Biosynthesis of Soluble Elastin by Pig Aortic Tissue in Vitro*

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SUMMARY

Incubation of normal newborn pig aorta with [14C]valine yielded a labeled protein that was isolated and purified with salt-soluble elastin from copper-deficient pig aorta as carrier. The labeled protein is soluble in strong neutral salt solution in the cold. It coacervates reversibly and can be separated into a hydrophobic phase by centrifugation at 32-37°C. The protein migrates on polyacrylamide gel electrophoresis in 6 M urea with a relative mobility of 0.066 ± 0.005. The molecular weight and hydrophobic properties identify the newly synthesized protein with soluble elastin. The comparably high specific activity of the insoluble elastin isolated from the incubation mixture indicates that the soluble protein is a rapidly cross-linked precursor of insoluble elastin.

A unique salt-soluble protein that resembles elastin in amino acid content has been extracted from the aortas of copper-deficient swine (1-3). Since it lacks the cross-links, desmosine and isodesmosine, of insoluble elastin, this protein has been presumed to be an elastin precursor that accumulates in the absence of a copper-containing lysyl amine oxidase (4). It has, thus far, not been isolated from normal tissues, however, and no direct evidence of its conversion to elastin has been reported. Evidence for the biosynthesis of soluble elastin by normal aortic tissue in vitro has now been adduced by the isolation of the radioactively labeled protein, using purified soluble elastin from copper-deficient aorta as carrier protein.

EXPERIMENTAL PROCEDURE

The essential steps of the experimental procedure are outlined in the flow diagram (Fig. 1). The labeled newborn pig aorta and the copper-deficient aorta were extracted together, and the crude salt extract was fractionated with slight modification of the method of Smith et al. (3) for the separation of soluble collagen, soluble elastin, and other soluble proteins. Insoluble elastin was separated from the extracted residue by autoclaving (5), and insoluble collagen was recovered in the gelatin supernatant.

Reagents—Urea was Schwarz-Mann, ultra pure label. Acrylamide, bis,1 N,N',N''-tetramethylethylenediamine, and ammonium persulfate were Bio-Rad, electrophoresis pure reagents. Deionized distilled water was used in all experimental work.

Triple-distilled constant boiling hydrochloric acid was used for the hydrolysis of proteins. L-[U-14C]Valine with a specific activity of 280 mCi per mmole and PCS-liquid scintillation fluid were obtained from Amersham-Searle. The internal counting standard was n-[14C]hexadecane-1 (Packard) with a specific activity of 1 μCi per g. Pepstatin (Lot. No. ME-68-A) was supplied by Dr. H. Umezawa, Microbial Chemistry Research Foundation and Institute of Microbial Chemistry, Tokyo. Porcine pepsin (2700 units per mg) was purchased from Sigma Chemical Company. Horse hemoglobin (2 times crystallized) was purchased from Miles Laboratories. Beef liver catalase (50,500 units per mg) was purchased from Worthington Biochemical Corporation. Penicillin-streptomycin (1:1) mixture (25,000 units per ml) was purchased from Microbiological Associates. Coomassie brilliant blue R 250 was purchased from Colab Laboratories, Inc.

Biological Material—Aortas were obtained from normal newborn pigs (aged 1 to 2 days) and from copper-deficient pigs reared in the laboratory. These were received from nearby farms before supplementation. A detailed account of conditions for production of copper deficiency has been published (6). The diet consisted of canned evaporated milk diluted with sulfide water and supplemented with iron. The deficient animals were killed by exsanguination under pentobarbital anesthesia at 2 to 3 months of age when their serum copper levels had fallen below 10 μg/100 ml. The thoracic aortas were removed, stripped of adventitia, and stored at −70°C until extracted. Newborn pigs were killed similarly to obtain viable aortic tissue. The thoracic aortas were removed with aseptic technique, weighed, and minced with sharp blades in sterile Krebs-Ringer solution and used immediately.

