stable. In a later paper Brookes *et al.* (1968) compared the rate of ring opening of 7-methylguanosine and 7-benzylguanosine at pH 9.5 at 37° and found them to have an identical half-life of 0.5 hr. It appears that the rate of ring opening is greatly affected by small changes in pH and temperature and thus comparisons are valid only under identical conditions.

Attempts to use high-resolution mass spectrometry proved disappointing. Dr. D. Daves examined all of the ethylguanosines obtained with ethyl iodide, with and without acetylation of the ribose moiety. In most cases the base was cleaved and dealkylation occurred. A similar observation was reported by Lawley and Jarman (1972) who used mass spectrometry to identify the products of reaction of adenine and guanine with propylene oxide. This was not the case for the methylguanosines. However the data obtained for the ethylguanosines were sufficient to assign mono- or dialkyl structures. This was particularly important in the case of 6-*O*,-*X*-diethylguanosine which showed, among others, ions at *m/e* 207 corresponding to diethylguanine whereas 6-*O*-ethylguanosine lacked this peak.

Acknowledgment

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Isolation and Characterization of Pancreatic Procarboxypeptidase B and Carboxypeptidase B of the African Lungfish†

Gerald R. Reeck‡ and Hans Neurath*

ABSTRACT: Procarboxypeptidase B of the African lungfish, *Protopterus aethiopicus*, has been purified by a combination of ion-exchange chromatography, affinity chromatography, and gel filtration and characterized. The monomeric zymogen has a molecular weight of 45,000 and its amino acid composition is similar to procarboxypeptidases B of other species. The amino-terminal sequence of 15 residues has been determined. The zymogen displays intrinsic peptidase and esterase activities which increase markedly upon activation with trypsin. The activation reaction proceeds *via* an intermediate and ultimately is accompanied by the release of a large peptide (approximately 10,000 daltons). The enzyme, carboxypeptidase B, has also been characterized by chemical and enzymatic methods which have revealed extensive similarities to the corresponding bovine, porcine, dogfish, and rat enzymes. The amino-terminal sequence of lungfish carboxypeptidase is homologous to those of bovine carboxypeptidases A and B.

As part of the study of the phylogenetic variations of the structure and function of certain proteases, we have undertaken an analysis of the pancreatic zymogens and enzymes of the African lungfish, *Protopterus aethiopicus* (Reeck *et al.*, 1970). A detailed study of trypsinogen and trypsin (Reeck and Neurath, 1972) revealed considerable basic similarity to the corresponding bovine and dogfish proteins but also

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an intriguingly unique structure of the activation peptide. In an extension of these comparative studies to the metalloexopeptidases, we present an investigation of lungfish carboxypeptidase B and its parent zymogen, procarboxypeptidase B. This zymogen–enzyme pair is of particular interest both because of the relative paucity of information about these proteins in other species and because, in contrast to bovine carboxypeptidase A (Brown *et al.*, 1963), the zymogen can be isolated as a monomer. We have therefore been able to directly analyze the physical and chemical consequences of activation without complications due to aggregation. This and other results of our investigation contribute to an accumulating body of knowledge of the evolutionary patterns of the pancreatic proteases.

Experimental Section

Materials

Sources of materials were the same as given by Reeck and Neurath (1972) except for the following.

Ammediol (2-amino-2-methyl-1,3-propandiol) was purchased from Sigma Chemical Co.

ϵ-Amino-*n*-caproyl-D-tryptophan methyl ester was synthesized according to the method of Horbett (1970). Sources of the materials used in the synthesis were as follows: carbobenzoxy-ϵ-amino-*n*-caproic acid, Cyclo Chemical Corp.; D-tryptophan methyl ester hydrochloride, Nutritional Biochemicals Corp.; and *N,N*-dicyclohexylcarbodiimide, Cyclo Chemical Corp.

Cyanogen bromide was obtained from Baker Chemical Co.

Benzoylglycyl-L-arginine was purchased from Mann Research Laboratories. Hippuryl-L-argininic acid was synthesized by R. L. Stevens of this laboratory, following the procedure of Sanders (1970).

Methods

Enzyme Kinetics. For the hydrolysis of benzoylglycyl-L-arginine by carboxypeptidase B, 50 μl of the enzyme (1.0×10^{-2} mg ml^{-1}) was added to 3.0 ml of the substrate (0.025 M Tris (pH 7.65), containing 0.1 M NaCl). Rate measurements were performed in triplicate at each of eight different substrate concentrations. The enzyme was stored at 4° in 0.01 M Tris (pH 8.0), containing 0.1 M NaCl. The same experimental procedures were used for rate measurements with hippuryl-L-argininic acid as substrate. Duplicate determinations were made at six different substrate concentrations. The results were interpreted by Michaelis–Menten kinetics. The kinetic constants were calculated from initial velocities plotted according to Eadie as previously described (Reeck *et al.*, 1971a). The kinetic parameters of procarboxypeptidase B toward benzoylglycyl-L-arginine and hippuryl-L-argininic acid were determined by duplicate measurements at five different substrate concentrations. The protein samples were 200 and 150 μl, respectively. The temperature for all measurements was 25°.

Scanning of Polyacrylamide Disc Gels. Gels were scanned at 625 nm with the Model 2411 adapter of the Gilford Model 2000 spectrophotometer. Peak areas were determined by tracing the scan onto paper which was then cut out and weighed. All peak areas were adjusted to give a constant value (in nanomoles) of the sum of zymogen and enzyme.

Ultracentrifugation. Sedimentation velocities were determined by centrifuging nine protein samples (2–16 mg ml^{-1}) at 60,000 or 68,000 rpm in a Spinco Model E ultracentrifuge. Sedimentation coefficients were determined by standard techniques.

Other methods were those given by Reeck and Neurath (1972).

