Trypsin-Like Neutral Protease Associated with Soluble Elastin†

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ABSTRACT: Isolation of tropoelastin is complicated by the presence of a neutral protease closely associated with tropoelastin that is capable of sequentially degrading tropoelastin to small peptides. Substrate and inhibitor specificities of this neutral protease associated with purified tropoelastin were examined. The enzyme displayed proteolytic activity against casein, and esterase activity was detected when assayed against *N*-tosyl-L-arginine methyl ester but not against *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester. No appreciable elastinolytic activity was detectable against either insoluble sodium dodecyl sulfate treated elastin or maleylated tropoelastin. The enzyme was not inhibited by the chymotrypsin inhibitor toluenesulfonylphenylalanine chloromethyl ketone. The enzyme was inhibited by phenylmethanesulfonyl fluoride and, to various degrees, by metal chelators. Tosyllysyl chloromethyl ketone, ε-aminocaproic acid, and Aprotinin (pancreatic trypsin inhibitor—Kunitz type), all inhibitors of trypsin-like enzymes, were very effective inhibitors, as were soybean trypsin inhibitor and human α-1-antitrypsin. These data suggest that the tropoelastin-associated enzyme is a neutral serine protease with trypsin-like specificity.

A major focus of elastin research has been the study of the structure and biosynthesis of tropoelastin (Sandberg et al., 1977), a salt-soluble elastin-like molecule (Sandberg et al., 1969) detected in pathological states in which the elastin cross-linking reactions are inhibited (Sandberg et al., 1969; Rucker et al., 1973; Miller et al., 1965; Pinell and Martin, 1968). Unlike mature, cross-linked elastin, tropoelastin is very susceptible to proteolytic attack (Mecham et al., 1976, 1977). This characteristic has made its isolation and characterization very difficult.

One method used to diminish proteolytic degradation of tropoelastin during its preparation is to include various enzyme inhibitors ($PhCH_2SO_2F$,[1] MalNEt, and EDTA) in the isolation buffers (Foster et al., 1975; Rucker et al., 1975). This, however, has proven only to be a palliative measure. Once the inhibitors are removed, tropoelastin again undergoes degradation when dissolved in a buffer at neutral pH. In this report, we further characterize a neutral serine protease with trypsin-like specificity associated with tropoelastin. More effective inhibitors of the enzyme are described which, when incorporated into a scheme for tropoelastin purification, allowed for the detection of proelastin, a high molecular weight species of soluble elastin (Foster et al., 1976; Rucker et al., 1977).

Materials and Methods

Materials

Chemicals and enzymes were obtained as follows: ε-aminocaproic acid, *o*-phenanthroline monohydrate, TLCK, TPCK, α,α-dipyridyl, $PhCH_2SO_2F$, tryptamine, Aprotinin (pancreatic trypsin inhibitor-Kunitz type), Plasmin, and Tos-ArgOMe from Sigma Chemical Co.; soybean trypsin inhibitor, human α-1-antitrypsin, TPCK-trypsin, and α-chymotrypsin from Worthington Biochemicals; diethyldithiocarbamic acid—diethylammonium salt from Pfalz and Bauer, Inc; penicillamine from Aldrich Chemical Co.; *N*-ethylmaleimide from Eastman; maleic anhydride from Pierce Chemical Co.; Agarose-soybean trypsin inhibitor and agarose-lysine from Miles-Yeda LTD; *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester from Mann Research Laboratories. $Ac(Ala)_4CH_2Cl$ was a gift from J. C. Powers, Georgia Institute of Technology. All other materials were of the highest quality available.

Methods

Isolation of Chick Tropoelastin. Tropoelastin was isolated from aortae of 7-day lathyritic chicks using the neutral salt extraction method described by Foster et al. (1976).

Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969) at room temperature overnight in 10% polyacrylamide gels at 2–3 mA/gel. The electrophoresis buffer was 0.1 M sodium phosphate (pH 7.2) containing 0.1% $NaDodSO_4$ and 0.5 M urea. Gels were stained with Coomassie brilliant blue. The molecular weights were estimated using a computer program which fit a logarithmic curve ($y = a + b \ln x$) to a set of data points supplied by the apparent molecular weights and distance of migration of the following proteins which were used as standards: bovine serum albumin (68 000), ovomucoid (45 000), pepsin (35 000), trypsin (23 300), and lysozyme (14 300) or ribonuclease (14 000).

Stoichiometry—Gel Scanning. The relative amount of protein in each band was determined by scanning the destained gel at 550 nm in a Beckman DU spectrophotometer Model 2400 equipped with a Gilford linear transport attachment and

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[1] Abbreviations used: TLCK, tosyllysyl chloromethyl ketone; TPCK, toluenesulfonylphenylalanyl chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tos-ArgOMe, *N*-tosyl-L-arginine methyl ester; *t*-BocAlaONp, *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester; MalNEt, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

integrating the area under the absorption curve. Care was taken to stain all gels for an equal period of time to minimize variations in protein dye absorption. The variation observed in calculating the concentrations of equivalent amounts of bovine serum albumin run on seven different gels was less than 5%.

