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Partial Characterization of a Tropoelastin Precursor Isolated from Chick Aorta†

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ABSTRACT: Evidence is presented that indicates tropoelastin is derived from a soluble elastin with a molecular weight of 95 000. Tropoelastin and its proposed precursor were isolated from the aortas of copper-deficient chicks. Although it is doubtful that the proposed precursor is an initial product of elastin translation, i.e., a proelastin, it is proposed to be at least a truncated form of proelastin that is converted to tropoelastin. The key to its isolation was the presence of α_1-antitrypsin at each step in the purification procedure. The first 11 amino acid residues at the NH_2 terminal of the proposed tropoelastin precursor (GGVPGAVPGGV) are the same as those for tropoelastin. Its amino acid composition is similar to that of tropoelastin, except for higher amounts of acidic amino acid residues. Further, the proposed precursor contains a limited number of aldehydic functions, presumably in the form of peptidyl allysine. This was taken as an indication that the proposed precursor serves as a substract for lysyl oxidase. Under the conditions used for the isolation, the precursor appeared to be in higher concentrations than tropoelastin in aorta extracts from copper-deficient chicks.

Tropoelastin is a soluble elastin with a molecular weight of ~72 000. It is generally accepted as the precursor to insoluble elastin, a highly cross-linked structural protein with the physical properties of an elastomer (Sandberg, 1976; Rucker & Tinker, 1977). Although there are reports that suggest tropoelastin is the only form of elastin secreted by cells capable of synthesizing elastin (Ryhänen et al., 1978; Burnett & Rosenbloom, 1979), Foster et al. (1977, 1978) have provided evidence for a soluble proelastin that appears modified to tropoelastin. They suggest that the molecular weight of proelastin is 120 000–140 000 (Foster et al., 1977). In comparison to tropoelastin, it contains more acidic and hydroxy amino acid residues. It also appears to contain histidine, cysteine, and methionine, i.e., amino acids typically not found in tropoelastin. Foster et al. (1977, 1978) isolated the proelastin from the aortas of lathyritic chicks. An important feature of their isolation was the use of high concentrations of proteolytic inhibitors. Without the use of proteolytic inhibitors during isolation, the predominant product was tropoelastin.

Using chicks fed copper-deficient diets, we have also reported the isolation of tropoelastin from chick aorta (Rucker et al., 1975) identical with the tropoelastin described by Foster et al. (1975). Both nutritional copper deficiency and lathyrism decrease cross-linking of insoluble elastin, which is necessary in order to isolate soluble elastins in quantities sufficient for characterization. Although we have yet to isolate a proelastin from the aorta of copper-deficient birds, the use of procedures similar to those described by Foster et al. (1977) resulted in the isolation of a soluble elastin with a molecular weight of 90 000–100 000. We feel that this form of soluble elastin may be an intermediate in the modification of proelastin to tropoelastin. This view is not inconsistent with that of Foster et. al. (1978), and it is in keeping with our previously reported observations on forms of soluble elastins which were identified by using radiochemical labeling procedures (Heng-Khoo et al., 1979; Rucker et al., 1977).

To be described are some of the properties of the proposed intermediate to tropoelastin. This form of soluble elastin possesses an amino acid composition similar to that of tropoelastin. Data obtained from amino acid sequencing indicate that its first 11 NH_2-terminal amino acid residues are identical with those of tropoelastin. Although the exact role of the proposed intermediate in elastin fibrogenesis is not clear, it does appear to be a predominant form of soluble elastin in aortas from copper-deficient chicks. This is an important point since for all previous reports using aortas from copper-deficient animals as a soluble elastin source only tropoelastin has been observed to be the predominant form in tissue extracts [cf. Rucker & Tinker (1977) and Sandberg (1976) and references cited therein].

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Experimental Methods

Preparation of Tropoelastin. For preparation of tropoelastin, 300 white Leghorn cockerels were fed a copper-deficient diet (Rucker et al., 1975) from the day of hatching for 21 days. The birds were killed and their aortas removed for the isolation of tropoelastin as previously described (Rucker et al., 1975). The tropoelastin was used primarily as an electrophoretic marker and standard. Other details regarding its properties may be found in recent reviews (Sandberg, 1976; Rucker & Tinker, 1977).

