Isolation and Amino-Terminal Sequence Analysis of Two Dissimilar Pancreatic Proelastases from the African Lungfish, *Protopterus aethiopicus*†

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ABSTRACT: Two proelastases, A and B, having molecular weights of about 25,000, were isolated in highly purified form from the pancreata of the African lungfish, *Protopterus aethiopicus*. Activation with trypsin yielded active enzymes having typical elastase specificity. These enzymes digested elastin and hydrolyzed the specific ester substrate Ac-$(Ala)_3$-OMe, but not Ac-Tyr-OEt or Tos-Arg-OMe. The elastolytic activity of elastase B and the affinity of the enzyme for Ac-$(Ala)_3$-OMe were higher than those of elastase A and resembled those of porcine elastase. Sequence analysis of proelastase A demonstrated that the "activation peptide" is very similar to that of the chymotrypsinogen family, especially that of chymotrypsinogen C. Amino-terminal sequence analysis of elastase A revealed a closer relationship to the chymotrypsinogen family, especially chymotrypsinogen C, than to either porcine elastase or lungfish elastase B. The sequence of the "activation peptide" of proelastase B had no obvious relationship to that of the zymogens of the other pancreatic serine proteases. The amino-terminal sequence of elastase B, however, resembles that of porcine elastase more closely than that of chymotrypsin, trypsin, or elastase A.

Although the structure and function of trypsinogen and chymotrypsinogen from various species have been extensively studied (for reviews, see Walsh, 1970, and Wilcox, 1970), less is known of the third group of vertebrate serine proteases, the elastases and their zymogens. So far only porcine proelastase and elastase have been investigated in detail. The primary and tertiary structures of elastase provide an explanation for its specificity and establish the homology of this enzyme to trypsin and chymotrypsin (for review, see Hartley and Shotton, 1971). Elastolytic activity has been found in the pancreata of other species and in some of these more than one type of elastase have been demonstrated (for review, see Shotton, 1970).

The pattern of evolution of pancreatic serine proteases is not completely understood. In each case studied, a zymogen is first synthesized and converted to the enzyme by proteolytic cleavage of an amino-terminal "activation peptide" (Neurath, 1957, 1964). Although the structures of the serine proteases bear similarities indicative of divergent evolution from a common ancestor, the activation peptides appear to conform to one of several archetypes, each characteristic of enzymes of a given specificity (Neurath *et al.*, 1973). Knowledge of the changes of the "activation peptides" in the course of evolution could provide additional information for an understanding of the evolution of enzyme specificity. So far, however, no activation peptide of a proelastase has been characterized. The present study describes two proelastases of the African lungfish, *Protopterus aethiopicus*, an animal which is thought to have diverged from mammals about 400 million years ago (Romer, 1966).

Materials and Methods

Three-times-crystallized α-chymotrypsin was obtained from Worthington Biochemical Corp. and crystalline porcine trypsin was a product of Novo Industri A/S. Pure porcine elastase was prepared according to Shotton (1970).

Acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Ac-$(Ala)_3$-OMe) and acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) were purchased from Cyclo Chemical Co. *p*-Nitrophenyl-*p'*-(ω-dimethylsulfonioacetamido)benzoate bromide, a specific active-site titrant of chymotrypsin (Wang and Shaw, 1972), was a gift of Dr. E. Shaw, and *N-tert*-butyloxycarbonylglycyl-L-leucyl-L-phenylalanylchloromethane (Boc-Gly-Leu-Phe-CH_2Cl) was a gift of Dr. K. Kurachi. 4-Vinylpyridine was vacuum distilled before use. Preswollen carboxymethylcellulose (CM-52) was purchased from Whatman Co.

A "stock solution" of radioactive diisopropyl fluorophosphate containing 1.0 mCi was prepared by mixing 0.4 g of pure diisopropyl fluorophosphate (Pierce Chemical Co.) with 0.167 mg of [^{14}C]diisopropyl fluorophosphate (New England Nuclear) in 0.5 ml of propylene glycol. The specific radioactivity was determined as described by Robinson *et al.* (1973).