Incubation Studies. Tropoelastin associated protease activity was detected by monitoring degradation of the 72 000 molecular weight tropoelastin to characteristic lower molecular weight products by electrophoresis in $NaDodSO_4$-polyacrylamide gels. Incubations of tropoelastin were routinely performed in 0.05 M phosphate buffer (pH 7.0) at 37 °C for 16 h. Tris (0.05 M) and 0.05 M borate buffers at pH 8.0 were also used in some studies. A stock solution of tropoelastin-enzyme complex was made fresh in distilled water (1 mg/0.1 mL) before each experiment. Ten microliters of this solution was then added to 0.1 mL of the incubation buffer. After the incubation period, 0.1 mL of gel sample buffer (0.01 M phosphate, pH 7.2, 0.1% $NaDodSO_4$, 0.1% in mercaptoethanol, 20% glycerol) was added to the incubation mixture and the combined solutions were heated in a boiling water bath for 5–10 min. The sample was then electrophoresed in $NaDodSO_4$-polyacrylamide gels.

Amino Acid Analyses. Samples for amino acid analysis were hydrolyzed under vacuum in constant boiling HCl at 110 °C for 16–24 h and analyzed using either a Jeolco 6AH or Beckman Model 119 automated amino acid analyzer. Values for serine and theonine are not corrected for losses due to hydrolysis. Tryptophan was estimated by the methods of Liu (1972) and Bencze and Schmid (1957). One-half-cystine was determined as cysteic acid by the method of Hirs (1967).

Inhibitor Studies. The effects of various inhibitors on tropoelastin degradation were studied by adding the inhibitors to the incubation buffer prior to the addition of the tropoelastin-enzyme complex. The inhibitors studied and their concentrations were: (1) *N*-ethylmaleimide, EDTA, α,α-dipyridyl, penicillamine, tryptamine, diethyldithiocarbamic acid, and *o*-phenanthroline, each at 1, 10, and 50 mM; (2) soybean trypsin inhibitor, human α-1-antitrypsin, and ovomucoid, 0.1 mg; (3) tosyl-L-lysine chloromethyl ketone hydrochloride, 1, 10, and 100 mM; (4) toluenesulfonylphenylalanine chloromethyl ketone, 1, 10, and 100 mM added in 0.01 mL of methanol or propanol; (5) ϵ-aminocaproic acid 1, 10, and 200 mM; (6) $AcAla_4CH_2Cl$ added as a suspension; (7) Aprotinin (purchased as a sterile solution containing 100 000 kallikrein inactivator units), 1 μL added to the incubation buffer; and (8) phenylmethanesulfonyl fluoride, 1, 10, and 100 mM added in 0.01 mL of methanol, isopropyl alcohol, or dimethylformamide.

Esterolytic Activity. Esterolytic activity against *p*-toluenesulfonyl-L-arginine methyl ester was measured using the method of Hummel (1959). A 0.01 M solution (0.3 mL) of Tos-ArgOMe was mixed with 2.6 mL of Tris buffer (0.046 M, pH 8.1), and 100 μL of the tropoelastin stock solution was added. The optical density of the solution at 247 nm was determined against a blank containing 2.6 mL of Tris buffer, 0.3 mL of Tos-ArgOMe, and 0.1 mL of distilled water.

Esterolytic activity was also measured against *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester essentially as described by Janoff (1969). A 0.2 mM solution of substrate was prepared by dissolving 6 mg of *t*-Boc-Ala-ONp in 1.0 mL of acetonitrile and diluting with 99 mL of 0.05 M sodium phosphate buffer (pH 8.0). Three milliliters of the substrate solution and 0.1 mL of the tropoelastin stock solution were mixed. Liberation of *p*-nitrophenol was followed spectrophotometrically at 400 nm. A standard curve using various amounts of pancreatic elastase was also determined.

Caseinolytic Activity. Proteolytic activity was determined using casein as substrate (Kunitz, 1947). The substrate solution was prepared by suspending 1 g of casein in 100 mL of 0.1 M phosphate buffer (pH 8.0) and heating for about 15 min in a boiling water bath until all the casein was dissolved.

In a typical enzyme assay, 50, 100, 150, and 300 μL of the tropoelastin stock solution were diluted to 1.0 mL with phosphate buffer and added to prewarmed substrate solution (37 °C). After exactly 20 min, 3.0 mL of 5% trichloroacetic acid was added, and the solution was throughly mixed and allowed to stand for at least 30 min at room temperature. The tubes were then centrifuged and the optical density of the supernatant was read at 280 nm against water. As a control, 50, 100, 150, and 300 μL of tropoelastin which had been boiled to inactivate the associated protease(s) were also assayed, as were varying amounts of TPCK-trypsin.

Elastinolytic Activity. Proteolytic activity against insoluble elastin was assayed using the method of Stone et al. (1977). One-milligram aliquots of tropoelastin-enzyme complex were incubated at 37 °C with $NaDodSO_4$-treated bovine *ligamentum nuchae* elastin which had been radiolabeled to a high specific activity with sodium borotritide. Elastinolytic activity in this system is detected as radioactive counts (solubilized peptides) released from the insoluble elastin substrate by proteolysis.