Preparation of the Putative Tropoelastin Precursor. The isolation procedure was a modification of that described by Foster et al. (1975). Four hundred chicks were fed a copper-deficient diet for 18 days, but in addition ϵ-aminohexanoic acid was added (0.2%). The birds were killed and the aortas removed and immediately immersed in liquid nitrogen. The tissue was pulverized by passing it in the presence of liquid nitrogen through a Wiley mill using a 1-mm mesh stainless steel sieve. The resulting powder was then suspended in 9 volumes of neutral salt buffer (0.5 M NaCl and 0.02 M sodium phosphate, pH 7.0) containing inhibitors (20 mM EDTA,[1] 5 mM BAPN, 5 mM *N*-ethylmaleimide, and 10 μg/mL α_1-antitrypsin) and extracted twice (3 h) with stirring. Following centrifugation (25000*g*, 1 h), the supernatant fractions were combined and the pellet was discarded. The supernatant was then filtered through Whatman No. 1 filter paper to remove insoluble lipid material.

For the first fractionation step, the pH was lowered to 4.0 by using 4 N acetic acid to precipitate salt-soluble collagen (Foster et al., 1975). The acidified mixture was allowed to stand for 2 h. The major precipitating fraction (mostly collagen) was removed by centrifugation (25000*g*, 30 min). The supernatant fraction was readjusted to pH 7.0, using 4 N NaOH. Sodium chloride was then added to 15% (w/v) to precipitate the bulk of the elastin. This mixture was also allowed to stand for 3–4 h (4 °C) before centrifugation (10000*g*, 60 min). The precipitate obtained at this point was resuspended in buffer plus inhibitors and stirred overnight. Undissolved material was removed by centrifugation (10000*g*, 30 min). The NaCl precipitation step was then repeated, but with two separate additions. NaCl was added to 5% (w/v), and a precipitate was recovered by centrifugation (10000*g*, 20 min). The remaining material in solution was then precipitated by the addition of NaCl to 15% and was further fractionated by gel filtration using a 2.6 × 80 cm column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden). In preparation for the gel filtration step, the precipitate was dissolved in 4–5 mL of 0.1 M Tris buffer (pH 7.8) containing 20 μg/mL α_1-antitrypsin (Calbiochemicals, La Jolla, CA). The sample was applied to the column and eluted by using 0.1 M Tris buffer containing 0.5 M NaCl and 10 μg/mL α_1-antitrypsin. The elution was monitored by the determination of protein (Bio-Rad protein determination kit, Bio-Rad Laboratories, Richmond, CA), estimates of turbidity at 41 °C, and radioactivity.

The rationale for estimating turbidity at 41 °C was based on the ability of soluble elastins to undergo heat-elicited coacervation (Sandberg, 1976; Urry et al., 1975). It was predetermined that a concentration of tropoelastin exceeding 25 μg/mL resulted in measurable turbidity at 600 nm and that the estimate of turbidity was linear with respect to elastin concentrations up to 300–400 μg/mL. The rationale for the estimation of radioactivity was from previous experience that [^{3}H]valine is readily incorporated into elastin-rich fractions from chick aorta following intraperitoneal injection. Forty of the 400 birds used for isolation of the precursor were each injected with 100 μCi of [^{3}H]valine 12 h prior to isolation. That the labeled arterial protein was mostly elastin was based on observations published previously (Heng-Khoo et al., 1979). Two major fractions of protein were collected following gel filtration (see Results). Protein in the two fractions was precipitated by the addition of NaCl (15%, w/v). Each of the precipitates was suspended in 3 mL of distilled water saturated with PMSF and dialyzed against a 300-volume excess of distilled water plus PMSF in preparation for storage (−70 °C).

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed according to the procedure of Furthmayr & Timpl (1971) or Sykes & Bailey (1971). The gels were 5% acrylamide with a ratio of methylenebis(acrylamide)/acrylamide of 1:38. Electrophoresis was performed at a constant current of 2 mA/tube (8–10 h) when the procedure of Furthmayr & Timpl was used or at 4 mA/tube (4 h) when the procedure of Sykes & Bailey (1971) was used. The gels were standardized for molecular weight estimates by using tropoelastin or bovine serum albumin, ovalbumin, and cytochrome *c* (Pierce Chemical Co., Rockford, IL). The gels containing the protein standards were run without reducing agents so that it was possible to detect bands corresponding to the bovine serum albumin and ovalbumin dimers.

