine was determined as cysteic acid after oxidation of 5–8 mg samples with performic acid for 11 hours at 0° (Hirs, 1956). Excess performic acid was removed com-

* Presented in part at the 46th Annual Meeting of the American Society of Biological Chemists, April, 1962, Atlantic City, N. J. (Cox *et al.*, 1962). This work has been supported by the National Institutes of Health (RG-4617) and by the American Cancer Society (P-79).

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[1] The following abbreviations are used: FDNP = 1-fluoro-2,4-dinitrobenzene; DNP = dinitrophenyl; Tris = tris(hydroxymethyl)aminomethane; HPLA = hippuryl-*dl*-phenyllactic acid.

pletely after oxidation by adding 25 to 30 ml of water to the reaction mixture and lyophilizing the sample. The dilution and drying procedure was repeated once before the sample was taken for hydrolysis.

Tryptophan was determined from the tyrosine-tryptophan ratio measured by the spectrophotometric method of Beaven and Holiday (1952) with a Beckman Model DK-1 recording spectrophotometer.

Total sulfur analysis was performed on a 17-mg sample of carefully washed and dried crystalline carboxypeptidase B by the Schöniger combustion method (Alicino, 1958).

Dinitrophenylation of the proteins was carried out in aqueous medium and the reaction was followed by continuous titration at *p*H 8.0 according to the method of Levy *et al.* (1955). Samples of 10–20 mg were taken for each reaction with FDNB. The DNP-protein was hydrolyzed in 2–3 ml of constant-boiling HCl in a sealed, evacuated ignition tube for 16 hours at 105°. Ether-soluble DNP-amino acids were separated by two-dimensional paper chromatography, with *t*-amyl alcohol saturated with 3% ammonia used in the first dimension and 1.5 M potassium phosphate buffer, *p*H 6.0, in the second. The spots of DNP-amino acids were cut out for quantitative determination (Fraenkel-Conrat *et al.*, 1955). After ether extraction, the aqueous phase of the acid hydrolysate was examined for the presence of DNP-arginine and of di-DNP-histidine (Biserte *et al.*, 1960). DNP-cysteic acid present in the aqueous phase of the acid hydrolysate of samples of oxidized procarboxypeptidase B was separated from ε-DNP-lysine by paper electrophoresis in 0.1 M pyridine formate buffer, *p*H 2.1, at 40 v cm for 50 minutes.

Results and Discussion

Amino Acid Composition.—The amino acid compositions of procarboxypeptidase B and carboxypeptidase B are summarized in Table I. The data have been normalized to the molecular weights—34,000 for carboxypeptidase B and 57,400 for procarboxypeptidase B—determined by sedimentation equilibrium (Wintersberger *et al.*, 1962).

The reproducibility of the preparation and of the analytical procedure is demonstrated by the data of Table II, which presents the amino acid

Table I

Amino-Acid Compositions of Procarboxypeptidase B and Carboxypeptidase B

	Procarboxypeptidase B			Carboxypeptidase B		
Amino Acid Residue	Amino Acid[a] Residues per 57,400 g	Nearest Integral Number of Amino Acid Residues	Integral Number of Residues × Respective Residue Molecular Weight	Amino Acid[b] Residues per 34,000 g	Nearest Integral Number of Amino Acid Residues	Integral Number of Residues × Respective Residue Molecular Weight
Aspartic acid	46.9	47	5409.7	25.9	26	2992.6
Threonine	37.3	37	3741.4	25.8	26	2629.1
Serine	44.4	44	3832.4	25.8	26	2264.6
Glutamic acid	47.3	47	6069.1	23.6	24	3099.1
Proline	19.4	19	1845.5	12.1	12	1165.6
Glycine	33.7	34	1940.4	21.4	21	1255.5
Alanine	32.8	33	2346.3	21.7	22	1564.2
½ Cystine	9.2	9	919.4	6.8	7	715.1
Valine	34.4	34	3371.1	13.7	14	1388.1
Methionine	9.6	10	1312.1	6.0	6	787.3
Isoleucine	22.4	22	2489.7	15.7	16	1810.7
Leucine	36.0	36	4074.1	20.2	20	2263.4
Tyrosine	27.2	27	4406.1	21.8	22	3590.2
Phenylalanine	21.9	22	3238.2	11.8	12	1766.3
Lysine	27.1	27	3461.1	16.7	17	2179.2
Histidine	16.9	17	2331.7	7.0	7	960.1
Arginine	25.5	26	4061.2	13.1	13	2030.6
Tryptophan	14.4	14	2607.1	9.9	10	1862.2
Amide N	(45.7)	(46)	(−46.0)	(22.9)	(23)	(−23.0)
Totals[c]	—	505	57,429	—	301	34,384