Maleylation of Tropoelastin. Maleic anhydride (2.5 mg) was added to 1 mg of tropoelastin dissolved in 0.6 mL of 0.05 M phosphate buffer (pH 8.3) at 4 °C. The reaction was allowed to proceed for 1 h by maintaining the pH at 8 with 0.1 N NaOH. Excess reagent was removed by gel filtration over a Bio-Gel P-2 column using 0.05 M phosphate buffer (pH 8.0) as the eluting buffer.

The extent of maleylation of the tropoelastin was judged by focusing the protein at its isoelectric point using a LKB 2117 Multiphor unit utilizing analytical thin-layer gel electrofocusing in a polyacrylamide gel (pH range 3.5–9.5).

To determine the effects of maleylation on the enzymatic degradation of tropoelastin, maleylated tropoelastin was incubated in phosphate buffer at pH 8. To differentiate between modification effects on the enzyme and substrate, an aliquot of unmaleylated tropoelastin-enzyme complex was incubated with the maleylated tropoelastin. As a control, boiled tropoelastin which had been shown not to degrade upon incubation was incubated with tropoelastin which had been shown to have active enzyme.

DEAE Chromatography. DEAE-cellulose (Whatman DE52, preswollen) was equilibrated in three changes of 0.05 M Tris–0.02 M lysine and poured as a slurry into a 1.7 × 12 cm column. The tropoelastin-enzyme complex was dissolved in the starting buffer (0.05 M Tris–0.2 M lysine, pH 8.0), applied to the column, and eluted using a stepwise gradient using successive 100 mL volumes of starting buffer containing no NaCl, 0.1 M NaCl, and 1.0 M NaCl at a flow rate of 30 mL/h. The optical density of the collected fractions was determined at 230 nm using a Beckman Model 24 spectrophotometer. Pooled fractions were dialyzed extensively against water at 4 °C and lyophilized. A portion of the lyophilized fractions was hydrolyzed and subjected to amino acid analysis. The fraction identified as tropoelastin by amino acid analysis was then reconstituted in 0.05 M phosphate buffer at pH 8 and incubated at 37 °C. In a second experiment, 0.2 M ϵ-aminocaproic acid was substituted for lysine in the DEAE buffers.

Affinity Chromatography. A column containing 20 mL of soybean trypsin inhibitor covalently bound to agarose was equilibrated at 4 °C with 0.2 M Tris (pH 7.8) containing 0.5

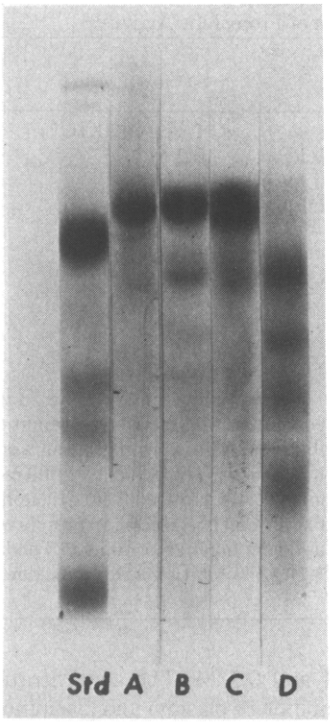

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of the tropoelastin used in this study (gel A). Gel D is tropoelastin after incubation at 37 °C (pH 8) for 12 h. The molecular weights of the stained bands are (from top to bottom) 57 000, 45 000, 36 000, and 24 500. Gel B is tropoelastin which was boiled to inactivate associated protease activity and then incubated as above. Gel C contains tropoelastin which was reduced and alkylated before incubation (Mecham et al., 1977), and gel Std contains rat tail collagen (>100 000 daltons), bovine serum albumin (68 000 daltons), pepsin (35 000 daltons), and ribonuclease (14 500 daltons). Gels were stained with Coomassie brilliant blue. The anode was at the bottom of the gels.

M NaCl. Bovine TPCK-trypsin was applied to the column in excess of the column capacity and washed with 0.1 N sodium acetate, pH 3, and the trypsin concentration determined spectrophotometrically at 280 nm ($E_{280}^{1\%} = 13.5$). The column was found to have a capacity of 3.3 mg of trypsin per mL of settled resin.

Tropoelastin (1–10 mg) was dissolved in 1 to 3 mL of starting buffer and layered on the top of the column. The flow rate was regulated at 30 mL/h and 5-mL fractions were collected. After approximately 3 h of collecting, the column was washed with 0.1 M sodium acetate at pH 3 to elute the retained protein. The optical density of the collected fractions was determined at 280 and 230 nm.