For isoelectric focusing, gels containing 7.5% acrylamide and 1% ampholyte (Bio-Lyte, Bio-Rad Laboratories, Richmond, CA) were used. The gels were prepared as described by Furthmayr & Timpl (1971) by using ampholyte (pH 4–9) instead of phosphate buffer and $NaDodSO_4$. L-Arginine and L-lysine were also added (0.06%) to stabilize the cathode end of the gels. The gels were ~10 cm long. The samples were applied mixed with 40% sucrose. NaOH or H_2SO_4 (0.5 M) was used as the cathode or anode solutions, respectively. A potential of 2 V/cm of gel was applied (200 V/10 tubes). Bromophenol blue, albumin, and cytochrome *c* were used as references in the isoelectric focusing gels. The pH gradient was also estimated (Bio-Rad pro-pHiler, Bio-Rad Laboratories, Richmond, CA). The gels were stained with Coomassie Brilliant Blue.

When it was necessary to determine radioactivity following electrophoresis, the gels were sliced into 2-mm contiguous slices. The slices were incubated overnight in 1 mL of Protosol and following dissolution were counted in 10 mL of Econofluor by using a Packard scintillation spectrometer (New England Nuclear, Boston, MA).

Amino Acid Analysis. The protein samples were hydrolyzed by using constant boiling HCl (5.7 N) in sealed tubes for 36 h at 110 °C. Amino acids in the hydrolysates were then chromatographed by using a Durrum D-500 analyzer.

NH_2-Terminal Amino Acid Sequence Determination. Edman degradations were performed by using a Beckman sequencer, Model 890C. The program used in sequence determination was Beckman Program 102974. In preparation for sequence determination, the protein sample (~1 mg) was dissolved in a small amount of 70% formic acid and then delivered to the spinning cup. The formic acid was removed by the vacuum steps and nitrogen flush used in sample application subroutine 02772 and by the heptafluorobutyric acid cleavage and butyl chloride extraction steps given by beginning automatic sequencing at the acid-cleavage step. Reagents were Beckman Sequanal grade. Amino acid phenylthiohydantoins

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BAPN, β-aminopropionitrile; PMSF, phenylmethanesulfonyl fluoride.

and their trimethylsilyl derivatives were identified by gas–liquid chromatography using a Hewlett-Packard 7620A gas chromatography system. Residues were also identified by thin-layer chromatography (Haugen et al., 1976).

Labeling in Vitro Soluble Elastins with [^3H]Valine. The studies in vitro were performed as described previously (Heng-Khoo et al., 1979). Briefly, chick aortas were minced (1-mm^3 segments) and then transferred to flasks containing Medium 199 (Grand Island Biochemical Co., Grand Island, NY) and [^{3}H]valine [50 μCi/mL (Amersham, Arlington Heights, IL); specific radioactivity 30 Ci/mol]. The flasks were immediately oxygenated with a mixture of O_2–CO_2 (95:5%). The tissue, suspended at 1 g/10 mL of medium, was incubated for varying times (cf. the legend for Figure 5). The tissue was chilled to 4 °C, washed with distilled water (10 °C), and homogenized in either 0.5 M acetic acid or 0.05 M phosphate buffer containing 0.9% NaCl (w/v) and α_1-antitrypsin (20 μg/mL) to terminate the incubations. In addition, samples of tissues were also extracted with sodium phosphate buffer (0.05 M) containing 6 M urea, 0.2% mercaptoethanol, and 5 mM *N*-ethylmaleimide. The ratio of tissue/buffer was 1:9 (w/v). [^{3}H]Valine not incorporated into protein was separated by passing aliquots (2 mL) of the labeled extracts through Sephadex PD-10 columns equilibrated with buffer or solutions appropriate for the electrophoretic systems used in a characterization. It is important to note that the time for sample preparation usually amounted to no more than 30 min.

Chemical Reduction with [^3H]$NaBH_4$ and Determination of Aldehydic Functions. Reduction of the elastin precursor was carried out by using 25 mCi of [^{3}H]$NaBH_4$ (specific activity adjusted to 25 mCi/mol; Amersham, Arlington Heights, IL). The reaction was carried out in 2.5 mL of 0.001 M EDTA, pH 9.0, at room temperature for 10 h with stirring. The tropoelastin precursor (100 μg) was first suspended in solution (0.5 mL) followed by the addition of [^{3}H]$NaBH_4$ in 2 mL of EDTA solution, pH 8.0. Following reduction, glacial acetic acid (2.5 mL) was added dropwise to destroy excess $NaBH_4$. Two milligrams of tropoelastin was then added as carrier. Tritium not incorporated into protein was removed by gel filtration using Sephadex G-10 columns (1 × 10 cm) with 0.5 acetic acid as eluant. In addition, the samples were taken to dryness to aid in the removal of exchangeable tritium. This step was repeated 3 times. A portion of the recovered protein was then hydrolyzed (5.6 N HCl, 24 h) and chromatographed by using a system for the estimation of basic amino acid residues and reducible nonacidic cross-linking amino acids (Green et al., 1973).