Results

Purification of Procarboxypeptidase B. The initial step was DE-52 cellulose chromatography of the acetone powder extract precisely as outlined by Reeck and Neurath (1972). The procarboxypeptidase B fraction from this column was concentrated to 100 ml by ultrafiltration using a UM-10 membrane, dialyzed against 5 mM potassium phosphate (pH 8.0), and lyophilized. The lyophilized powder (1500 mg of protein) was dissolved in 50 ml of distilled water and brought to 10^{-3} M DFP by the addition of 50 μl of 1 M DFP in isopropyl alcohol. The sample was then applied to a 5 × 120 cm column of Sephadex G-100 which had been equilibrated with 5 mM ammediol (pH 9.0), containing 5 mM NaCl, and eluted by the further application of this buffer at 72 ml hr^{-1}. The elution profiles from this and subsequent columns have been presented elsewhere (Reeck, 1971). Procarboxypeptidase B emerged between elution volumes of 640 and 1000 ml (V_e = 800 ml), as did chymotrypsinogen and trypsinogen contaminants. This fraction was pumped onto a 2.5 × 40 cm column of DE-52 cellulose equilibrated with 5 mM ammediol (pH 9.0), containing 5 mM NaCl. A linear NaCl gradient from 0.005 to 0.10 M in a total volume of 4 l. of ammediol buffer was applied to elute the proteins. A chymotrypsinogen peak appeared at 0.04 M NaCl but trailed into a second chymotrypsinogen peak eluted with 0.07 M NaCl, coincident with the elution of procarboxypeptidase B. No matter how shallow a gradient was employed, a substantial amount of chymotrypsinogen still emerged with the procarboxypeptidase B peak.

The procarboxypeptidase B fraction was concentrated to 77 ml by ultrafiltration and mixed with 77 μl of 1 M DFP. The sample was dialyzed against 0.05 M Tris (pH 8.0) for 8 hr and applied to a 2.5 × 35 cm column of ϵ-amino-*n*-caproyl-D-tryptophan methyl ester–Sepharose (Trp-OMe-Sepharose). This modified Sepharose column contained 10 μmoles of inhibitor/ml of Sepharose. As shown in Figure 1, neither procarboxypeptidase B nor chymotrypsinogen was eluted on application of the initial buffer, 0.05 M Tris (pH 8.0). Chymotrypsinogen eluted only with the application of 0.1 M sodium acetate (pH 3.0), as did any procarboxypeptidase A present at this stage. The procarboxypeptidase B fraction, eluted with 0.05 M Tris (pH 8.0), containing 0.10 M NaCl, was concentrated to 44 ml by ultrafiltration, dialyzed against 0.01 M Tris (pH 8.0) and lyophilized. Examination of this material by analytical polyacrylamide disc gel electrophoresis revealed a substantial contaminant which was removed by gel filtration. Three separate preparations of procarboxypeptidase B from 100 g of acetone powder, each of which had been processed as described above, were pooled and dissolved in 20 ml of water and applied to a 5 × 120 cm column of Sephadex G-100 equilibrated with 0.01 M Tris (pH 8.0). In contrast to its behavior in the earlier gel filtration step, procarboxypeptidase B emerged in a peak centered at V_e = 980 ml. The majority of the procarboxypeptidase B was thus separated from contaminating protein, which appeared brown colored in the fractions eluted before the zymogen.

A summary of the purification of lungfish procarboxypeptidase B is presented in Table I.

TABLE I: Purification of Procarboxypeptidase B.[a]

Purification Step	Total Protein[b] (mg)	Sp Act. (ΔA min^{-1} mg^{-1})	Purification	Total Act. (ΔA min^{-1})	Recov (%)
Extract	12,300	0.27	1.0	3200	100
Sample for first DE-52 column	9,800	0.32	1.2	3100	97
Sample for first G-100 column	1,510	1.72	6.4	2600	81
Sample from first G-100 column	1,000	2.30	8.5	2300	72
Sample from second DE-52	394	4.80	17.8	1900	59
Sample for second G-100 column	135	9.64	35.7	1300	41
Sample from second G-100 column	95	10.7	39.6	1000	31

[a] From 100 g of acetone powder. [b] Determined by absorbance at 280 nm applying the extinction coefficient of purified procarboxypeptidase B. The value for the extract has been corrected for absorbance of nucleic acids at 280 nm using Warburg–Christian factors (Warburg, 1957).

Physicochemical Characterization of Lungfish Procarboxypeptidase B. POLYACRYLAMIDE DISC GEL ELECTROPHORESIS. Procarboxypeptidase B prepared by the above procedure appeared to be nearly homogeneous when examined by analytical polyacrylamide disc gel electrophoresis as can be seen in the leftmost gel in Figure 6 (*vide infra*). This material was judged to be sufficiently pure for all purposes except the determination of the amino acid composition. For amino acid analysis, the slight impurities, estimated by scanning to be approximately 5%, could be removed by chromatography on a DE-52 cellulose column at pH 9.0, as shown in Figure 2. (The significance of the data given in Figure 2 is discussed below.) The first 60% of the protein eluted from such a column is pure as judged by polyacrylamide disc gel electrophoresis (Figure 3A).

SODIUM DODECYL SULFATE GEL ELECTROPHORESIS. Procarboxypeptidase B was subjected to polyacrylamide electrophoresis in the presence of 0.1% sodium dodecyl sulfate as further evidence for its purity and for the determination of its molecular weight. A single protein band was seen corresponding to a molecular weight of 45,000 (Figure 4).