The retained and nonretained fractions were dialyzed against water (4 °C) and lyophilized. An aliquot was hydrolyzed and its amino acid composition determined. Enzymatic activity in the fraction identified by amino acid analysis and $NaDodSO_4$–polyacrylamide gel electrophoresis as tropoelastin (that fraction not retained on the column) was investigated by: (a) incubating the tropoelastin in phosphate buffer; and (b) analyzing for esterolytic activity with Tos-ArgOMe. Tos-ArgOMe was also used as a substrate for the retained fraction.

In another experiment, tropoelastin–enzyme complex was passed over a lysine-bound agarose column in phosphate buffer (0.3 M, pH 8.0). Elution was carried out using 0.2 M ϵ-aminocaproic acid (pH 8.0). All steps were carried out at room temperature.

Results

The tropoelastin used in this study showed only a single band (70 000 molecular weight) on $NaDodSO_4$ gels (see Figure 1, gel A). The amino acid composition has been given elsewhere (Foster et al., 1975; Mecham et al., 1976).

Degradation of the tropoelastin was observed when the protein was incubated in phosphate or Tris buffer at neutral pH (Mecham et al., 1976). Figure 2 shows the densitometry tracings of $NaDodSO_4$ gels, indicating the degradation pattern after 1, 3, 7, and 14 h incubations (37 °C). The first degradation product to appear has a molecular weight of 57 000. This is then followed by appearance of four lower bands with molecular weights of approximately 45 000, 36 000, 24 500, and 13 000–14 000. After 30 h of incubation, all the resultant peptides ran with the tracking dye and, hence, were of molecular weights less than 15 000.

Enzyme activity was quantitated by measuring the disappearance of 72 000 molecular weight tropoelastin by scanning the stained $NaDodSO_4$ gel and integrating the area under the absorption curve. The ratio of the peak area under the incubated tropoelastin to the area of a control sample (an equivalent amount of tropoelastin before incubation) expresses the relative enzyme activity, or in the case of inhibitor studies, multiplying the ratio by 100 gives percent inhibition. In the absence of enzyme (or with complete inhibition), the ratio

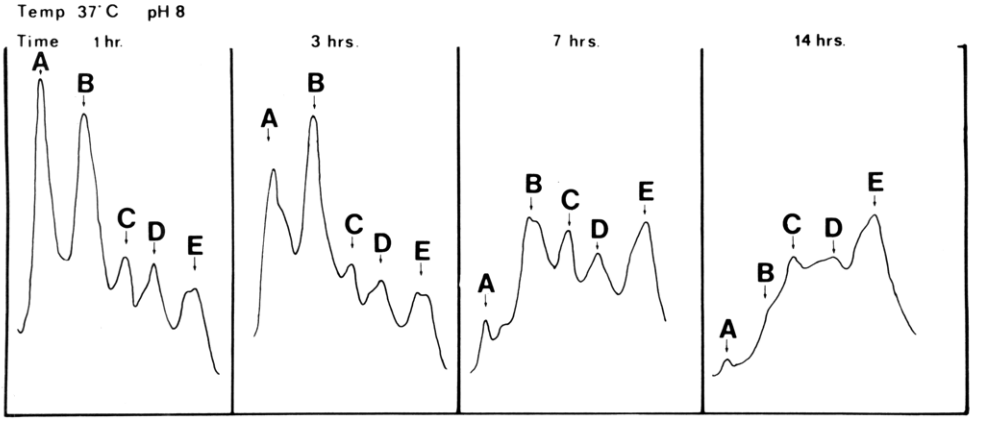

FIGURE 2: Densitometry tracings (550 nm) of Coomassie brilliant blue stained sodium dodecyl sulfate–polyacrylamide gels showing the tropoelastin degradation sequence after 1, 3, 7, and 14 h of incubation (see Methods). After 30 h, no material other than a small amount of the 24 500 molecular weight species was detectable. The apparent molecular weight of each fraction is: (peak A) 72 000, (peak B) 57 000, (peak C) 45 000, (peak D) 36 000, and (peak E) 24 500.

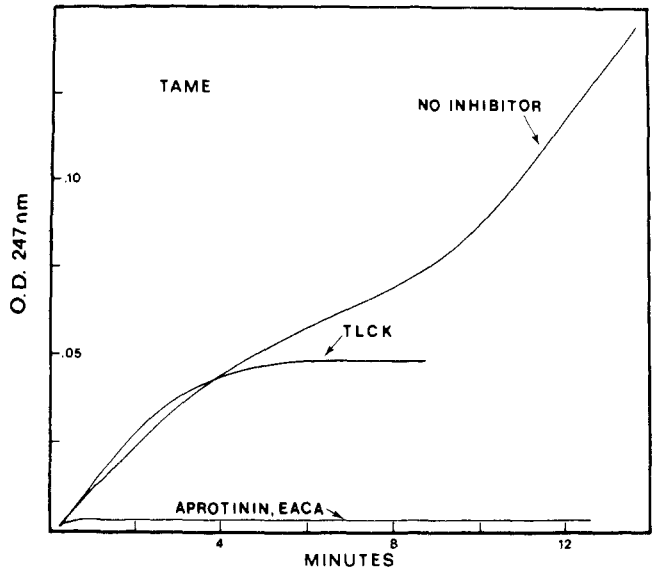

FIGURE 3: Tracing of spectrophotometer graph showing hydrolysis of tosylarginine methyl ester by the tropoelastin-enzyme complex. Also shown are the effects of adding 0.1 M ϵ-aminocaproic acid (EACA), aprotinin (1 unit), and 0.01 M tosyllysine chloromethyl ketone (TLCK) to the incubation mixture at zero time.

would be approximately 1; in the presence of active enzyme (or with partial or no inhibition), the ratio would be less than 1.