A spectrophotometric assay was used for the determination of total aldehydic functions (Paz et al., 1965). Tropoelastin and butanal were used as standards, and $NaBH_4$-reduced tropoelastin and butanal were used as reagent blanks. By using microcuvettes (total volume 0.2 mL), we observed reasonable color yields after reaction of *N*-methylbenzothiazolone hydrazine with 50 μg of tropoelastin or the tropoelastin precursor (see Results).

Results

Purification of Elastin. Success in isolating the tropoelastin precursor appeared most related to the inclusion of α_1-antitrypsin in each of the isolation steps. Without α_1-antitrypsin the final elastin product was tropoelastin and related cleavage products. Since ion-exchange chromotography was not included as part of the isolation protocol (Abraham & Carnes, 1978a), α_1-antitrypsin could be included as an inhibitor. It appeared to be more effective than high concentrations of PMSF or *N*-ethylmaleimide [cf. Mecham & Foster (1977)].

Table I: Steps in Purification of the Tropoelastin Precursor

step	fraction	protein recovd[a] (mg)	total radio-act (10^{-3} × dpm)	sp act. [10^{-3} × (dpm/mg)]	recov-ery[a] (%)
1	original extract	593	47.7	0.08	100
2	acidification	278	37.2	0.13	78
3	first NaCl precipitation	58	17.2	0.25	36
4	second NaCl precipitation[b]	33	15.8	0.48	33
5	Sephacryl S-200[c]				
	fraction A	7	3.5	0.50	
	fraction B	6.5	2.9	0.48	

[a] The protein recovered at each step is indicated in addition to the recovery of radioactivity. Four hundred chicks were used, of which 40 were injected with 100 μCi of [^{3}H]valine/bird 12 h prior to the purification. [b] The step 4 fraction represents the precipitate after the addition of NaCl to 15% following the removal of material that was precipitated with 5% NaCl (see text). [c] Fractions A and B are defined in Figure 1.

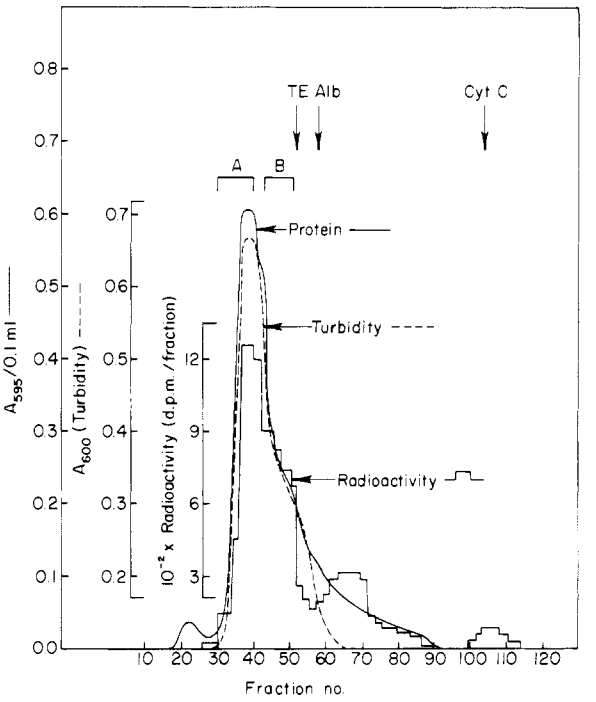

FIGURE 1: Elution of the aorta protein fraction obtained by NaCl precipitation from a column of Sephacryl S-200. Total protein (—), turbity (---) (difference between A_{600}(35 °C) $-A_{600}$(4 °C)), and radioactivity were determined. Fractions A and B were taken for further characterization.

Samples of the protein fractions were taken at each step and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Assessment of the gels indicated a distribution of protein almost identical with that described by Foster et al. (1975), except that the tropoelastin precursor (90 000–100 000 daltons) persisted throughout isolation.

