CROSSLINKAGE OF SALT-SOLUBLE ELASTIN IN VITRO*

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SUMMARY

Radioactively labeled soluble elastin, synthesized in vitro by viable copper-deficient pig aorta in a culture medium containing L - [4,5-^{3}H] lysine, was incubated with normal newborn pig aorta. The insoluble residue, after extraction of the aorta with cold 0.5M NaCl at pH 7.4, was reduced with $NaBH_4$. Insoluble elastin, prepared from this by autoclaving after extraction with guanidine, was hydrolyzed with HCl and the hydrolysate was chromatographed on Aminex A-5. Among the radioactive residues eluted in the basic region, four elastin crosslinks (isodesmosine, desmosine, lysinonorleucine and merodesmosine) were identified by comparison with known standards on the Beckman amino acid analyzer. This provides the first direct evidence that soluble elastin is a precursor of insoluble elastin.

A soluble protein with an amino acid composition resembling elastin has been extracted from the aortas of copper-deficient swine (1,2). Biosynthesis of this protein in vitro has been accomplished (3) but dynamic proof that it is a precursor of insoluble elastin has been lacking. The kinetics of incorporation of radioactive amino acids into insoluble elastin proceeds so rapidly that the chase of a pulse-label from the soluble into the insoluble protein could not be demonstrated. Specific inhibition of elastin crosslinkage by β-aminoproprionitrile or penicillamine has not succeeded. For these reasons we have undertaken to demonstrate the incorporation of a radioactively labeled soluble elastin from copper-deficient aorta into the insoluble elastin of normal

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aorta. The identification of labeled, lysine-derived crosslinks in the elastin hydrolysate indicates that this has been achieved.

MATERIALS AND METHODS

Biologic material. Thoracic aortas obtained from one day old pigs and from pigs raised on a diet deficient in copper by the method of Shields et al (4), were killed by exsanguination under pentobarbital anesthesia. The aortas were removed and finely minced under aseptic conditions, weighed and placed in sterile Eagle's minimal essential medium (MEM). The MEM was adjusted to pH 7.4 with 0.02M NaH_2PO_4 and contained ascorbic acid (100 µg/ml) and a mixture of penicillin-streptomycin (2,400 units/ml). MEM (B) had the same composition excepting that it lacked lysine.

Biosynthesis of [^{3}H]lysine-labeled soluble elastin. A copper-deficient aorta, weighing 8.1 grams, was incubated 24 hours at 37° with gentle shaking in MEM (B) plus 2 millicuries of L-[4,5-^{3}H]lysine. The incubated mixture was then decanted and the soluble elastin was prepared from the aortic residue using the method of Smith and Carnes (3). Briefly, this method consists of : extracting the aortic residue in cold 0.5M NaCl, pH 7.4; removing the soluble collagen from a clarified extract by precipitating and centrifuging at pH 4.0 in 1M NaCl, (using pepstatin as a protease inhibitor); and removing the soluble elastin from the collagen-free extract by coacervating and centrifuging this solution at 32° in 1M NaCl, pH 8.0. Soluble elastin, collected as a viscous pellet, was redissolved in the cold and again coacervated and collected by centrifugation to insure a constant specific activity. The purity of the soluble elastin was further tested by discontinuous gel electrophoresis in 6M urea at low pH.

Incubation of labeled salt-soluble elastin with newborn pig aorta. Soluble elastin, 5.1 mg, having a specific activity of 6.78×10^4 cpm per mg was suspended in 5 ml of MEM with one gram of newborn pig aorta and incubated at 37° for 24 hours with gentle shaking. The incubation was stopped by quick freezing in liquid nitrogen. The mixture was thawed and decanted; the remaining aortic residue was used to prepare insoluble elastin.