When tropoelastin was incubated in Tris buffer which contained $CaCl_2$ at a concentration normally used to enhance the rate of pancreatic trypsin (0.011 M), no degradation occurred. This was also the case when borate buffer was used for incubations.

Inhibitor Studies. In order to better characterize the enzyme involved in tropoelastin degradation, various inhibitors were used as described in the Materials and Methods section. In general, the inhibitors which act as metal chelators showed moderate inhibition at high concentrations (greater than 10 mM) but were only marginally effective at lower concentrations. The inhibitors which are specific for chymotryptic-like enzymes (TPCK and tryptamine) were ineffectual. The specific irreversible elastase inhibitor $Ac(Ala)_4CH_2Cl$ (Powers and Tuhy, 1973) had no effect at low or high concentrations. However, because this inhibitor remained somewhat insoluble in the incubation buffer, 0.1 mg of pancreatic elastase was incubated with the inhibitor as a control. The $Ac(Ala)_4CH_2Cl$ completely inhibited the elastase (as assayed against *t*-BocAlaONp) even though much of the inhibitor did not go into solution.

Phenylmethanesulfonyl fluoride was able to inhibit tropoelastin degradation at concentrations above 50 mM when dissolved in propanol and added to the incubation mixture. Inhibition was marginal at a concentration of 10 mM and absent at 1 mM. When $PhCH_2SO_2F$ was dissolved in dimethylformamide, inhibition was excellent, even at concentrations less than 10 mM. Dimethylformamide itself had no inhibitory effect at the concentration used.

The most effective inhibitors were TLCK, ϵ-aminocaproic acid, and Aprotinin, all inhibitors of trypsin-like enzymes (see Figure 3 and Table I). Trypsin inhibitor from egg white (ovomucoid) was an exception, however, and appeared to have little or no inhibitory effects. Aprotinin appeared to be the most potent inhibitor as judged using the tropoelastin-$NaDodSO_4$ gel assay and by kinetic studies using Tos-ArgOMe. ϵ-Aminocaproic acid was also an excellent inhibitor at concentrations

TABLE I: Inhibition of Proteolytic Activity.[a]

Inhibitor	Concn	% inhibition (range)
Aprotinin	1 μL (100 KIU[b])	98-100
ϵ-Aminocaproic acid	0.2 M	98-100
Human α-1-antitrypsin	0.01 mg	95-100
Soybean trypsin inhibitor	0.01 mg	95-100
TLCK	0.01 M	85-95
$PhCH_2SO_2F$	0.01 M	50-95[c]
MalNEt	0.01 M	70-85
Metal chelators	0.01 M	30-55
TPCK	0.01 M	0
Chicken ovomucoid	0.1 mg	5%
$Ac(Ala)_4CH_2Cl$	0.01 M	0

[a] Expressed as percent 72 000 molecular weight tropoelastin remaining after incubations. Assays for inhibition were performed at pH 8 as described in Methods. The percent 72 000 molecular weight tropoelastin remaining was determined from densitometry at 550 nm of sodium dodecyl sulfate gels stained for protein with Coomassie brilliant blue. [b] Kallikrein inactivator units. [c] The large variability observed for $PhCH_2SO_2F$ inhibition is felt to be due to solubility effects.

between 10 mM and 0.2 M. The concentration, 0.2 M, is suggested for inhibition of plasmin and plasminogen activation (Plow and Edgington, 1975). Human α-1-antitrypsin and soybean trypsin inhibitor were also potent inhibitors, although their specificities are not restricted to trypsin-like enzymes.

Incubation of Maleylated Tropoelastin. Tropoelastin is a basic protein with a p*I* of between 9 and 11. The maleylated tropoelastin, however, isoelectrically focused at a pH of approximately 4 as a single sharp band, indicating successful modification of the ϵ-amino groups.

When maleylated tropoelastin was incubated at pH 8 (37 °C) for 16 h, relatively little degradation was detected (see Figure 4, gel A), as was the case when unmaleylated tropoelastin known to have active enzyme was incubated with the maleylated sample (Figure 4, gel B). The lower molecular weight bands observable in gel B are presumed to be the products of the unmaleylated sample. The amount of material in the 72 000 band determined by scanning the gel corresponds to the amount of maleylated sample included in the assay. When unmaleylated tropoelastin was incubated with tropoelastin which had been boiled to inactivate the associated protease activity, no 72 000 molecular weight protein was apparent after a 16-h incubation.

Caseinolytic Activity. The tropoelastin-enzyme complex demonstrated proteolytic activity toward casein. No activity was present with heat inactivated tropoelastin.