Table I shows the recovery of protein and [^{3}H]valine incorporated into protein for each step in the purification. The fractions designated A and B (step 5) in Table I were obtained after gel filtration followed by NaCl precipitation (Figure 1). The recovery of soluble elastin was 33 mg, or 1.5 mg/g of tissue, at step 4. This estimate was based on the amino acid composition of the material at this step, which was very similar to that of tropoelastin. Previous recoveries of tropoelastin (Tom et al., 1977) from copper-deficient chicks have amounted to 2–3 mg of tropoelastin/gram of fresh aorta. With respect

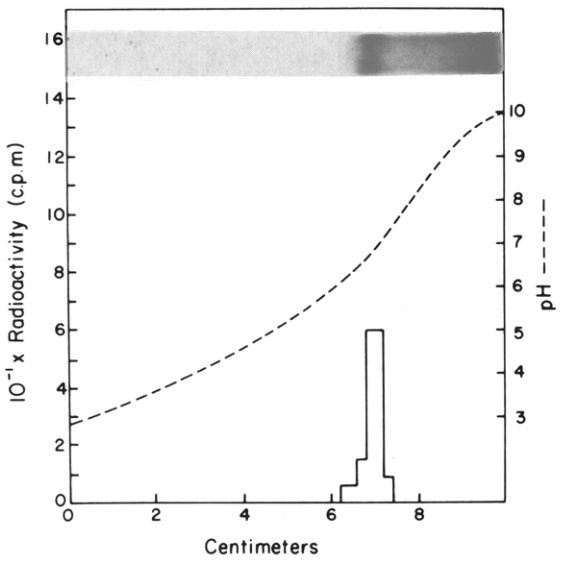

FIGURE 2: Isoelectic focusing of the tropoelastin precursor. A stained gel is shown in addition to the mobility of [^{3}H]valine-labeled protein in a corresponding gel. Two hundred micrograms of protein was applied.

to the recovery of protein labeled with [^{3}H]valine, 33% of the radioactivity was recovered as soluble elastin at step 4. This represented 40–50% of the anticipated yield as radioactively labeled soluble elastin, since from previous experiments we have observed that ~60–75% of the total [^{3}H]valine incorporated into soluble protein from copper-deficient chick aorta appears as elastin (Heng-Khoo et al., 1979). As shown in Figure 1, most of the radioactivity eluted as a globular protein with a molecular weight of 90 000–100 000 upon gel filtration. A shoulder corresponding to a molecular weight of 70 000–80 000 and a small peak corresponding to protein with a molecular weight near 25 000–30 000 were also observed. Turbidity resulting from the incubation of fractions at 35 °C (5 min) is also indicated. Incubation at 4 °C resulted in clear solutions.

The leading edge of the peak (A) and the shoulder (B) were taken for characterization. Sufficient material was not isolated for characterization of the fraction eluting near a molecular weight of 25 000–30 000. As a final purification step, protein in the fractions designated A and B was precipitated with NaCl (15%).

Isoelectric Focusing and NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. The putative tropoelastin precursor migrated as a single band in polyacrylamide gels containing NaDodSO$_4$, corresponding to a globular protein with a molecular weight of 95 000. The precursor focused in polyacrylamide gels containing ampholyte (pH 4–7) at pH 7.0 (Figure 2). The inclusion of urea in the gels did not alter its mobility. The behavior was felt to be anomalous, however, since a protein with the composition of the precursor (cf. Table II) would be expected to focus between pH 10 and 11. Tropoelastin focused with an apparent p*I* of 8.4. This was also viewed as anomalous behavior, but is in keeping with observations by Roensch et al. (1972) on the behavior of tropoelastin in isoelectric focusing gels.

Chemical Composition and NH$_2$-Terminal Sequence Data. The amino acid compositions for the tropoelastin precursor and tropoelastin are given in Table II. The NH$_2$-terminal amino acid residues of the precursor and its comparison to tropoelastin are given in Figure 3. The NH$_2$-terminal amino acid sequences for both tropoelastin and the precursor were determined to be the same. Recoveries at each step in the

Table II: Amino Acid Composition[a] of Tropoelastin and the Putative Tropoelastin Precursor

amino acid	tropoelastin precursor	tropoelastin	amino acid	tropoelastin precursor[b]	tropoelastin[c]
Lys	40	42	Gly	315	335
His	trace	0	Ala	165	175
Arg	12	6	$^1/_2$-Cys	0	0
Hyp	8	8	Val	169	177
Asp	15	5	Met	0	0
Thr	17	10	Ile	16	18
Ser	13	8	Leu	44	54
Glu	23	13	Tyr	12	11
Pro	121	127	Phe	25	30

[a] Expressed as mol/1000 mol of amino acid residues. [b] 90 000–100 000 daltons. [c] 72 000 daltons.