SEDIMENTATION VELOCITY STUDIES. The sedimentation constant, $s_{20,w}$, was determined at a series of protein concentra-

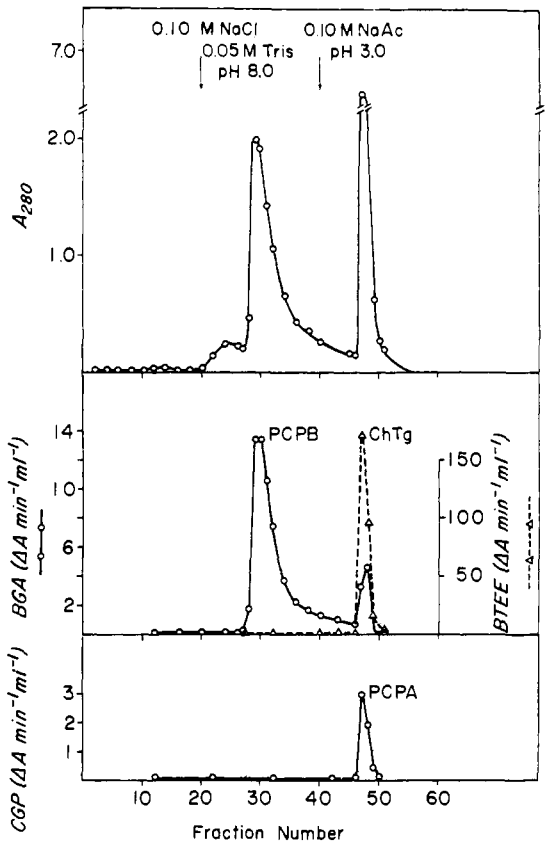

FIGURE 1: Chromatography of procarboxypeptidase B on Trp-OMe-Sepharose. The fraction volume was 20 ml. Fractions 1–20 were eluted with 0.05 M Tris (pH 8.0). Other details are given in the text. In order to prevent the possible loss of metal from the procarboxypeptidases and concomitant inactivation, the fractions eluted by 0.1 M sodium acetate were adjusted to pH 8.0 immediately upon their elution. They were subsequently incubated with bovine trypsin as described in Methods before assays were performed for carboxypeptidase activities.

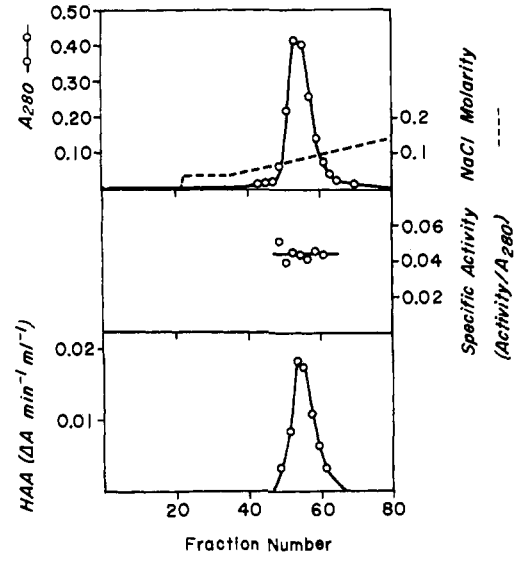

FIGURE 2: Chromatography of purified procarboxypeptidase B on a 2.5 × 40 cm column of DE-52 cellulose previously equilibrated with 5 mM ammediol (pH 9.0), containing 5 mM NaCl. The fraction volume was 10 ml. The absorbance of the effluent was monitored as the column was developed; no absorbance was detected before tube 40. Activities were determined without prior activation with trypsin.

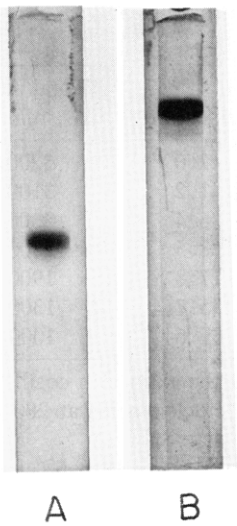

FIGURE 3: Analytical disc gel electrophoresis (pH 8.3). (A) Of lungfish procarboxypeptidase B. Migration was from top to bottom (cathode to anode). The gel was severed at the dye front prior to staining and destaining. (B) Of lungfish carboxypeptidase B. Migration was from top to bottom (cathode to anode). The gel was severed at the dye front prior to staining.

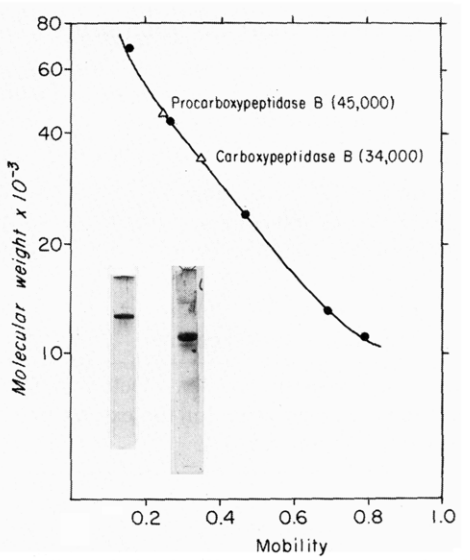

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of lungfish procarboxypeptidase B (left gel) and carboxypeptidase B (right gel). Standard proteins used for molecular weight determinations were bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen (25,700), and the B and C chains of chymotrypsin (11,000 and 13,000).

tions. Least-squares analysis of the data gave a straight line extrapolating to $s^0_{20,w} = 4.04 \pm 0.08$ S and having a slope of -0.038 ± 0.009 S mg^{-1} ml^{-1}. The schlieren optical pattern of a 15.7-mg ml^{-1} solution of the zymogen after centrifugation at 68,008 rpm for 150 min was entirely symmetrical, providing qualitative evidence for the size homogeneity of the preparation.

HIGH-SPEED SEDIMENTATION EQUILIBRIUM ANALYSIS. Molecular weight moments as a function of protein concentration were determined as outlined in the Methods section of the paper by Reeck *et al.* (1971a). A monomer molecular weight of 45,000 ± 1000 was calculated from the data.

EXTINCTION COEFFICIENT DETERMINATION. A solution of procarboxypeptidase B ($A_{280} = 7.89$) was centrifuged in a synthetic boundary cell at 4000 rpm until the fringes observed in the Rayleigh interference optical system were fully resolved. A total of 19.62 fringes was counted in a microcomparator across the protein boundary. Assuming a refractive index factor of 4.04 (Doty and Edsall, 1951), the protein concentration was determined to be 4.86 mg ml^{-1}. Thus $A^{1cm}_{1\%} = 16.2$ at 280 nm.