Preparation of reduced insoluble elastin hydrolysate. The aortic residue was extracted with 10 ml of 0.5M NaCl, buffered with 0.02M phosphate, pH 7.4 and containing 0.5% 2-mercaptoethanol, overnight at 4°. The washed aortic residue was reduced for 12 hours at room temperature by the method of Starcher et al (5) by resuspending it in 0.5M Tris-HCl, pH 7.2, and slowly adding 200 mg of $NaBH_4$ over a period of one hour. Reduction was stopped by dialyzing against running distilled water at room temperature for 24 hours. The reduced aortic residue was then extracted four times in 10 ml of 5M guanidine containing 0.05M dithiothreitol, 0.1M Tris, pH 8.5, and 0.1% EDTA at room temperature for 24 hours to remove microfibrils from the elastic tissue (6). The extracted residue was then thoroughly washed with distilled water and autoclaved in an excess of distilled water for one hour at 15 p.s.i. four times. The autoclaved residue was then extracted with 100% ethanol and with ether and air dried. This isolated elastin was suspended in constant boiling HCl, sealed under nitrogen and hydrolyzed at 110° for 72 hours. The hydrolysate was filtered, flash evaporated and brought to a 5 ml volume with distilled water. Aliquots were used immediately for counting, protein determination and ion exchange.

Cation exchange of the reduced elastin hydrolysate. A slight modification of the cation exchange method of Green et al (7) was used to isolate known lysine-derived crosslinks from the elastin hydrolysate. This method fractionates the basic polyfunctional amino acids on a column of Aminex A-5 cation exchange resin maintained at 50°, using volatile pyridine-acetate buffers. Pyridine-acetate, 0.15M, pH 4.5 was used as the first buffer; the ionic strength was raised to 0.3M in the second buffer and to 1.0M in the third buffer, pH 5.0. The modifications consisted of using a shorter and wider column (1.6 x 15.9 cm); a slower flow rate (30 ml per hour); elution of desmosines with the second buffer instead of the first; collection of larger fractions (3.3 ml); and location of the lysine-derived crosslinks by radioactivity. The radioactive lysine-derived crosslinks were identified on the Beckman amino acid analyzer (model 120B), using both the long column (53.3 x 0.9 cm) containing Beckman PA-28 resin and the short column (16.5 cm x 0.9 cm) containing Beckman PA-35 resin. The eluting sodium citrate buffers were pumped at a flow rate of 50 ml/hr. Desmosines were eluted from the short column with 0.38M sodium citrate, pH 4.26, at 31°. Lysinonorleucine was eluted from the short column using 0.38M sodium citrate, pH 4.50, at 59° as suggested by Sandberg (8). Merodesmosine was eluted from the long column at 56° in the second buffer of a two buffer system; the first buffer (104 ml) consisted of 0.2M sodium citrate, pH 4.25, and the second buffer used was 0.35M sodium citrate, pH 5.28. Desmosine, isodesmosine and merodesmosine standards were generously provided by Dr. Barry L. Starcher. Lysinonorleucine was prepared from L. nuchae elastin (Worthington Biochemical Corporation) hydrolysate by the method of Green et al (7).

Scintillation counting. Aliquots of radioactive samples were counted in 10 ml xylene-based scintillation fluid in a Beckman LS-250 scintillation counter with an efficiency of 35%.

Protein determination. Protein was estimated from the nitrogen content of the elastin hydrolysate. The nitrogen was determined using the micro-Dumas technique with a Coleman nitrogen analyzer.

RESULTS

The purified radioactive soluble elastin gave a single sharp band by disc electrophoresis. Approximately one-seventh the radioactivity (52,000 cpm) contained in this protein was recovered in the insoluble elastin (49.5 mg) isolated from the incubation mixture. The radioactive profile of an hydrolysate of the reduced elastin eluted from Aminex A-5 is shown in Fig. 1. Two peaks eluted in the lower ionic strength buffer (0.15 M), correspond to acidic or neutral lysine derivatives. Eight peaks, in addition to lysine, eluted in the higher ionic strength buffer (0.3 M), comprise the basic lysine derivatives. Thus far, four of these peaks (Nos. 1, 2, 6 and 7) have been identified as elastin crosslinks by comparison with standards, utilizing Beckman physiologic columns. Fig. 2 gives a portion of the elution profile of an acid hydrolysate of elastin