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Tropoelastin Precursor (first 11 residues):

 1   2   3   4   5   6   7   8   9  10  11
Gly-Gly-Val-Pro-Gly-Ala-Val-Pro-Gly-Gly-Val-...


Tropoelastin (first 26 residues):

 1   2   3   4   5   6   7   8   9  10  11  12  13
Gly-Gly-Val-Pro-Gly-Ala-Val-Pro-Gly-Gly-Val-Pro-Gly-
14  15  16  17  18  19  20  21  22  23  24  24  26
Gly-Val-Phe-Phe-Pro-Gly-Ala-Gly-Leu-Gly-Gly-Leu-Gly-...
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FIGURE 3: NH$_2$-terminal amino acid sequences for the tropoelastin precursor and tropoelastin.

sequence were similar to those described previously for tropoelastin (Rucker et al., 1975).

The elastin intermediate also appeared to contain aldehydic functions. When butanal was used as a standard, it was estimated that the intermediate contained between 4 and 6 mol of aldehyde/1000 mol of amino acid residues. This value corresponds to the amount of allysine present in tropoelastin from lathyritic chicks (Foster et al., 1975). Further, it was of concern that the tropoelastin precursor did not represent cross-linked tropoelastin, i.e., a dimer that had undergone limited proteolysis (Abraham & Carnes, 1978b; Partridge & Whiting, 1979). The tropoelastin precursor was reduced with [^{3}H]NaBH$_4$ in order to stabilize potential aldol or Schiff-base condensation products. Shown in Figure 4 is the elution of tritiated amino acids in the precursor after hydrolysis and ion-exchange chromatography (Green et al., 1973). Reduction with 25 mCi of [^{3}H]NaBH$_4$ resulted in an incorporation of ~5 × 10^5 dpm as tritiated amino acids. Application of 88 000 dpm for each column run resulted in a >95% recovery of the radioactivity as neutral-acidic residues with the remaining labeled components distributed as given in Figure 4. These results were taken as evidence that the cross-links in elastin that arise from Schiff-base condensation were not present in amounts sufficient to account for dimerization. For example, radioactivity corresponding to the mobility of lysinonorleucine or reduced merodesmosine was not observed in amounts significantly above background [cf. Abraham & Carnes (1978b)].

Radiochemical Labeling in Vivo and in Vitro. The specific radioactivity of the elastins initially extracted from aortas of birds injected with [^{3}H]valine was not sufficiently high so that it could be directly characterized by using polyacrylamide gel techniques (80 dpm/mg, Table I). However, radioactivity as tropoelastin and the tropoelastin precursor could be identified following purification (cf. Figure 2). When arterial tissue was incubated in vitro, components containing [^{3}H]valine corresponding to the precursor and tropoelastin were easily observed

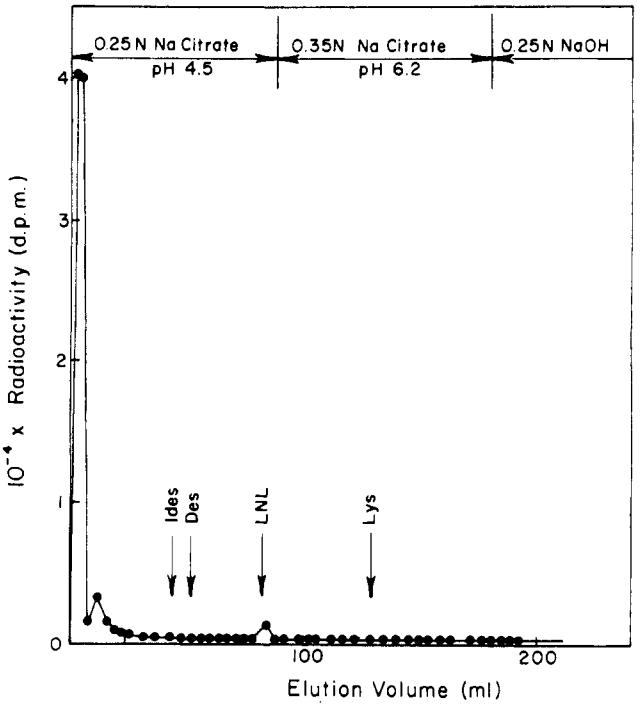

FIGURE 4: Elution profile of tritiated amino acids from a column of Aminex A-5 resin. The tropoelastin precursor was first reduced with [^{3}H]$NaBH_4$ (see text). Following reduction, the protein was hydrolyzed in preparation for chromatography. The conditions for chromatography were essentially the same as those described by Green et al. (1973), except that sodium citrate buffer was used instead of pyridine acetate for the elution. Each fraction represents 1 mL of eluant at a flow rate of 1 mL/min. (Ides) isodesmosine; (Des) desmosine; (LNL) lysinonorleucine; (Lys) lysine.