AMINO ACID COMPOSITION. Table II presents the amino acid composition of lungfish procarboxypeptidase B, derived from duplicate analyses after 24-, 48-, 72-, and 96-hr hydrolysis as described previously (Reeck and Neurath, 1972). The composition greatly resembles that of dogfish procarboxypeptidase B (Prahl and Neurath, 1966) as well as those of the bovine (Cox *et al.*, 1962) and rat (Sanders, 1970) zymogens.

AMINO-TERMINAL SEQUENCE. The amino acid sequence of the amino-terminal portion of procarboxypeptidase B was determined using the Beckman Sequencer (Hermodson *et al.*, 1971). The sequence is Glu-Pro-Thr-Pro-Arg-Ser-Phe-Asn-Gly-Asp-Lys-Val-Phe-Arg-Val- and constitutes the first sequence information about a portion of the activation peptide of a procarboxypeptidase B.

Enzymatic Characterization of Lungfish Procarboxypeptidase B. In light of the recent discoveries that dogfish and bovine procarboxypeptidase A (Lacko and Neurath, 1970; Uren *et al.*, 1972) possess significant enzymatic activities toward substrates of carboxypeptidase A, it was of interest to examine lungfish procarboxypeptidase B for catalytic activity.

In preliminary measurements, this zymogen displayed low but measurable activity toward benzoylglycyl-L-arginine and hippuryl-L-argininic acid. It is difficult to rule out by physical techniques the possibility that such activity, approximately 1% of that of the enzyme, was due to the presence of a small amount of carboxypeptidase B in the zymogen preparation. The following experiment, based on the different chromatographic behaviors of the zymogen and the enzyme, was designed to eliminate this possibility.

Procarboxypeptidase B was dialyzed for 16 hr against 5 mM ammediol (pH 9.0) containing 0.01 M NaCl and 10^{-4} M $ZnCl_2$ and then for 3 hr against the same buffer lacking only $ZnCl_2$. The purpose of this dialysis was to ensure that any metal bound to the zymogen was zinc and thus to make the results of kinetic measurements directly comparable to those of lungfish carboxypeptidase B. The sample was applied to a 2.5 × 40 cm column of DE-52 cellulose previously equilibrated with 5 mM ammediol (pH 9.0), containing 5 mM NaCl. Then 100 ml of 30 mM NaCl in 5 mM ammediol was applied, followed by a linear gradient from 0.06 to 0.15 M NaCl in a total volume of 500 ml. Any carboxypeptidase B in the preparation would be eluted during the application of 30 mM NaCl (Figure 10, *vide infra*). No detectable amount of protein appeared before the emergence of procarboxypeptidase B (Figure 2). Immediately after its elution, the activity of fraction 54 toward 1.0 mM hippuryl-L-argininic acid was measured and then aliquots of this fraction were subjected to kinetic characterization. The activities of several other fractions toward 1.0 mM hippuryl-L-argininic acid were determined as well. As is shown in Figure 2, the specific activity of these fractions is essentially constant.

The initial velocities of the hydrolysis of hippuryl-L-argininic acid and benzoylglycyl-L-arginine were plotted according to Eadie. From these plots, the kinetic constants given in Table III were calculated. After all these measurements had been completed (within 24 hr of the elution of the protein

TABLE II: Amino Acid Compositions of Lungfish Procarboxypeptidase B, Carboxypeptidase B, and of Activation Peptides of Several Procarboxypeptidases.

			Activation Peptides[a]				
	Lungfish pCPB[b] (Residues/ 45,000 Daltons)	Lungfish CPB[c] (Residues/ 34,000 Daltons)	Isolated Peptide (Residues/ 10,000 Daltons)	Lungfish pCPB (by Diff[d] of Columns 1 + 2)	Dogfish pCPB[e]	Dogfish pCPA[f,g]	Bovine CPA[h] Fraction I[i]
Asp	46.0	32.6	13.7	13.4	15	8	5
Thr	26	24	1.6[m]	1	3	5	0
Ser	31	32	2.3[m]	0	2	6	0
Glu	40.0	21.0	15.9	18.0	15	20	12
Pro	17.8	12.9	2.79	2.9	3	0	4
Gly	23.9	22.0	3.87	1.9	6	3	4
Ala	33.4	27.2	5.40	6.2	7	0	4
Cys/2	7.20[j]	7.8[j]		0	2	0	
Val	22.7	13.3	10.7[n]	9.4	10	6	6
Met	6.29[k]	4.08[k]	2.92	2.2	0	5	1
Ile	18.7	15.2	2.24[n]	3.5	4	6	2
Leu	27.9	19.1	7.62	8.8	8	8	7
Tyr	25.0	22.2	2.42	2.8	0	3	1
Phe	16.4	11.9	5.86	4.5	4	3	2
Trp	10.6[l]	7.56[l]		3.0	3	0	
Lys	20.3	15.3	4.17	5.0	6	2	2
His	7.33	4.17	3.07	3.2	1	2	1
Arg	16.4	13.2	2.29	2.2	6	2	3

[a] Except for the third column, these values result from subtraction of carboxypeptidase composition from the corresponding procarboxypeptidase composition. [b] pCPB = procarboxypeptidase B. [c] CPB = carboxypeptidase B. [d] Excluding the N-terminal pentapeptide of procarboxypeptidase B. [e] From Prahl and Neurath (1966). [f] pCPA = procarboxypeptidase A. [g] From Lacko and Neurath (1970). [h] CPA = carboxypeptidase A. [i] From Freisheim *et al.* (1967). [j] Determined as cysteic acid after performic acid oxidation. [k] Determined as methionine sulfone after performic acid oxidation. [l] Determined by the method of Edelhoch (1967). [m] 24-hr values. [n] 72-hr values.