Enzymatic Assays. To 1 ml of proelastase A solution (0.3–1.0 mg) was added 1 ml of 0.2 M Tris-HCl buffer (pH 8.0) containing 0.1 M $CaCl_2$ and sufficient porcine trypsin to yield a zymogen : enzyme weight ratio of 5:1. The solutions were incubated at 0° for 3 hr and the esterolytic activity was determined at 25° in a pH-Stat using Ac-$(Ala)_3$-OMe (7 mM), a specific elastase substrate (Gertler and Hofmann, 1970). In the case of proelastase B the zymogen : enzyme ratio was 10:1 and the incubation time 1.5 hr.

The activation mixtures were also assayed for chymotrypsin using Ac-Tyr-OEt (10 mM) as a substrate and for elastase using the orcein–elastin method as described by Sachar *et al.* (1955).

Purification Procedure. The starting material for the purification was the cationic protein fraction of the preparation of lungfish trypsinogen which was not adsorbed on DEAE-

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received October 26, 1973.* This work was supported by the National Institutes of Health (GM 15731). C. d. H. was a recipient of a fellowship from the Stiftung für Stipendien auf dem Gebiete der Chemie, Basel, Switzerland.

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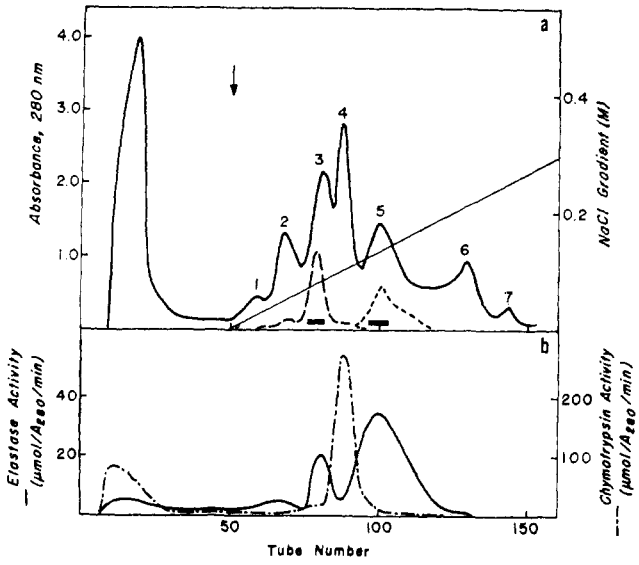

FIGURE 1: Chromatography of the cationic proteins from the pancreas of African lungfish on carboxymethylcellulose columns (see text for experimental details). (a) (——) Absorbance of cationic proteins eluted from the initial chromatography column, (— — —) rechromatography of peak fraction 3, (- - - - - -) rechromatography of peak fraction 5, (——) tubes pooled after rechromatography of peak fraction 3 (proelastase A) and peak fraction 5 (proelastase B). (b) Enzymatic activities after activation of the zymogens eluted from the initial chromatography column. (——) Elastase activity [Ac-(Ala)$_3$-OMe], (—·—) chymotrypsin activity [Ac-Tyr-OEt]. The activities are given in μmol of substrate hydrolyzed per min per absorbance unit of protein (1 absorbance unit of protein represents 1 ml of a solution of protein with $A_{280}^{1\,cm} = 1.0$).

cellulose (Reeck and Neurath, 1972). After lyophilization, 1200 mg was dissolved in 100 ml of 0.01 M sodium phosphate–citrate buffer (pH 6.0) (Gomori, 1955) and applied to a carboxymethylcellulose column (2.5 × 30 cm) previously equilibrated with the same buffer. The column was initially eluted with that buffer (600 ml) and then with 2 l. of a mixture of buffers forming a linear gradient of 0–0.3 M NaCl. The flow rate was 90 ml/hr and the fraction volume, 14 ml. Fractions were assayed for proelastase and chymotrypsinogen. Two peak fractions containing proelastase (see Figure 1) were separately pooled (tubes 78–82 and 97–104), dialyzed against distilled water, lyophilized, and rechromatographed under identical conditions. Fractions having the highest proelastase activity were pooled again (see Figure 1), dialyzed, and lyophilized. All procedures were carried out at 4°.