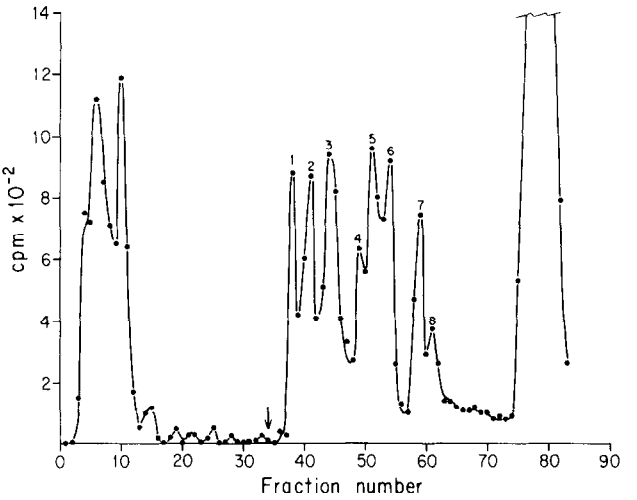

Fig. 1. Elution profile of radioactive hydrolysis products of borohydride-reduced elastin from newborn pig aorta that had been incubated with [^{3}H] lysine-labeled soluble elastin. Chromatography was performed on Aminex A-5 resin using pyridine-acetate buffers, pH 4.5. The arrow marks the point at which the buffer was changed from 0.15M to 0.3M. Fractions 3-12 contain the acidic and neutral lysine derivatives, fractions 35-62 contain the basic lysine derivatives, and fractions 75-82 contain lysine. Peaks 1, 2, 6, and 7 have been identified with known elastin crosslinks (see Figs. 2 and 3).

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from commercial Ligamentum nuchae on the Beckman PA-35 column by which isodesmosine and desmosine are resolved (top curve). Superimposed curves of separate elutions of peaks 1 and 2 from the Aminex A-5 chromatogram (Fig. 1) on the same column are shown (bottom curves), indicating their identity with isodesmosine and desmosine. Similarly, Fig. 3 (A & C) shows the identity between elution curves of a lysinonorleucine standard and peak 7 from the Beckman PA-35 column and (B & D) between a standard merodesmosine and peak 6 from the Beckman PA-28 column.

DISCUSSION

Lysine has been shown to be incorporated into the chief crosslinks of elastin (9, 10, 11). Recent studies have provided indirect evidence for the existence in elastic tissues of a soluble protein precursor of elastin (1, 3, 12, 13). In copper deficiency (1, 2, 14, 15, 16) and in

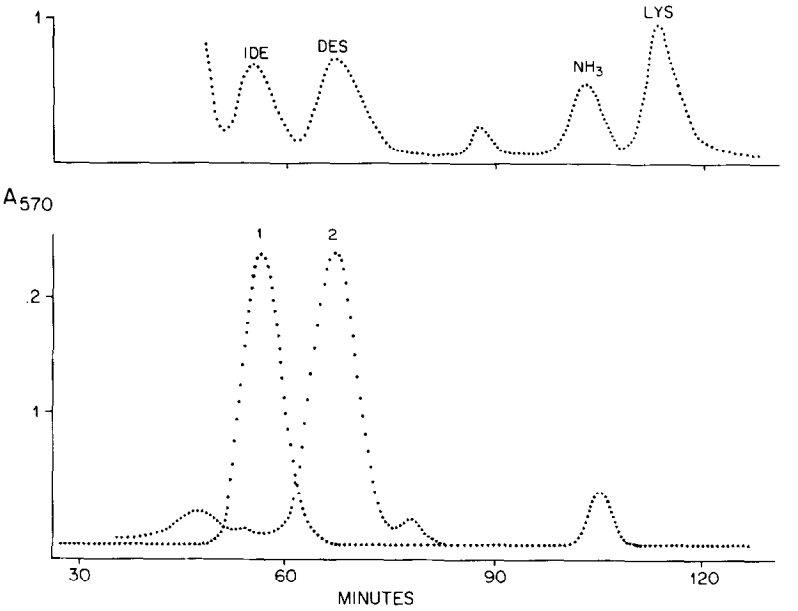

Fig. 2. Elution profile of ninhydrin reaction products from HCl hydrolysates of bovine L. nuchae elastin (top) and of reduced aortic elastin of newborn pig (bottom). Chromatography was performed on 16.5 cm PA-35 column of Beckman amino acid analyzer using conditions for separation of physiologic basic amino acids (see text). Identity of peaks in top profile was established by comparison with standards. Bottom profile is composite tracing of separate elutions of peaks 1 and 2 shown in Fig. 1.