TABLE III: Kinetic Constants for the Hydrolysis of Benzoylglycyl-L-arginine and Hippuryl-L-argininic Acid by Procarboxypeptidase B and Carboxypeptidase B.[a]

	BGA[b]		HAA[c]	
	K_m (mM)	k_0 (sec^{-1})	K_m (mM)	k_0 (sec^{-1})
Procarboxypeptidase B	0.62 ± 0.12	0.35 ± 0.04	0.40 ± 0.07	0.85 ± 0.08
Carboxypeptidase B	0.32 ± 0.01	86 ± 2	0.053 ± 0.006	171 ± 13

[a] All measurements were performed at 25°. Solvent: 0.025 M Tris (pH 7.65), containing 0.1 M NaCl. [b] BGA = benzoylglycyl-L-arginine. [c] HAA = hippuryl-L-argininic acid.

from the column), the activity of fraction 54 toward 1.0 mM hippuryl-L-argininic acid was once again measured and determined to be 44.2 ΔA min^{-1} ml^{-1}, not significantly higher than the original activity of the same fraction, 42.8 ΔA min^{-1} ml^{-1}.

These data indicate that procarboxypeptidase B does indeed possess significant, if low, catalytic activity toward substrates of carboxypeptidase B. The enzymatically active species does not appear to have been generated by partial activation of procarboxypeptidase B after its elution from the column, since the activity was present immediately upon the emergence of the protein from the column and did not increase in the next 24 hr. The simplest explanation of the data is that the protein exhibiting enzymatic activity is in fact procarboxypeptidase B.

Furthermore, the kinetic constants which characterize the catalytic activity associated with the zymogen differ from those of carboxypeptidase B (Table III). These differences are not only in k_0 (which could be explained on the basis of contamination with carboxypeptidase B) but in K_m as well. This finding is further evidence that the activity could not be due to contaminating carboxypeptidase B.

Activation of Lungfish Procarboxypeptidase B. KINETICS OF TRYPTIC ACTIVATION. The activation of procarboxypeptidase B by lungfish and bovine trypsins was measured at an

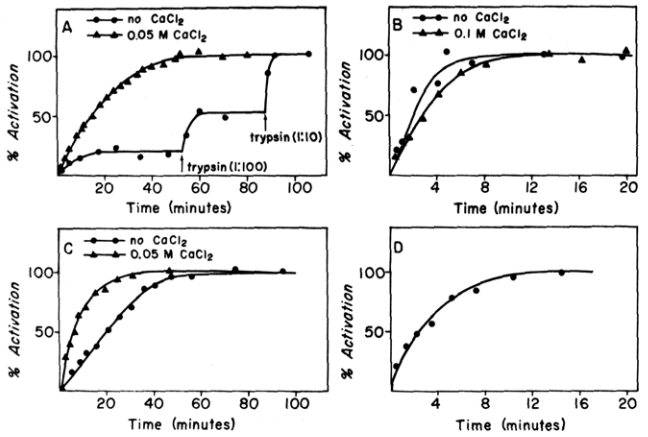

FIGURE 5: Activation of lungfish procarboxypeptidase B by lungfish and bovine trypsins. All activations were performed at a zymogen concentration of 5 mg ml^{-1} in 0.01 M Tris (pH 8.0), at 25°. (A) Activations by bovine trypsin (1:100 molar ratio) in the presence and absence of calcium chloride. (B) Activations by bovine trypsin (1:10 molar ratio) in the presence and absence of calcium chloride. (C) Activations by lungfish trypsin (1:100 molar ratio) in the presence and absence of calcium chloride. (D) Activation by lungfish trypsin (1:10 molar ratio) in the absence of calcium chloride.

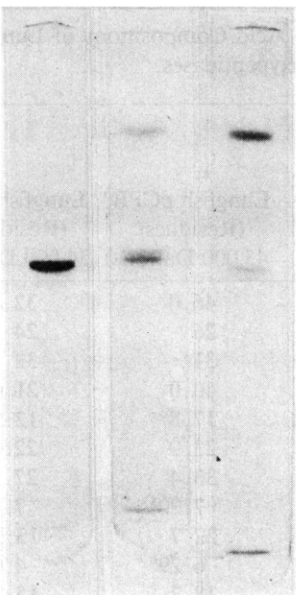

FIGURE 6: Disc gel electrophoresis (pH 8.3) of the activation mixture of lungfish procarboxypeptidase B. Details of the experiment are given in the text. From left to right the gels are aliquots of the activation mixture after 0.12- and 30-min incubation with trypsin. Electrophoresis is from top to bottom (cathode to anode); gels were severed at the dye front prior to staining. Uncomplexed soybean trypsin inhibitor migrates very near the dye front, essentially identical to the activation peptide, and is faintly visible in the leftmost gel.

initial zymogen concentration of 5 mg ml^{-1} in 0.01 M Tris (pH 8.0) at 25°. The same final activity toward benzoyl-glycyl-L-arginine was achieved with both trypsins in the presence or absence of calcium, *i.e.*, 58 ΔA min^{-1} ml^{-1}. This value corresponds to the activity which would result from quantitative conversion of the zymogen to the enzyme, based on the specific activity of the enzyme.

Figure 5A,B shows the time courses of activation by bovine trypsin at molar ratios of enzyme to zymogen of 1:100 and 1:10. At the 1:100 level only partial activation (20%) was achieved in the absence of $CaCl_2$. Addition of a second aliquot of trypsin after activation had leveled off resulted in the appearance of another 30% of the potential carboxypeptidase activity. Complete activation could be achieved by addition of sufficient trypsin to bring the molar ratio of enzyme to zymogen to 1:10. The partial activation by the lower trypsin concentration was apparently due to the instability of bovine trypsin in the absence of calcium. When activation was more rapid (1:10) no significant difference in the rate of appearance of activity in the presence or absence of calcium chloride was observed.

The activation by lungfish trypsin (Reeck and Neurath, 1972) is shown in Figure 5C,D. Lungfish trypsin does not require calcium for stability at neutral pH and complete activation was observed at a 1:100 ratio of enzyme to zymogen in the absence of calcium. The rate of activation appears to be somewhat faster in the presence of 0.05 M $CaCl_2$. The rate is comparable to the activation of procarboxypeptidase B by 1:100 levels of bovine trypsin in the presence of calcium. The rather weak effect of calcium on the activation of lungfish procarboxypeptidase B contrasts sharply with the strong stimulatory effect of this ion on the activation of dogfish procarboxypeptidase A (Lacko and Neurath, 1970).