Electrophoresis on Cellulose Acetate Membranes. Electrophoresis was performed on cellulose acetate membranes in a Beckman microzone electrophoresis system Model R-100 using 0.08 M collidine acetate buffer (pH 6.9). Samples of 0.75 μl (15 μg of protein) were applied and subjected to electrophoresis for 30 min at 400 V. The protein was detected by staining with Ponceau S.

Sodium Dodecyl Sulfate Gel Electrophoresis. Protein samples were denatured by adding them to a boiling solution of 1% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% β-mercaptoethanol. After 10 min at that temperature the samples were cooled and applied to gels following the general procedure of Weber *et al.* (1972). Molecular weights were calculated using beef liver catalase (mol wt 58,000/subunit), ovalbumin (mol wt 43,000), bovine chymotrypsinogen A (mol wt 25,700), and chicken egg-white lysozyme (mol wt 14,400) for calibration of a standard curve.

Reaction of Elastases with [^{14}C]Diisopropyl Fluorophosphate. To a solution (2 ml) of activated proelastase (4 mg) in 0.1 N Tris-HCl buffer (pH 8.0), 2 μl of [^{14}C]diisopropyl fluorophosphate stock solution were added. The enzyme was completely inactivated in 15 min at room temperature. After desalting on a column (0.9 × 20 cm) of Sephadex G-25 with 1 mM HCl, the protein concentration was estimated from the absorbance at 280 nm (see Table II) and the radioactivity was measured with a Packard Model 3003 TriCarb scintillation counter using an Aquasol (New England Nuclear) scintillant. After correcting for the trypsin added to activate proelastase, the specific incorporation of [^{14}C]diisopropyl fluorophosphate into elastase was calculated.

Pyridylethylation. The procedure of Friedman *et al.* (1970) was adapted to serine proteases by dissolving 5–25 mg of protein in 1 ml of 6 M guanidine hydrochloride containing dithioerythritol (68.3 mg, 0.44 mmol) previously adjusted to pH 3.5 with formic acid. After 2 hr at room temperature, Tris (15.8 mg, 0.13 mmol) was added. The pH was then adjusted to 7.5 with concentrated HCl. After 4 hr 100 μl (0.93 mmol) of 4-vinylpyridine was added. After 2 hr the reaction mixture was exhaustively dialyzed against 5% acetic acid and lyophilized.

Sequence Analysis. Amino-terminal sequences of the proteins were determined by automated Edman degradation using a Beckman Sequencer Model 890A. The methods were those of Edman and Begg (1967) as modified by Hermodson *et al.* (1972). Stepwise yield was estimated by comparison of gas chromatographic peak heights of identical amino acids separated by a given number of degradative cycles. The stepwise yield was 94–96% in all analyses.

Results

Purification. Reeck *et al.* (1970) first described the presence of two cationic proelastases in the pancreas of the African lungfish. These two proelastases, A and B, were isolated as summarized in Figure 1 and Table I. Seven chromatographic protein peaks were obtained during gradient elution (see solid line in Figure 1a). Peak fractions 2 and 4 contained mainly potential chymotrypsin activity and peak fractions 3 and 5, potential elastase activity. Further purification of these fractions was achieved by repeating the chromatography under identical conditions (see dashed lines in Figure 1a). Only 50% of the activity applied to the column was recovered in each case, indicating some inactivation during chromatography.

TABLE I: Recovery of Esterase Activity toward Ac-(Ala)$_3$-OMe During Purification of Proelastases from the African Lungfish.[a]

Fraction	mg	Activity: Sp (μmol/mg per min)	Activity: Total (μmol/min)	Yield (%)
DEAE-cellulose, void volume	1200	9	10,800	100
CM-cellulose, fraction 3	72	35	2,520	23.4
Rechromatography of fraction 3 (proelastase A)	30	41	1,230	11.4
CM-cellulose, fraction 5	85	40	3,400	31.4
Rechromatography of fraction 5 (proelastase B)	35	51	1,790	16.6

[a] $A_{280}^{0.1\%}$, uncorrected for moisture, of the lyophilized proelastase A was 1.55 and of proelastase B, 1.05.