Elastinolytic Activity. The tropoelastin-enzyme complex showed no activity against insoluble $NaDodSO_4$-treated radiolabeled elastin. The sensitivity of the assay was such that 10 ng of pancreatic elastase equivalents could be detected (Stone et al., 1977), or approximately a 0.0001% contaminant of pancreatic elastase in the 1 mg of tropoelastin assayed.

Artificial Substrates. Artificial substrates were used to further characterize the specificity and, to some degree, the kinetics of the tropoelastin-associated enzyme. No activity was demonstrated against *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester, a substrate analogue commonly used for elastase and elatase-related enzymes. Activity was detected with Tos-ArgOMe, a trypsin substrate (see Figure 3). The kinetics of the reaction with Tos-ArgOMe were different than trypsin, however, with the tropoelastin enzyme showing a biphasic curve.

Affinity Chromatography. In an attempt to separate the

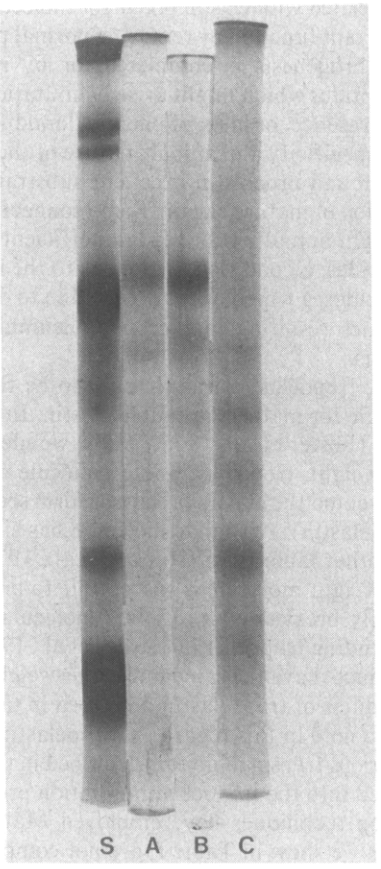

FIGURE 4: Sodium dodecyl sulfate electrophoresis of maleylated tropoelastin incubated at pH 8 (37 °C) for 16 h (Gel A). Gel B is maleylated and unmaleylated tropoelastin incubated together, and gel C is heat inactivated tropoelastin incubated with tropoelastin containing active enzyme. Gel S contains the following standard proteins: rat tail collagen, bovine serum albumin, and lysozyme. The anode was at the bottom of the gels.

enzyme from the tropoelastin–enzyme complex, tropoelastin was passed through an agarose–soybean trypsin inhibitor affinity column. The first protein peak eluted with the void volume and proved by amino acid analysis to be tropoelastin. The amino acid analysis of the second peak is given in Table II. The molecular weight of this retained material was 45 000 ± 4000 by $NaDodSO_4$ gel electrophoresis. When assayed against Tos-ArgOMe, both the tropoelastin which eluted with the void volume and the material retained by the column showed activity, although in both cases the rate was dramatically reduced from the rate exhibited by the starting material. Also, when the tropoelastin from the void volume peak was incubated at neutral pH, degradation occurred. Hence, the soybean trypsin inhibitor affinity column was not effective at removing all the proteolytic activity from the tropoelastin.

No material was retained by the lysine–agarose column using the conditions described in the Materials and Methods section.

DEAE-Cellulose Chromatography. $NaDodSO_4$ electrophoresis of tropoelastin after DEAE chromatography indicated lower molecular weight peptides in addition to the 72 000 molecular weight parent molecule. When this fraction was incubated at neutral pH, no further degradation occurred, indicating effectual removal of the associated protease by the DEAE column. The lower molecular weight peptides in the sample apparently resulted from degradation which occurred before or during the chromatography process, a condition which was alleviated when ε-aminocaproic acid was substituted for lysine in the chromatography buffers.

TABLE II: Amino Acid Composition of the Protein Fraction Retained by the Agarose–Soybean Trypsin Inhibitor Affinity Column.

Amino acid	Residues per 1000 residues
Hyp	0
Asp	117
Thr	71
Ser	83
Glu	91
Pro	57
Gly	104
Ala	86
½-Cys	43
Val	41
Met	17
Ile	36
Leu	63
Tyr	27
Phe	24
Trp	25[a] (29[b])
Hyl	0
Lys	59
His	23
Arg	33

[a] Determined chemically using the method of Liu (1972). [b] Determined spectrophotometrically using the method of Bencze and Schmid (1957).

Discussion

The data presented herein, as well as in a previous communication (Mecham et al., 1976), provide evidence for trypsin-like protease activity associated with purified tropoelastin. This activity is maximal at neutral pH (between 7 and 9) and results in degradation of tropoelastin in a very specific manner; first forming a 57 000 molecular weight product, followed by the appearance of fragments having molecular weights of 45 000, 36 000, 24 500, and 13 000–14 000. This fragmentation pattern is specific and reproducible, a property which can be used as a sensitive assay for the enzyme.