ELECTROPHORETIC ANALYSIS OF THE ACTIVATION PROCESS. In order to examine the nature and number of protein species present during the activation of lungfish procarboxypeptidase B, the activation mixture was examined at various stages of activation by analytical polyacrylamide disc gel electrophoresis. Procarboxypeptidase B (5 mg ml^{-1} in 0.01 M Tris (pH 8.0), containing 0.05 M $CaCl_2$) was activated at 25° by bovine trypsin at a concentration of 0.025 mg ml^{-1}. At 12 subsequent times, a 5-μl aliquot was removed and added to 20 μl of 0.03-mg ml^{-1} soybean trypsin inhibitor to stop the reaction. The samples were examined by disc gel electrophoresis. Representative gels are shown in Figure 6.

Procarboxypeptidase B disappears entirely during the reaction and three other species appear. The protein migrating with a mobility of 0.20 corresponds to carboxypeptidase B (see Figure 3B) and its intensity increases in proportion to the appearance of activity (see Figure 7A,B). A second species which appears during the reaction has a very high mobility and migrates essentially as fast as the dye front. This species probably corresponds to the activation peptide, which, being relatively small (about 10,000 daltons) and acidic (*vide infra*), would be expected to have a high mobility. This band appears to have reached a maximum intensity before activation was complete (Figure 7C). This could reasonably be due to degradation of the peptide to fragments too small to remain in the gel during the staining and destaining process.

This experiment also clearly demonstrated the presence in the activation mixture of a third new species with a mobility just slightly lower than that of procarboxypeptidase. This protein, not present at all in an unactivated sample of zymogen, can be detected by scanning the gels as early as 3 min after addition of trypsin. After approximately 18-min incubation, the new protein is present in an amount equal to the zymogen. At subsequent times there is more of the new species than the zymogen, although the sum of these proteins is constantly decreasing. Thus this protein appears to be an intermediate in the activation process.

The activation process was further examined by gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. In this experiment, lungfish trypsin was used

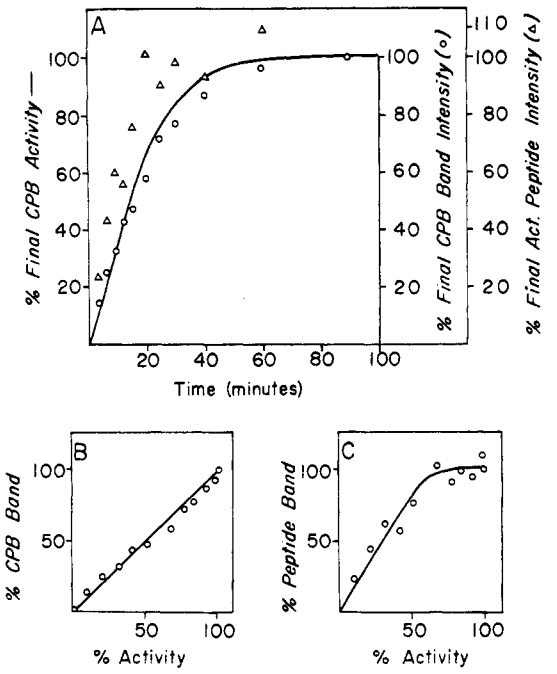

FIGURE 7: Analysis of the activation of lungfish procarboxypeptidase B. The data are derived from the gels shown in Figure 6, which were scanned as described in Methods.

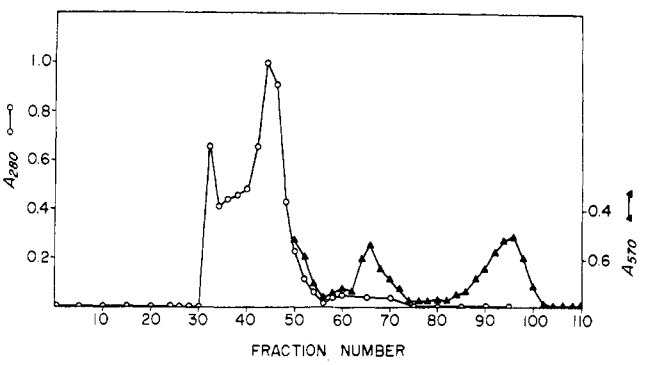

FIGURE 8: Gel filtration of the activation mixture on Sephadex G-75. The fraction volume was 5 ml. The absorbance at 570 nm was determined after alkaline hydrolysis and reaction with ninhydrin.

to activate procarboxypeptidase B because the reaction proceeds in the absence of calcium. Inclusion of calcium would interfere with the gels since calcium dodecyl sulfate is insoluble. Using molar ratios of enzyme to zymogen of 1:100 and 1:10 and a zymogen concentration of 5 mg ml^{-1} in 0.01 M Tris (pH 8.0), 25°, the activation was stopped at various stages by removing a 10-μl aliquot of the reaction mixture and mixing it with 50 μl of 10% acetic acid containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. After incubation for 1 hr the samples were immersed in boiling water for 1 min and lyophilized. The lyophilized powder was dissolved in 50 μl of 0.01 M sodium phosphate (pH 7.0), containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. The samples were incubated at 37° for 2 hr and the electrophoreses begun. During the course of the reaction a band corresponding to a molecular weight of 45,000 disappeared and a band corresponding to a molecular weight of 35,000 appeared. Furthermore, a band corresponding to a molecular weight of approximately 10,000 appeared during the activation.

ISOLATION OF THE ACTIVATION PEPTIDE. To 50 mg of procarboxypeptidase B in 10 ml of 0.01 M Tris (pH 8.0) was added lungfish trypsin at a molar ratio of 1:100. After 30-min incubation at 25° (75% activation), 1 ml of glacial acetic acid was added to stop the reaction and the sample was applied to a 2.5 × 110 cm column of Sephadex G-75 equilibrated with 5% acetic acid. The elution of ninhydrin-positive material and absorbance at 280 nm are shown in Figure 8. After elution of protein peaks near the void volume of the column, a ninhydrin-positive peak eluted (K_{av} = 0.45) as well as a broad, second ninhydrin-positive peak which required nearly the total bed volume of the column for its elution (K_{av} = 0.84).