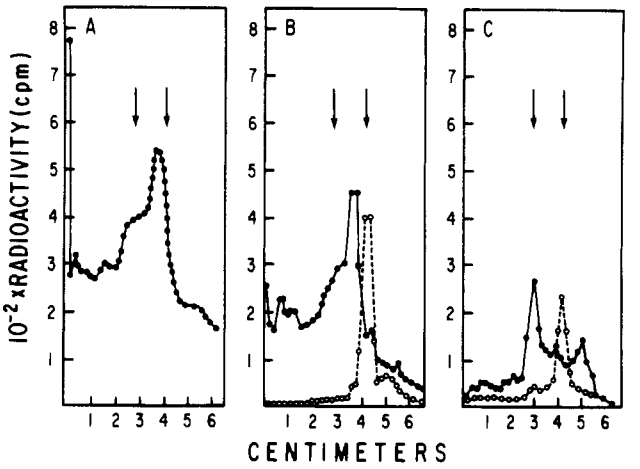

FIGURE 5: Distribution of radioactivity from chick arterial extracts after electrophoresis in $NaDodSO_4$–polyacrylamide gels. The arrows correspond to the mobility of globular proteins with molecular weights of 70000 or 100000. Part A corresponds to [^{3}H]valine-labeled protein extracted from chick aorta using 0.1 M phosphate buffer, pH 7.0, containing 6 M urea, 5 mM *N*-ethylmaleimide, and 0.2% mercaptoethanol. The aortas were incubated in tissue culture for 20 min prior to extraction (see text). Part B represents the mobility of [^{3}H]valine-labeled urea-soluble protein (part A) following separation into acetic acid soluble (broken line) and insoluble (solid line) fractions. The peak represented by the broken line most closely corresponds to the mobility of tropoelastin. Part C represents protein extracted into phosphate buffer containing 0.9% NaCl and 20 μg/mL α_1-antitrypsin following a 20-min incubation of chick aorta in the presence of [^{3}H]valine. The solid line represents the distribution of radioactivity immediately following incubation. The broken line represents the distribution of radioactivity following an additional 20 min of incubation in medium containing no [^{3}H]valine and cycloheximide [cf. Heng-Khoo et al. (1979)]. Further, without α_1-antitrypsin in the solutions used for extraction or when 0.5 M acetic acid was used for extraction, the mobility of the labeled protein in the gels was identical with that indicated by the broken line.

(parts A–C of Figure 5). Soluble elastin with a molecular weight of 95000 appeared to be rapidly converted to tropoelastin. It was only detected when α_1-antitrypsin was present in the extraction buffers (Figure 5C). Extraction of the tissue with phosphate buffer or 0.5 M acetic acid consistently resulted in a single major peak that corresponded to the mobility of tropoelastin.

When urea and reducing agents were included in the extraction solutions (parts A and B of Figure 5), high molecular weight proteins or protein aggregates appeared as the major products. Most appeared to possess molecular weights greater than tropoelastin. Furthermore, when urea was included in the extraction buffer, a component was typically observed that migrated as a broad band corresponding to a molecular weight of 72000–76000. Upon dialysis against acetic acid it was possible to fraction this component into two fractions corresponding to protein with molecular weights of 74000–76000 or 70000–72000 (Figure 5C). The higher molecular weight material was not soluble in acetic acid and was recovered by centrifugation following the dialysis step. It could be solubilized, however, by dissolution into 0.1 M phosphate buffer, pH 7.0, containing 1 M urea.

Discussion

Evidence has been presented that suggests tropoelastin arises from the cleavage of a soluble elastin with a molecular weight of 95000. The precursor appears closely related to tropoelastin and under appropriate conditions appears to be the major form of soluble elastin in aorta extracts from copper-deficient chicks. Foster et al. (1978) have also suggested that tropoelastin arises from a high molecular weight form of soluble elastin (120000–140000) that is metabolized through a 90000–100000 intermediate. Further, the observations that aldehydic functions are present in the proposed intermediate and that the intermediate is only observed in high concentrations when cross-linking is blocked suggest that it serves as a substrate for lysyl oxidase. Similar to tropoelastin, the protein could be easily separated from other proteins by using solutions containing no denaturants. In contrast, extraction with buffers containing urea solutions under reducing conditions resulted in the dissolution of mostly high molecular weight components that were difficult to resolve.