That the enzyme is a serine protease is demonstrated by the ability of $PhCH_2SO_2F$ to inhibit tropoelastin degradation. A trypsin-like specificity for the enzyme is indicated by the inhibition of tropoelastin degradation by TLCK (an active site directed chloromethyl ketone inhibitor of trypsin and many trypsin-like enzymes), by the inhibition with ε-aminocaproic acid (an inhibitor of plasmin and plasminogen activation), and Aprotinin (a lung kallikrein inhibitor). The trypsin-like specificity is further supported by the enzymes' ability to hydrolyze Tos-ArgOMe and by the resistance of maleylated tropoelastin to enzymatic degradation.

Soybean trypsin inhibitor (a naturally occurring inhibitor which inhibits trypsin-, chymotrypsin-, and, to some extent, elastase-like enzymes) is an excellent inhibitor of tropoelastin degradation, as is human α-1-antitrypsin.

The failure of $Ac(Ala)_4CH_2Cl$ (a potent inhibitor of porcine elastase) to inhibit tropoelastin degradation, along with the failure to detect esterase activity when the tropoelastin–enzyme complex was assayed with the elastase substrate *t*-Boc-AlaONp, would argue against an elastase-like enzyme. The tropoelastin–enzyme complex also failed to demonstrate elastinolytic activity when incubated with radiolabeled $NaDodSO_4$-treated elastin. The failure of TPCK and tryptamine

to inhibit degradation suggests that the enzyme is not chymotrypsin like.

When tropoelastin was incubated in borate buffer, or when $CaCl_2$ was added to Tris buffer at a concentration normally used to stabilize trypsin (0.011 M), the rate of tropoelastin degradation was dramatically reduced. There are a number of possible explanations to account for this decrease in the rate of proteolysis, one being a conformational change induced in the tropoelastin substrate by the borate and calcium ions, thus rendering proteolysis and/or enzyme–substrate binding unfavorable. Circular dichroism studies suggest that Ca^{2+} influences the conformational properties of tropoelastin (H. Kagan, unpublished results). While the effects of borate on tropoelastin have not been investigated, borate has been shown to elicit a conformational change in bovine serum albumin which changes its susceptibility to enzymatic degradation (Aoki and Foster, 1975). Substrate conformation may, therefore, play an important role in modulating the turnover of elastin precursor molecules in vivo.

A difficult problem encountered in this study was the development of a sensitive assay for proteolytic activity. The tropoelastin-associated enzyme did show activity when assayed against casein and Tos-ArgOMe, but in both cases large amounts of enzyme were required. Preference for lysine over arginine is, in fact, suggested by the maleylation studies which show a resistance of tropoelastin to degradation after the lysine ϵ-amino groups are maleylated. Arginine residues would not be affected by the modification reaction and, hence, should still be susceptible to proteolytic attack. This is not conclusive evidence for lysine specificity, however, since inhibition by maleylation may simply reflect a decrease in enzyme–substrate binding resulting from the change in the charge distribution on the maleylated tropoelastin.

The most sensitive qualitative assay appears to be the use of $NaDodSO_4$–polyacrylamide gel electrophoresis to detect the degradation products of tropoelastin. This technique also lends specificity to the assay in that the production of the five discrete degradation products, i.e., peptide fragments having molecular weights of 57 000, 45 000, 36 000, 24 500, 13 000–14 000, is reproducible and appears to be specific for this enzyme. No other enzyme studied to date (trypsin, elastase, chymotrypsin, plasmin) produces the same fragmentation pattern (unpublished results). At present, the methodology of the tropoelastin assay does not lend itself to a thorough kinetic study of the tropoelastin-associated enzyme.

The biphasic rate curve obtained when the tropoelastin–enzyme complex was assayed against Tos-ArgOMe is suggestive of the presence of two different enzymes (Segel, 1975). However, endogenous substrate (tropoelastin) added with the enzyme may be affecting the reaction velocity.

The fact that proteolysis can be demonstrated in tropoelastin preparation isolated from various sources and by various techniques (Mecham et al., 1976) suggests a high affinity of the enzyme for the tropoelastin substrate. For this reason, purification of the enzyme has been difficult. Affinity chromatography techniques employing soybean trypsin inhibitor bound to agarose are ineffectual at completely removing proteolytic activity from the tropoelastin. DEAE-cellulose chromatography, on the other hand, appears to be very efficient at removing the bound enzyme and should, therefore, be included in tropoelastin purification techniques. Degradation of the tropoelastin before or during chromatography can be prevented by incorporating 0.2 M ϵ-aminocaproic acid into the chromatography buffers.

A role for the enzyme suggested by the data presented in this communication is one of tropoelastin degradation. A degradative enzyme associated with elastin precursor molecules could serve a very important function by removing normal precursor peptides when fibrogenesis is completed, or by removing nonfunctional peptides which might arise by mutation, denaturation, or the presence, or lack, of chemical modifications. The trypsin-like specificity and anionic nature of the enzyme make tropoelastin and proelastin excellent substrates while precluding digestion of mature elastin. Some connective tissue disease states might actually result from inefficient removal of precursor peptides, a condition analogous to the inherited mucopolysaccharidoses which appear to be due to defects in hydrolytic enzymes resulting in excessive accumulation of normal substances.