We have focused our attention on the peak having a K_{av} of 0.45 since this value indicates a molecular weight of 10,000–11,000 (Gel Filtration in Theory and Practice, Pharmacia). Furthermore, a polypeptide chain of bovine carboxypeptidase B for which we determined a molecular weight of 10,000 eluted from this same column at a nearly identical position (Reeck *et al.*, 1971a). The polypeptide in this fraction is thus too small to be procarboxypeptidase B, carboxypeptidase B, or trypsin and corresponds in size to the polypeptide observed on sodium dodecyl sulfate gel electrophoresis during the activation of procarboxypeptidase B.

Fractions 63–72 were combined, concentrated to 2 ml by rotary evaporation, and subjected to amino acid and sequenator analyses. The amino acid composition derived from duplicate analyses after 24- and 72-hr hydrolysis (Table II, column 3) agrees well with the difference between the compositions of procarboxypeptidase B and carboxypeptidase B (Table II, column 4) assuming the absence of the amino-terminal pentapeptide of the zymogen (*vide infra*). The activation peptide is notable for its high content of the acidic amino acids, which is characteristic of the activation peptide regions of other procarboxypeptidases.

After one cycle of the Edman degradation in a sequenator the main PTH-amino acid observed was serine but several other amino acids were observed in significant amounts, including isoleucine, valine, phenylalanine, glycine, and glutamic acid. The level of secondary amino acids decreased greatly in the second cycle which gave phenylalanine as the predominant amino acid and approximately 15% valine. The subsequent sequence of major amino acids (about 85% on each turn) was Asn-Gly-Asp-Lys-Val-Phe-Arg-Val. The predominant sequence thus corresponds to residues 6–15 of the amino-