Partridge & Whiting (1979) have suggested that urea or reducing conditions facilitate the association of soluble elastins with other matrix proteins. For example, using techniques applicable to the resolution of complex protein mixtures, they found it difficult to separate soluble elastin from acidic glycoproteins when urea and reducing agents were included in the initial buffers used for tissue extraction. This point is emphasized, because in preliminary experiments on the isolation of high molecular weight forms of soluble elastin we have also found it difficult to separate soluble elastins from other arterial proteins in the presence of reducing agents or denaturants. Further, we have observed that tropoelastin rapidly degrades in 6 M urea in keeping with the observation of Mecham & Foster (1978). Even relatively pure preparations of tropoelastin [>90% using the criteria suggested by Sandberg (1967)] are rapidly degraded to peptides ranging in molecular weights from 12000 to 58000 if stored in physiological buffers. This phenomenon appears to be the result of proteinases bound to soluble elastin that persist throughout isolation (Mecham & Foster, 1978).

Subsequently, the possibility that soluble elastin may form stable complexes with other proteins has represented a concern throughout our attempts to identify products as possible

precursors to tropoelastin. Also, the possibility that soluble elastins with molecular weights greater than 70000 may represent intermolecularly cross-linked forms of tropoelastin that have been partially degraded has been a concern. From the data related to composition and NH_2-terminal amino acid sequences, it is clear that the proposed intermediate is not contaminated with typical microfibrillar or structural glycoproteins. Although the question that the intermediate may be a cross-linked form of tropoelastin was not completely resolved (e.g., potential aldol condensation products were not identified), it was clear that Schiff-base derived cross-links were not present in sufficient amounts to indicate dimerization (Abraham & Carnes, 1978b). Further, we have examined tropoelastin stored for long periods of time (months) with no evidence of significant cross-linking during storage. Also, previous evidence from the radiochemical labeling studies in vitro has not suggested that tropoelastin or related peptides are immediately cross-linked to form a product with a molecular weight of 90000–100000 (Heng-Khoo et al., 1979).

Since several of the previous attempts to identify tropoelastin precursors have been unsuccessful (Ryhänen et al., 1978; Burnett & Rosenbloom, 1979), we felt it also important to point out that [^{3}H]valine is rapidly incorporated into at least one other matrix protein with a mobility in $NaDodSO_4$–polyacrylamide gels near that for tropoelastin. Although the extent to which this protein shares chemical properties with tropoelastin was not assessed, its presence complicates the interpretation of experiments in which tropoelastin synthesis has been partially assessed on the mobility of radiochemically labeled products in polyacrylamide gels containing $NaDodSO_4$ or by gel filtration. For example, in one of our previous communications we found it difficult to establish a precursor–product relationship between the 95000-dalton soluble elastin and tropoelastin (Rucker et al., 1977). It is now clear that one of the reasons for the difficulty was the comigration of the urea-soluble component with tropoelastin in $NaDodSO_4$–acrylamide gels. Also, this observation may have some bearing with respect to earlier reports that have indicated a range of molecular weights for radiochemically labeled tropoelastin from 68000 to 76000 [cf. Rucker & Tinker (1977) and references cited therein].

Of interest is the role of the precursor in relationship to the overall regulation of elastic fiber formation. Evidence for its modification to tropoelastin suggests steps in elastin fiber assembly somewhat analogous to those observed in the assembly of collagen fibers. Because of its relatively high concentrations in chick arterial extracts, it might also be speculated that it is incorporated into fibrous elastin in a manner similar to that suggested for tropoelastin (Rucker & Tinker, 1977). Such speculations, however, require that certain inconsistencies be resolved. Our observations, although consistent with selected features of soluble elastin synthesis reported by Foster et al. (1978), are at present inconsistent with observations from studies on the translation of soluble elastin from mRNA obtained from embryonic chick aorta cells. Although we could argue that rapid proteolysis has compromised the observations in these studies, Ryhänen et al. (1978) and Burnett & Rosenbloom (1979) have shown that chick aortic RNA does not significantly affect the incorporation of radioactive valine into soluble elastins that are significantly larger than tropoelastin. Obviously, it will also be essential to identify the factors that underly the apparent differences in the nature of elastins synthesized when translation or cell culture systems vs. in vivo techniques are used as the instruments for investigation.

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