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lathyrism (17) a soluble protein accumulates which has an amino acid composition related to that of elastin, differing principally in the lack of lysine-derived crosslinks and in the presence of an excess of lysine. The lysyl groups of this soluble protein have been labeled by incubating viable aorta from copper-deficient pigs in culture medium containing [4,5-^{3}H]lysine. When this protein was purified and incubated in turn with normal newborn pig aorta in complete, nonradioactive culture medium, labeled crosslinks derived from the soluble protein were incorporated into the insoluble elastin. The identification of tritium-labeled isodesmosine, desmosine, merodesmosine and lysinonorleucine (18) in the insoluble elastin indicates that these crosslinks were derived from preformed soluble elastin, thus supporting the previously unproven

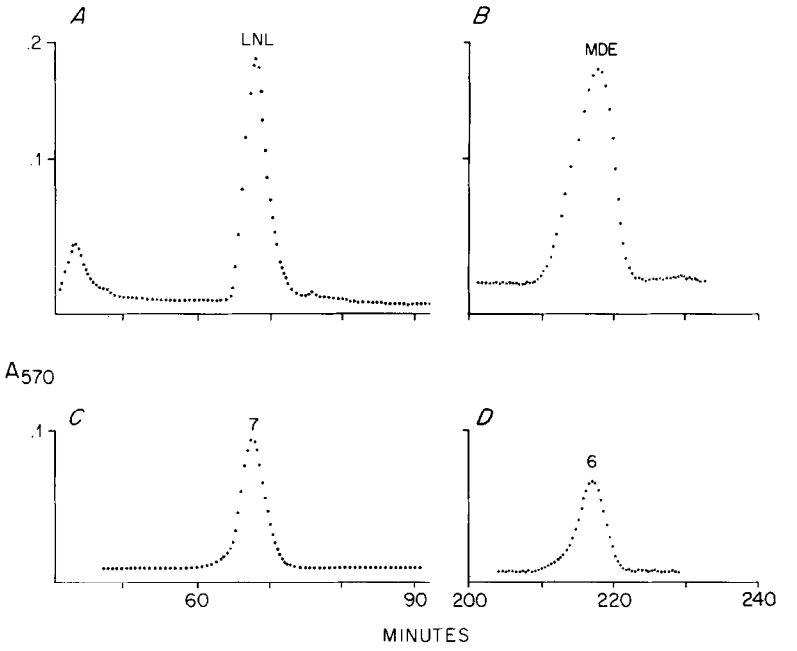

Fig. 3. Elution patterns of lysinonorleucine (LNL) and merodesmosine (MDE) standards (top) and of peaks 6 and 7 of reduced aortic elastin (bottom) shown in Fig. 1. Curves A and C depict elution from the 15.5 cm PA-35 column of the Beckman amino acid analyzer and curves B and D, from the 53.3 cm PA-28 column, using conditions that resolve the standards from other amino acids of elastin (see text).

hypothesis that this protein is an intermediate in the formation of insoluble elastin.

New synthesis of soluble elastin and incorporation of amino acids into insoluble elastin have been shown to occur in comparable incubation of newborn pig aorta (3). It is likely, therefore, that synthesis of new elastin from amino acids has occurred during the incubation with the labeled soluble elastin. It is possible that some of the labeled soluble elastin could have been degraded to amino acids which were then incorporated into the newly formed insoluble elastin. However, in consideration of the large pool of nonradioactive lysine (2μmoles) in the culture medium it appears unlikely that much of the labeled crosslinks could have been derived by this indirect pathway.

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