[a] Average of four duplicate pairs of samples hydrolyzed for 24, 48, 72, and 96 hours. [b] Average of columns I and II of Table II. Values for threonine and serine were obtained by extrapolation to zero time hydrolysis. Maximum values are given for valine and isoleucine; these maxima were reached after 72 hours of hydrolysis. Half-cystine was determined as cysteic acid after performic acid oxidation of separate samples. Tryptophan was determined spectrophotometrically on unhydrolyzed samples. [c] Calculated molecular weights have been corrected by the addition of one mole of water to account for the terminal residues of the chain and, in the case of carboxypeptidase B, for the presence of one atom of zinc.

compositions of two samples of carboxypeptidase B derived from two different preparations of procarboxypeptidase B. Sample I had been crystallized twice, sample II three times. Each column is a summary of multiple analyses, as noted in the legend to Table II. Single amino acid analyses were done on 24-hour hydrolysates of several other preparations of carboxypeptidase B, and they all agreed with the complete determinations given in Table II, indicating that the preparative procedure was entirely reproducible according to this criterion. The values for preparations I and II are in good agreement, with the exception of glutamic acid, for which a difference of one residue is observed.

Total sulfur analysis gave a value of 12.9 gram atoms of sulfur per mole of carboxypeptidase B, assuming a molecular weight of 34,000 for the protein. This result is in excellent agreement with the sulfur content expected from the analytical values for half-cystine (7) and methionine (6).

The amino acid composition of carboxypeptidase B differs from that of bovine carboxypeptidase A (Smith and Stockell, 1954; Bargetzi *et al.*, 1962) with respect to every amino acid, as shown in Table III. The two enzymes are strikingly different in their content of half-cystine and methionine; whereas carboxypeptidase B contains 7 half-cystine and 6 methionine residues, the corresponding values for carboxypeptidase A are 2 and 3. This difference is of special interest in the light of the significance of a single thiol of carboxypeptidase A in the catalytic activity of that enzyme (Vallee *et al.*, 1960; Walsh *et al.*, 1962). The amino acid composition of bovine carboxypeptidase B also differs substantially from that of the porcine carboxypeptidase B given by Folk *et al.* (1960) (Table III). The porcine enzyme, like bovine carboxypeptidase B, contains 13 atoms of sulfur, but these are distributed between 8 half-cystine and 5 methionine residues.

Metal Analysis.—Spectrographic analyses of a crystalline preparation of bovine carboxypeptidase B by methods previously described (Vallee and Neurath, 1955) yielded one gram atom of zinc per mole of enzyme, and no significant amount of any other metal.[2] In the light of this result and of the fact that the enzyme is inhibited by the metal-chelating agent 1,10-phenanthroline (Wintersberger *et al.*, 1962), it seems likely that bovine pancreatic carboxypeptidase B, in analogy with bovine carboxypeptidase A (Vallee and Neurath, 1955) and porcine carboxypeptidase B (Folk *et al.*, 1960), is a zinc metalloenzyme.

Amino-terminal Residues.—The amino-terminal residue of procarboxypeptidase B could not be conclusively identified. No ether-soluble α-DNP-amino acid was found in significant amount in hydrolysates of dinitrophenylated procarboxypeptidase B. Only traces of DNP-serine and

TABLE II

AMINO-ACID COMPOSITION OF BOVINE CARBOXYPEPTIDASE B

Column I is the average of four samples of preparation I hydrolyzed for 24, 48, 72, and 96 hours respectively. Column II is the average of three samples of preparation II hydrolyzed for 24, 48, and 112 hours respectively. The data for threonine, serine, valine, isoleucine, half-cystine, and tryptophan were obtained as noted in footnote *b* of Table I.

Amino Acid Residue	Amino Acid Residues per 34,000 g	
	I	II
Aspartic acid	25.9	25.9
Threonine	25.9	25.7
Serine	25.7	25.9
Glutamic acid	22.8	24.3
Proline	11.9	12.3
Glycine	21.4	21.4
Alanine	21.7	21.6
½ Cystine	6.8	6.8
Valine	13.9	13.5
Methionine	6.0	6.0
Isoleucine	15.9	15.5
Leucine	20.3	20.1
Tyrosine	22.0	21.6
Phenylalanine	11.9	11.7
Lysine	16.8	16.5
Histidine	7.0	7.0
Arginine	13.3	12.9
Tryptophan	10.0	9.8
Amide N	23.2	22.6

TABLE III

COMPARISON OF THE AMINO ACID COMPOSITIONS OF THREE CARBOXYPEPTIDASES

Amino Acid Residue	Bovine Carboxypeptidase B	Bovine Carboxypeptidase A[a]	Porcine Carboxypeptidase B[b]
Aspartic acid	26	28	32
Threonine	26	27	30
Serine	26	33	18
Glutamic acid	24	26	25
Proline	12	10	13
Glycine	21	23	23
Alanine	22	20	25
½ Cystine	7	2	8
Valine	14	16	11
Methionine	6	3	5
Isoleucine	16	20	17
Leucine	20	24	23
Tyrosine	22	19	20
Phenylalanine	12	16	12
Lysine	17	15	18
Histidine	7	8	6
Arginine	13	11	10
Tryptophan	10	8	9
Total	301	309	305

[a] Data of Bargetzi *et al.*, 1962. [b] Data of Folk *et al.*, 1960.