Similar instability of porcine proelastase has been reported by Gertler and Birk (1970). After the second chromatography, the preparations were checked for purity by electrophoresis on cellulose acetate membrane at pH 6.9. They were visually estimated to be at least 90% pure. Sodium dodecyl sulfate gel electrophoresis showed a single band in each case corresponding to a molecular weight of about 25,000. The high purity of the preparations was further confirmed by sequenator analysis, which gave one sequence only, in a yield of about 90% in the first degradation step. It should be noted, however, that peak fraction 4 contained a chymotrypsinogen (Figure 1b), which after purification, had a blocked amino-terminal residue (A. Gertler and C. de Haën, unpublished results). Contamination by this material would, therefore, not be detected by sequenator analysis.

Some preparations of purified proelastase still contained up to 10% chymotrypsinogen as measured after activation with trypsin by active-site titration with the specific chymotryptic titrant *p*-nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide (Wang and Shaw, 1972). This result was confirmed by measuring the contaminating chymotrypsin activity, assuming identical specific activity for both lungfish and bovine α-chymotrypsin. Since this activity could be completely inhibited by Boc-Gly-Leu-Phe-chloromethane (Kurachi *et al.*, 1973) without affecting the activity toward Ac-$(Ala)_3$OMe or orcein–elastin, it was concluded that lungfish elastases do not hydrolyze Ac-Tyr-OEt. Furthermore, no activity toward Tos-Arg-OMe could be detected other than that due to added trypsin.

Activity Studies. To find optimal conditions for activation of the proelastases a set of experiments were carried out, in which the zymogens were activated by porcine trypsin at pH 8.0, 0°. Five different zymogen to trypsin weight ratios were chosen, varying from 50:1 to 5:1, and the activation process was followed up to 24 hr. Maximal activation of proelastase A was obtained after 3-hr incubation of the zymogen with trypsin (weight ratio 5:1) and maximal activation of proelastase B was obtained after 1.5 hr of incubation with a 10:1 zymogen to trypsin mixture. The same maximal activities could be obtained with higher zymogen to trypsin ratios after longer times of activation. Maximal activity remained constant for at least 24 hr, thus indicating that the esterase activity toward Ac-$(Ala)_3$-OMe was completely unaffected by the presence of porcine trypsin in the activation mixture. Prior to activation esterolytic activities of the proelastases A and B were only 2.2 and 0.8% those of the activated enzymes, and may have been due to traces of activated zymogens. In Table II some properties of the activated proelastases are summarized and compared to those of porcine elastase and bovine α-chymotrypsin. The esterolytic and elastolytic activities of the activated proelastases were considerably lower than those of porcine elastase. Although electrophoretic analyses both on cellulose acetate and sodium dodecyl sulfate gels and sequenator analyses both before and after activation under the conditions given in the following chapter indicated a high degree of homogeneity of the zymogens, incorporation of [^{14}C]diisopropyl fluorophosphate was low (Table II). Nonstoichiometric incorporation was probably caused by the presence of nonactivatable (denatured) zymogen rather than by contamination with foreign proteins or by partial enzymatic digestion.

The elastolytic activity of elastase A was significantly lower than that of elastase B, although V_{max} for ester hydrolysis and the extent of incorporation of [^{14}C]diisopropyl fluorophosphate were quite similar. However, elastolytic activity of proteases is known to be dependent both on substrate specificity and on the ability to be adsorbed by elastin (Gertler, 1971a,b). Elastase A has approximately sixfold lower affinity for Ac-$(Ala)_3$-OMe and is also a more acidic protein than elastase B. Both properties would be expected to lower the elastolytic activity.

TABLE II: Comparative Esterolytic and Elastolytic Activities of Activated Lungfish Proelastases, Porcine Elastase, and Bovine α-Chymotrypsin.

Enzyme	Substrate: Ac-$(Ala)_3$-OMe V_{max}[a,b]	Ac-$(Ala)_3$-OMe K_m[a] (mM)	Orcein–Elastin[c]	Incorporation [^{14}C]-DFP (mol/mol)[d]
Activated proelastase A	47	1.12	0.11	0.47
Activated proelastase B	53	0.19	0.45	0.43
Porcine elastase	168	0.43	1.00	1.00
Bovine α-chymotrypsin	11	13.70	0.00	0.86

[a] By extrapolation from Eadie–Hofstee plots. The concentration range of the substrate was 0.7–7.0 mM for the elastases and 0.4–20.0 mM for bovine α-chymotrypsin. [b] In μmol per mg per min. [c] In arbitrary units, relative to porcine elastase. [d] Relative to porcine elastase, assuming a molecular weight of 25,000. The concentrations of the proelastases were calculated from the absorption values reported in Table I assuming 10% water content. DFP = diisopropyl fluorophosphate.