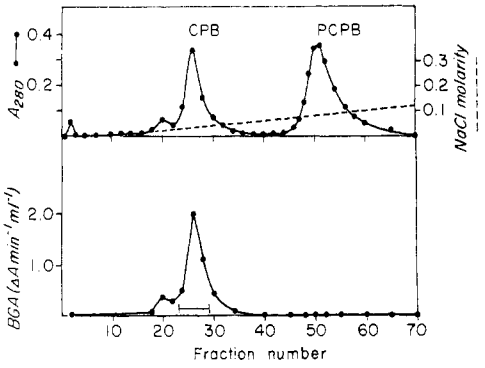

FIGURE 9: Chromatography of carboxypeptidase B and procarboxypeptidase B on DE-52 cellulose. After partial activation of procarboxypeptidase B as described in the text, trypsin was inactivated by DFP and the sample dialyzed against 5 mM ammediol (pH 9.0). The column (1.5 × 20 cm) was previously equilibrated with the same ammediol buffer. The fraction volume was 20 ml.

```
                                                                  15
Bovine CPA_α   Ala-Arg-Ser-Thr-Asn-Thr-Phe-Asn-Tyr-Ala-Thr-Tyr-His-Thr-Leu-
Lungfish CPB                        Tyr-Ser-Tyr-Glu-Lys-Tyr-Asn-Thr-Trp-
Bovine CPB                    Thr-Thr-Gly-His-Ser-Tyr-Glu-Lys-Tyr-Asn-Asn-Trp-

                                                                  30
Bovine CPA_α   Asp-Glu-Ile-Tyr-Asp-Phe-Met-Asp-Leu-Leu-Val-Ala-Gln-His-Pro-
Lungfish CPB   Asp-Glu-Ile-Ala-Ala-Trp-Thr-X -Glu-Ile- X -Ala-Ala-Asn-Pro-
Bovine CPB     Glu-Thr-Ile-Glu-Ala-Trp-Thr-Glu-Gln-Val-Ala-Ser-Glu-Asn-Pro-
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FIGURE 10: Comparison of the amino-terminal sequences of bovine carboxypeptidase A_α (Bradshaw *et al.*, 1969), lungfish carboxypeptidase B, and bovine carboxypeptidase B (Reeck *et al.*, 1971b). The identity of the residues marked "X," positions 23 and 26, is unknown.

terminal sequence of the zymogen. On the basis of its molecular weight and amino acid composition, the present peptide can be identified with the entire polypeptide segment of the zymogen extending to the point of cleavage during activation but exclusive of the amino-terminal pentapeptide.

Characterization of Lungfish Carboxypeptidase B. PREPARATION AND PHYSICOCHEMICAL PROPERTIES. Lungfish carboxypeptidase B was isolated by DE-52 chromatography of activated procarboxypeptidase B. Procarboxypeptidase B (5 mg ml^{-1} in 0.01 M Tris (pH 8.0), containing 0.05 M NaCl) was activated by bovine trypsin at a 1:100 molar ratio of enzyme to zymogen. The chromatographic system used for the isolation of carboxypeptidase B from a partially activated sample is shown in Figure 9. Insufficient quantities precluded the characterization of the minor component of carboxypeptidase B. The major component, constituting about 90% of the total carboxypeptidase B, was judged to be homogeneous by analytical polyacrylamide disc gel electrophoresis (Figure 3B) and sodium dodecyl sulfate gel electrophoresis (Figure 4). The latter technique yielded a molecular weight of 34,000 for the enzyme, very similar to the molecular weights previously determined for bovine (Wintersberger *et al.*, 1962), porcine (Folk *et al.*, 1960), dogfish (Prahl and Neurath, 1966), and rat (Sanders, 1970) carboxypeptidase B. These values are 34,000, 34,300, 35,000–37,000, and 35,000, respectively.

Using this isolation procedure 18 mg of carboxypeptidase B was recovered from 30 mg of activated procarboxypeptidase B, a yield of 75% of the theoretical value. Attempts to crystallize the enzyme by dialysis against 0.01 M Tris (pH 8.0), conditions which result in the crystallization of bovine and dogfish carboxypeptidase B, were unsuccessful because the lungfish enzyme remained soluble under these conditions.

The amino acid composition of carboxypeptidase B, derived from duplicate amino acid analyses after 24-, 48-, 72-, and 96-hr hydrolysis, is given in Table II. The composition bears marked similarities to the bovine (Cox *et al.*, 1962), porcine (Folk *et al.*, 1960), dogfish (Prahl and Neurath, 1966), and rat (Sanders, 1970) enzymes.

The extinction coefficient of carboxypeptidase B was determined (Walsh and Brown, 1962) to be $A^{1\,cm}_{0.1\%} = 2.2$.

The amino-terminal sequence of lungfish carboxypeptidase B was determined using the Beckman Sequencer. The data are illustrated in Figure 10. The sequence displays considerable similarity to those of bovine carboxypeptidases B and A.

ENZYMATIC PROPERTIES. Carboxypeptidase B (0.5 mg ml^{-1}) was dialyzed for 48 hr against 0.01 M Tris (pH 8.0), containing 0.10 M NaCl and 10^{-4} M $ZnCl_2$, and subsequently for 12 hr against the same buffer lacking only $ZnCl_2$. For kinetic measurements the enzyme was then diluted 50-fold with 0.01 M Tris (pH 8.0), containing 0.10 M NaCl.

Table III presents the kinetic parameters for the hydrolysis of benzoylglycyl-L-arginine and hippuryl-L-argininic acid. The specific activity toward 1.0 mM hippuryl-D,L-phenyllactic acid was 38 μmoles min^{-1} mg^{-1}.

Carboxypeptidase B was inhibited by the metal chelating agents *o*-phenanthroline and EDTA. To 1 ml of the enzyme (0.5 mg ml^{-1}) in 0.005 M ammediol (pH 9.0), containing 0.04 M NaCl, was added 0.1 ml of 0.1 M EDTA (pH 7.65). Of the activity toward benzoylglycyl-L-arginine, 50% was lost in 14 hr and only 4% remained after 72 hr at room temperature. (A control lacking only EDTA was entirely stable during this incubation.) Incubation of the same amount of enzyme solution with 10 μl of the EDTA solution resulted in a loss of only 12% of the activity in 120 hr. Inhibition by 1 mM *o*-phenanthroline was much faster, 50% of the activity being lost in 2 hr.

Discussion

The purification of lungfish procarboxypeptidase B was complicated by the difficulty of its separation from chymotrypsinogen. Indeed, by conventional techniques we were unable to prepare procarboxypeptidase B entirely free from chymotrypsinogen. For this purpose we took advantage of the tighter binding of chymotrypsinogen to a column of Trp-OMe-Sepharose (Figure 1). The behavior of chymotrypsinogen and procarboxypeptidase B during the purification of the latter protein strongly suggests that they bind to one another in a manner somewhat reminiscent of fractions I, II, and III of bovine procarboxypeptidase A (Brown *et al.*, 1963) but clearly not as tightly.

By two independent techniques the molecular weight of purified lungfish procarboxypeptidase B has been established as 45,000. The known molecular weights of monomeric procarboxypeptidases A and B from various species are presented in Table IV. The molecular weight of bovine procarboxypeptidase B, previously reported to be 55,000 (Wintersberger *et al.*, 1962), has been reexamined in this laboratory by R. L. Stevens and R. D. Wade (unpublished observations). A variety of techniques gave values from 42,000 to 47,000 with no indication of a higher monomer molecular weight. The value of 55,000 for the rat zymogen is suspect because of heterogeneity in the ultracentrifuge (Sanders, 1970). Thus the most probable explanation of the rather wide variation in the molecular weights of procarboxypeptidases B would seem to be inaccuracies in the previously reported values for the bovine and rat zymogens.

The isolation of lungfish procarboxypeptidase B as a monomer and recent technical advances permitted a more detailed characterization of the physicochemical changes accompanying the activation of this zymogen than was possible for any other procarboxypeptidase. The most likely sequence of chemical events in the activation involves two sequential steps. In the first, the amino-terminal pentapeptide of the zymogen, Glu-Pro-Thr-Pro-Arg-, is released. The resulting protein intermediate is similar to the zymogen in molecular weight and does not have significantly greater catalytic activity. The latter fact is clear from the assays shown in Figure 9; essentially no catalytic activity was

TABLE IV: Molecular Weights of Monomeric Procarboxypeptidases.

Protein	Mol W	Reference
Succinyl fraction I of bovine procarboxypeptidase A	40,200	Freisheim *et al.* (1967)
Dogfish procarboxypeptidase A	44,000	Lacko and Neurath (1970)
Dogfish procarboxypeptidase B	45,000	Prahl and Neurath (1966)
Lungfish procarboxypeptidase B	45,000	This work
Rat procarboxypeptidase B	55,000	Sanders (1970)
Bovine procarboxypeptidase B	57,000	Wintersberger *et al.* (1962)
	42,000–47,000	R. L. Stevens and R. D. Wade (unpublished experiments)

found in the fractions of the peak eluted with 0.08 M NaCl, which according to analytical disc gel electrophoresis contains both procarboxypeptidase B and the intermediate. The second step of activation involves the release of the amino-terminal polypeptide of the intermediate (approximately 10,000 in molecular weight) and produces a large increase in catalytic activity with the formation of carboxypeptidase B. After its release the large peptide is degraded by trypsin and probably carboxypeptidase B as well.

Lungfish procarboxypeptidase B possesses significant intrinsic enzymatic activity toward substrates of carboxypeptidase B. This activity is not due to contaminating enzyme since it eluted from DE-52 cellulose with the zymogen and differs in kinetic constants from the carboxypeptidase B (Table III). Similar observations have previously been reported for dogfish procarboxypeptidase A (Lacko and Neurath, 1970) and bovine procarboxypeptidase A S5 (Uren *et al.*, 1972). While it is difficult to ascribe a physical meaning to the steady-state constants, in all likelihood the active site of the enzyme is closely approximated in the zymogen. A similar conclusion has been reached from crystallographic studies of chymotrypsinogen and chymotrypsin (Freer *et al.*, 1970). The significance and structural necessity of the large activation peptide (8000–10,000 daltons) in the case of procarboxypeptidase remain to be clarified in further investigations.

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