Until recently, tropoelastin was thought to be the direct precursor molecule for mature, insoluble elastin. In previous communications (Foster et al., 1976, 1977), we identified a high molecular weight, tropoelastin-like molecule which we postulated represented the elastin precursor form secreted by the cell (i.e., proelastin). A similar molecule has since been identified by another laboratory (Rucker et al., 1977). This high molecular weight molecule is susceptible to proteolytic attack and readily breaks down to lower molecular weight components, including tropoelastin (Foster et al., 1976). The degradation products have the same molecular weights as the degradation products of tropoelastin described in this paper. It is important to note in this regard that proelastin was not detected until various trypsin inhibitors described in this report were incorporated into the tropoelastin isolation procedures. Previous isolation techniques have employed MalNEt and EDTA which, as we show in Table I, are not completely effective at inhibiting tropoelastin degradation.

An alternative role suggested by these data for the tropoelastin and apparently proelastin-associated enzyme might be that of "processing" or preparing proelastin for subsequent incorporation into the fibril by the cleavage of register or aligning peptides from the molecule once cross-link formation has begun. In the event that proelastin is not incorporated, the enzyme might serve to degrade the precursor molecules, or fragments thereof.

The actual biological significance of the tropoelastin-associated enzyme is unknown. A major question is whether the enzyme is part of the elastin metabolic machinery or an extraneous enzyme present as an artifact of tissue preparation or protein purification. This is a difficult question to answer conclusively. However, the tight binding of the enzyme to tropoelastin and the demonstration of concomitant synthesis and secretion of tropoelastin with enzyme in chick aortae and rabbit aortic smooth muscle cell culture (manuscript in preparation) suggests a prominent role for the enzyme in elastin metabolism.

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M_2 Isozyme of Pyruvate Kinase from Human Kidney as the Product of a Separate Gene: Its Purification and Characterization†

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ABSTRACT: The M_2 isozyme of pyruvate kinase has been purified from human kidney. The procedure involved conventional enzyme purification steps plus an affinity chromatography step utilizing the interaction between the dye, Cibracron blue F3GA, and the pyruvate kinase isozyme. During the purification it was observed that the M_2 isozyme is very unstable in the absence of fructose 1,6-bisphosphate. In addition, the electrophoretic mobility of the isozyme in polyacrylamide disc gels at pH 9.3 is greatly affected by the presence or absence of this glycolytic intermediate. The final enzyme product had a specific activity of 127 units/mg of protein and represented a 470-fold purification over the crude extract. The high purity of the enzyme preparation was established by polyacrylamide disc gel electrophoresis in the presence and absence of sodium dodecyl sulfate, by sedimentation velocity and equilibrium analyses, and by NH_2-terminal analysis. Characterization of the purified human M_2 isozyme showed that it is a tetramer of 206 700 daltons with a sedimentation coefficient ($s_{20,w}$) of 9.25 S. Sodium dodecyl sulfate gel electrophoresis indicated that the isozyme consists of four subunits of very similar or identical molecular weight. NH_2-terminal analysis suggested that the peptide chains of the enzyme are blocked. The M_2 isozyme cross-reacts with antiserum prepared against the human M_1 isozyme. The amino acid composition of the M_2 isozyme is distinct from that of the M_1 or R isozymes. Based on the amino acid compositions of the purified M_1 and M_2 isozymes we have concluded that they represent the products of separate genes rather than different molecular forms of the same gene product as others have recently proposed.

The M_2 isozyme[1] of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) occurs in human and rat tissues as a major component in kidney and a minor component in liver (Bigley et al., 1968; Imamura and Tanaka, 1972). It is the only pyruvate kinase isozyme found in rat liver cells in culture (Walker and Potter, 1973) and predominates in rat and human hepatomas (Farina et al., 1974; Balinsky et al., 1973a,b), regenerating liver (Bonney et al., 1973; Garnett et al., 1974), and fetal tissue (Imamura and Tanaka, 1972; Balinsky et al., 1973a,b).

From their studies of the pyruvate kinase isozyme distribution in differentiating human tissues Marie et al. (1976) suggested that the M_1 and M_2 isozymes represent different molecular forms of the same gene product.

We have previously described the purification of the R (Chern et al., 1972) and M_1 isozymes (Harkins et al., 1977) of pyruvate kinase from human erythrocytes and skeletal muscle, respectively. In this paper we describe the purification of the M_2 isozyme from human kidney and determination of its amino acid composition. This has allowed us to compare the amino acid compositions of all three purified isozymes, M_1, M_2, and R, and to conclude that M_1 and M_2 represent the products of separate genes.

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[1] We use the generally accepted L, M_1, M_2, and R nomenclature in this paper to represent the major pyruvate kinase isozyme present in liver, skeletal muscle, kidney, and erythrocytes, respectively.