Sequence Analysis. The amino-terminal sequences of proelastases A and B were determined both before and after reduction and pyridylethylation. Regardless of modification only one sequence was observed in each case (Figure 2). Occasionally a preparation of proelastase A contained in addition to the intact zymogen some material lacking the amino-terminal residue, presumably due to the action of a contaminating aminopeptidase in the course of the isolation. The yields of phenylthiohydantoyl amino acids in the first several turns of sequenator analyses exceeded 80% with respect to protein applied to the cup.

Sequenator analysis of proelastase B yielded a consecutive sequence up to position 31 (27 sequenator turns), and permitted the identification of certain residues as far as residue 46 (44 sequenator turns). Sequenator analysis of proelastase A yielded a much shorter sequence, presumably due to the five prolyl residues and one Asn-Gly sequence present among the first 28 residues (Hermodson *et al.*, 1972).

Samples of both proelastases A and B were maximally activated using a zymogen to trypsin weight ratio of 20:1. Thereafter the pH was dropped to 3 with formic acid and the proteins were desalted on Sephadex G-25, lyophilized, and analyzed on the sequenator. Only one sequence was observed in each case, starting with Val-Val and corresponding to the sequences starting at position 16. This showed that activation was the result of cleavage of the bond between Arg-15 and Val-16, as expected on the basis of sequence homology. Furthermore, since no additional splits in the elastases could be detected, it showed that the observed specificities were those of the intact enzyme. Thus the possibility that the observed specificities were due to additional cleavages in the polypeptide chain, as for example in the case of pseudotrypsin (Keil-Dlouhá *et al.*, 1971), could be ruled out.