[2] Establishment of bovine carboxypeptidase B as a zinc metalloenzyme requires in addition metal analyses of the fractions attending isolation of the protein, as was described for bovine carboxypeptidase A (Vallee and Neurath, 1955). Such measurements are in progress and will be reported in detail elsewhere.

DNP-aspartic acid, accounting for less than 0.03 residues per mole, were obtained. The aqueous phase of the hydrolysate contained neither di-DNP-histidine nor DNP-arginine. When the protein was denatured prior to dinitrophenylation, the hydrolysate contained an ether-soluble, brownish-yellow substance; this substance behaved on chromatography like the product reported by Bettelheim (1955) to arise during dinitrophenylation and hydrolysis of chymotrypsinogen A, which contains N-terminal half-cystine. Dinitrophenylation of performic acid–oxidized procarboxypeptidase B yielded 0.1 residue of DNP-cysteic acid per mole of protein and 0.07 residues of DNP-serine. No DNP-cysteic acid was obtained after oxidation and hydrolysis of DNP-procarboxypeptidase B. The data indicate that procarboxypeptidase B may contain N-terminal half-cystine, but the evidence is not conclusive.

In contrast, the results of analogous experiments with bovine carboxypeptidase B seem clearcut. Dinitrophenylation and hydrolysis of carboxypeptidase B yielded 0.6 residues of DNP-threonine per mole of protein (without any correction for destruction during hydrolysis). The only other DNP-amino acid observed was a trace of DNP-aspartic acid (0.02 residues per mole). The amino-terminal residue in porcine carboxypeptidase A is also threonine (Folk *et al.*, 1961); that in bovine carboxypeptidase A is asparagine (Thompson, 1953; Coombs and Omote, 1962).

Further Studies on the Activation of Bovine Procarboxypeptidase B.—The accompanying paper (Wintersberger *et al.*, 1962) has described some features of the trypsin-catalyzed activation of procarboxypeptidase B. It was noted that the activation reaction appeared to proceed in two steps. The first step was complete in one minute at a trypsin-procarboxypeptidase mole ratio of 1:10 and yielded a material with 60–70% of maximal enzymic activity and a sedimentation coefficient identical with that of the proenzyme. The second stage proceeded much more slowly, full activity being reached after 2–3 hours. The product of the second stage of the reaction had a lower sedimentation coefficient than the proenzyme, and it was this product which was crystallized and characterized as carboxypeptidase B. A material similar in activity and sedimentation behavior to the "first-stage" product was also obtained after incubation for 2 hours at a trypsin-procarboxypeptidase mole ratio of 1:1000. The second stage of the reaction, leading to the development of full activity, did not occur at an appreciable rate under these conditions.

The extreme rapidity of the first step of the activation process suggested that the reaction might involve the cleavage by trypsin of only a few particularly susceptible bonds of the procarboxypeptidase molecule. It seemed of interest to test this possibility by attempting to identify the bonds involved. From the known specificity of trypsin (Hofmann and Bergmann, 1939), it might be expected that the reaction would result in the production of fragments containing C-terminal arginine or lysine. Since the product of the reaction has carboxypeptidase B activity, such C-terminal residues would be rapidly released as free amino acids (Folk, 1956). The general experimental procedure, then, was to follow the rapid first stage of the activation reaction by the development of enzymic activity, by the appearance of new N-terminal residues, and by the release of free lysine or arginine.

Procarboxypeptidase B (12 mg/ml in 0.1 M ammonium acetate, *p*H 7.5) was activated at 25° at a trypsin-procarboxypeptidase mole ratio of 1:100. After 10, 20, 50, and 90 seconds, 0.5-ml aliquots were removed and the activation reaction was stopped by rapid mixing with 0.1 ml of a solution containing a two-fold molar excess of soybean trypsin inhibitor over the trypsin in the aliquot. Carboxypeptidase B activity was measured with HPLA as substrate. Each aliquot was incubated at 25° for one additional hour after the addition of the trypsin inhibitor, to allow carboxypeptidase B to release any C-terminal basic amino acid formed by the action of trypsin.