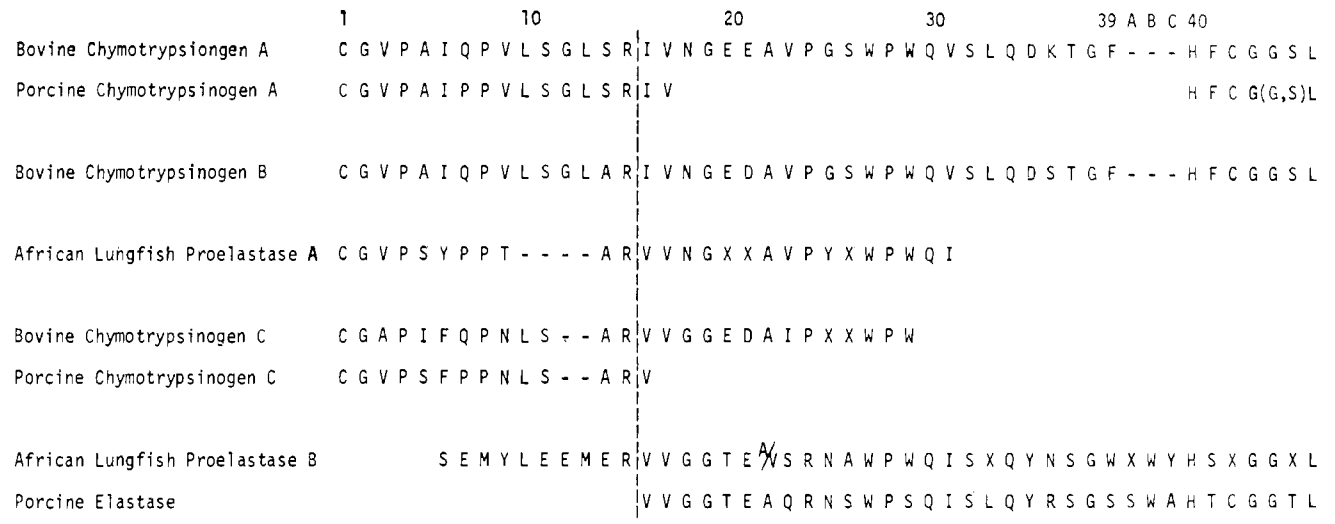

FIGURE 2: Alignment of amino-terminal sequences of proelastase A and B from the African lungfish with those of porcine elastase and bovine and porcine chymotrypsinogens. The sequence of bovine chymotrypsinogen C (subunit II) is from Uren and Neurath (1972); all other published data are from Dayhoff (1972). The one letter code is: A = Ala, R = Arg, N = Asn, D = Asp, C = Cys, Q = Gln, E = Glu, G = Gly, H = His, I = Ile, L = Leu, K = Lys, M = Met, F = Phe, P = Pro, S = Ser, T = Thr, W = Trp, Y = Tyr, V = Val. Unidenitified residues are indicated by X. A/V indicates an allotypic replacement. Dashes are indicating alignment gaps. The numeration of residue positions is that traditional for bovine chymotrypsinogen A. The broken vertical line indicates the position of activation.

It should be noted that both alanine and valine were found in approximately equal amounts at position 22, both in native and activated proelastase B, indicating microheterogeneity.

Discussion

Two proelastases, A and B, each having a molecular weight of approximately 25,000 were isolated from the pancreata of the African lungfish. After tryptic activation both zymogens exhibited activity toward the specific elastase substrates, orcein-elastin and Ac-$(Ala)_3$-OMe, but the B enzyme had a higher activity toward these substrates than the A enzyme. Neither of them was active toward substrates for trypsin (Tos-Arg-OMe) or chymotrypsin (Ac-Tyr-OEt). Both enzymes were inhibited by diisopropyl fluorophosphate but neither by Boc-Gly-Leu-Phe-CH_2Cl, a specific chymotrypsin inhibitor (Kurachi *et al.*, 1973). Amino-terminal sequence analysis yielded in addition to the sequence of the activation peptides an amino-terminal sequence of the active enzyme which established that the two proteins are homologous to other pancreatic serine proteases. Figure 2 shows an alignment of the sequences of the two proelastases with porcine elastase and with bovine and porcine chymotrypsinogens. The particular alignment presented is based on a more extensive inquiry into the evolution of the serine proteases, which will be described in a forthcoming publication (C. de Haën, H. Neurath and D. C. Teller, in preparation).

Proelastase B yielded an activation peptide which displayed no obvious structural similarity to any of the known activation peptides of other pancreatic serine proteases. However, the cluster of acidic residues preceding the point of activation (Arg_{15}-Val_{16}) is reminiscent of the activation peptides (Reeck and Neurath, 1972) of the trypsinogens. The length of the activation peptide is similar to that suggested for porcine proelastase on the basis of differences in amino acid composition between zymogen and enzyme (Gertler and Birk, 1970) but the amino acid compositions of the two proelastase activation peptides are rather dissimilar. It may be noted, however, that an arginyl residue precedes the bond cleaved during activation, just as suggested for porcine proelastase (Gertler and Birk, 1970). The length of the chain from the amino terminus of the active enzyme (Val_{16}) to the position corresponding to the first half-cystinyl residue in porcine elastase (position 42) is the same in both the lungfish B and the porcine enzyme and is three residues longer than in chymotrypsin, and five residues longer than in trypsin. Considering only positions identified within the region of residues 16–41, there are 10 minimum base differences (MBD) between proelastase B and porcine elastase, but 18–19 MBD between proelastase B and both chymotrypsins or trypsins. The sequence homology, the chain length in the amino-terminal region of the active enzyme and the similar specificity and kinetic parameters all indicate that lungfish proelastase B and porcine proelastase evolved from a common ancestor having elastase specificity.

African lungfish proelastase A yielded the activation peptide given in Figure 2. Despite the difference in length, the alignment of this activation peptide with those of the chymotrypsinogens was unambiguous due to a high degree of relatedness. In particular, the presence of an amino-terminal half-cystinyl residue in lungfish proelastase A suggests that this zymogen may contain a disulfide bridge homologous to that which links residues 1 and 122 in the chymotrypsinogens. Amino acid composition of porcine proelastase (Gertler and Birk, 1970) and the sequence of the active enzyme (Hartley and Shotton, 1971) definitively preclude such a bridge in porcine elastase. Lungfish proelastase B also lacks this disulfide bond.