In order to determine free amino acids, Dowex 50 x 4, 20 to 50 mesh, previously equilibrated with citrate buffer, *p*H 3, was washed with 0.01 M acetic acid, and 0.5 ml of the washed resin was added to each aliquot of the activation mixture. After the suspension was stirred for 5 minutes, the resin was allowed to settle, and the supernatant was removed. The resin was washed ten times with 0.5-ml portions of 0.01 M acetic acid to remove protein. The first supernatant was combined with the first two acetic acid washes, and the solution was lyophilized. The lyophilized material was dinitrophenylated, hydrolyzed, and examined for the presence of DNP-amino acids.

The resin was washed with three 0.5-ml portions of 1 M ammonia in order to elute adsorbed amino acids. The ammonia eluate was lyophilized and the residue was subjected to paper electrophoresis in pyridine acetate buffer, *p*H 6.5, at 40 v/cm for 60 minutes. The separated amino acids were determined quantitatively by the method of Connell *et al.* (1955).

The results of this experiment are shown in Figure 1. The only N-terminal residue which appeared during the rapid first step of the activation was threonine. C-terminal arginine also appeared as the activation reaction proceeded. The appearance of N-terminal threonine and of C-terminal arginine was parallel to the development of activity against HPLA. In addition, a smaller amount of C-terminal lysine was found, but its concentration was no more than a third of that of arginine.

In control experiments, unactivated procarboxypeptidase was incubated with carboxypeptidase B or with carboxypeptidase A; no amino acid was released in significant amounts.

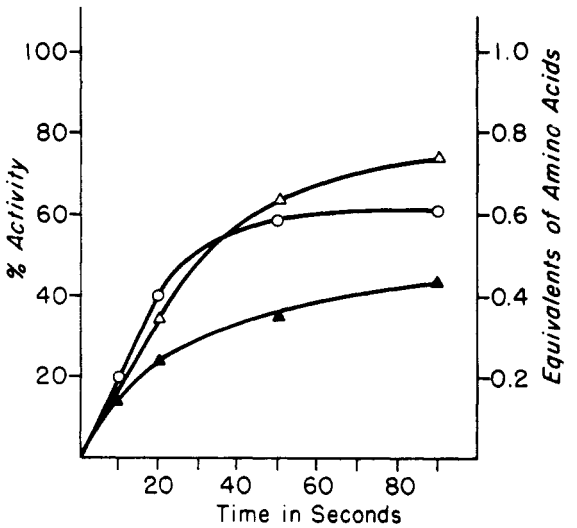

FIG. 1.—Activation of procarboxypeptidase B. Mole ratio of trypsin to procarboxypeptidase, 1:100. Circles represent carboxypeptidase B activity against HPLA, open triangles represent free arginine released, and filled triangles represent DNP-threonine. For further details, see text.

CONCLUSIONS

The present experimental data lend strong support to the view that the first stage of the tryptic activation of procarboxypeptidase B involves the hydrolysis of an arginyl-threonyl bond in the single polypeptide chain which constitutes this zymogen. This primary chemical event gives rise to a protein having approximately 60–80% of the enzymatic activity of the fully activated, crystalline enzyme. In this rapid, initial stage of activation, the newly formed carboxypeptidase B rapidly releases the C-terminal arginine formed during proteolysis, with the result that the appearance of enzymatic activity, the appearance of the amino-terminal threonine, and the release of the C-terminal arginine all occur simultaneously. In analogy with the activation of trypsinogen and chymotrypsinogen (Neurath, 1957) it may be surmised that the appearance of enzymatic activity is the result of changes in tertiary configuration brought about by the single hydrolytic event just described.

The second step of activation of procarboxypeptidase B occurs more slowly and is accompanied by fragmentation of the molecule, giving rise to a species having maximum enzymatic activity and a molecular weight of approximately 34,000 as compared to 57,400 for the zymogen. The structural relation of the fully activated enzyme to the zymogen cannot be elucidated from the present data, since the amino-terminal group of the zymogen has not yet been identified. Since procarboxypeptidase B appears to consist of a single polypeptide chain, the appearance of an amino-terminal threonine residue in the fully activated enzyme may suggest that the zymogen and the crystalline enzyme differ in the amino terminal sequence. However, if the crystalline enzyme should contain, in addition, the same FDNB-unreactive amino-terminal residue which exists in procarboxypeptidase B, the inert fragment released during full activation would have originated from the carboxyl-terminal sequence of the zymogen.

ACKNOWLEDGMENT

We are indebted to Dr. Bert L. Vallee for the metal analyses quoted in this paper, which will be published in full elsewhere. Our thanks are due also to Dr. Kenneth A. Walsh for his help and advice in connection with the sulfur analyses, and to Dr. Makoto Yamasaki and Mr. James R. Brown for advice in the determination of amino-terminal residues. The assistance of Mr. Peter Schneider in performing the amino acid analyses, and of Mrs. Elizabeth Edwards and Miss Sung Lee in the preparation of the proteins, are also gratefully acknowledged.

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