Unfortunately, only limited sequence data could be obtained beyond the point of activation (Arg_{15}-Val_{16}). Comparing, nonetheless, those residues that have been identified among residues 16–31, it appears that lungfish elastase A is more closely related to the chymotrypsins (3 MBD) than to the trypsins (15–16 MBD), porcine elastase (8 MBD) or lungfish elastase B (6 MBD). This order of relatedness corresponds to that of the respective activation peptides. More precisely, the number of minimum base differences which separates the activation peptide of proelastase A from those of porcine and bovine chymotrypsinogen C is smaller (average 3.5 MBD) than the number of MBD separating it from those of chymotrypsinogens A and B (average 5.8 MBD). This comparison suggested to us that proelastase A might be more closely related to chymotrypsinogen C than to chymotrypsinogen A or

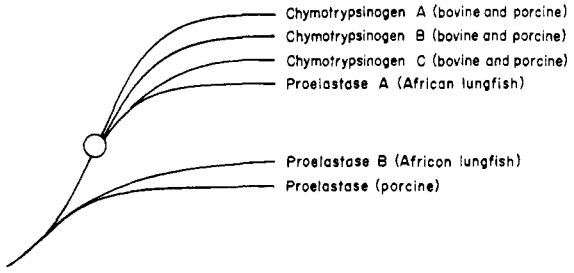

FIGURE 3: Evolutionary relationships of chymotrypsinogens and proelastases.

B. This suggestion is further supported by the similar location of the gaps (positions 10–13 and 12–13) necessary to align proelastase A and chymotrypsinogen C with chymotrypsinogens A and B. Moreover, when the activation peptides of proelastase A and those of porcine and bovine chymotrypsinogen C are contrasted, only single base changes result; interconversion of the activation peptides of proelastase A and those of chymotrypsinogen A or B requires several double base changes. According to the stoichastic model of evolution (Holmquist *et al.*, 1972) this fact also indicates closer relationships of proelastase A with chymotrypsinogen C than with chymotrypsinogens A or B.

These findings together with the kinetic properties of the two enzymes suggested to us the evolutionary tree shown in Figure 3. According to this scheme four possibilities exist. (a) Proelastase A is derived from the gene previously coding for chymotrypsinogen C. (b) Proelastase A appeared after duplication of the gene coding for chymotrypsinogen C. (c) Chymotrypsinogen C is derived from the gene previously coding for proelastase A. (d) Chymotrypsinogen C appeared after duplication of the gene coding for proelastase A.

In cases a and b, proelastase A acquired elastolytic specificity independent of proelastase B or porcine elastase. In cases c and d, chymotrypsinogen C acquired chymotryptic specificity independent of chymotrypsinogens A and B. It is interesting to note that in any case at least two parallel specificity changes must have taken place. These presumably require relatively small changes in the binding site of the enzyme, as may be inferred from a comparison of bovine trypsin, chymotrypsin, and porcine elastase (Hartley and Shotton, 1971). *In vitro* assays using substrates such as Ac-$(Ala)_3$-OMe and elastin do not necessarily reflect those physiological functions of the enzymes which are maintained by natural selection. Thus, the independent specificity changes observed may not be the result of similar selection pressures.

We have described in this report a new type of pancreatic serine protease, proelastase A. Should this enzyme be found in other species too, it will represent a new type of serine protease that combines specificity of elastase with structural similarity to chymotrypsinogens, and could be called *allo*-elastase. Recently a new serine protease from the hepatopancreas of the fiddler crab has been described having both tryptic and collagenolytic specificity (Eisen *et al.*, 1973). It appears, therefore, that more than the three digestive serine proteases trypsin, chymotrypsin, and elastase may exist. In view of these findings caution should be exercised in assigning new enzymes to the known classes of serine proteases and in assessing the evolution of function of digestive enzymes solely on the basis of their specificity.

Acknowledgments

We thank Dr. Hans Neurath and Dr. Kenneth A. Walsh for support, encouragement, and advice, and Lowell H. Ericsson for the sequenator analyses.

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