Isotopic Labeling—Each minced newborn pig aorta, weighing about 1 g wet, was incubated for 2 hours at 37°C with gentle shaking in 10 volumes of sterile Krebs-Ringer solution containing 50 μCi of L-[U-14C]valine. The incubation mixture was then centrifuged for 10 min at 1005 × g at 23°C, and the aortic residue was briefly washed twice in the same way with phosphate-buffered NaCl solution pH 7.4 at 37°C. The combined supernatants were saved. The aortic residues were extracted immediately.

Preparation of Crude Aortic Extract—All operations were carried out at 4°C except where noted otherwise. The frozen copper-deficient aorta was ground in a Latapie grinder. This was combined with an equal weight of the washed, labeled residue of newborn pig aorta. The combined aortic tissues were suspended in 10 volumes of 0.5 M NaCl in 0.02 M phosphate buffer, pH 7.2, and 0.5% 2-mercaptoethanol and extracted with shaking overnight. The mixture was then centrifuged at 90,000 × g for 45

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1 The abbreviation used is: bis, N,N'-methylenebisacrylamide.
Preparation of Soluble Elastin—The crude aortic residue was washed exhaustively in the extracting buffer and then in distilled water at 23°. The washed residue was repeatedly autoclaved (five times) in fresh distilled water (15 pounds pressure) until the supernatant fluid was free of protein by absorbance at 280 nm. The combined supernatants containing the gelatin were dried at 95° and stored in a desiccator at 4°. The residue of insoluble elastin was washed sequentially with butanol, acetone-ether (1:1), and ether, air-dried, and stored in a desiccator at 4°.

Protein Analysis—Protein was estimated by the Lowry method using α-elastin as a standard.† Proteins were hydrolyzed in triplicate-distilled constant boiling HCl in vacuo for 20 hours at 110°.

Disc Electrophoresis—The disc electrophoresis method of Riesfeld et al. (11) was used with 0.6 m urea in the gels (1). Potassium, 0.06 N (K+), was used as the leading ion. β-Alanine, 0.35 m, was used as the trailing ion. The separating gel (0.5 × 4 cm) was buffered at pH 4.3 with potassium acetate, 0.375 m (CH₃COO⁻); the pH fell to 3.8 during electrophoresis. The sample and spacer gels (0.5 × 1 cm) were buffered at pH 6.7 with potassium acetate, 0.062 m (CH₃COO⁻). Safranin 0 was used as a tracking dye (12). The separating gels were subjected to electrophoresis to remove excess persulfate before adding the stacking and sample gels which were polymerized with riboflavin. The separating gel had a total gel concentration of 7.7% (w/v) and the ratio of bis to acrylamide monomer was 1:38.5. The sample and stacking gels had a total gel concentration of 3.125% (w/v) and a ratio of bis to acrylamide monomer of 1:4. The electrophoresis was carried out at 4° and 2 ma per tube until the tracking dye reached the bottom of the separating gel. Soluble elastin was concentrated at 37° in the acrylamide gel in 1 m NaCl buffered with 0.02 m phosphate at pH 8.0. The concentrated protein was visualized by dark field illumination. Soluble elastin and other proteins were also fixed in the gels in 5% trichloroacetic acid and stained with Coomassie brilliant blue. The relative mobilities of the proteins were determined by the method of Rodbard and Chrambach (13).

Scintillation Counting—Protein solutions containing [14C]valine were counted in 10 ml of xylene-based liquid scintillation fluid until 2,000 or more counts were reached to minimize counting errors. Ten microliters of the hexadecane solution, having an activity of 17,211 dpm, were then added to each vial as an internal standard, and the vials were recounted. Disc electrophoresis gels were sliced into 1-mm sections manually or with a gel slicer, and the slices were digested for 4 hours with 0.5 ml of 30% H₂O₂ at 55° in capped scintillation vials. Peroxide was removed by adding 35 μl of catalase, containing 5,000 units of activity, to each vial and incubating overnight at 23°. The peroxide-treated slices were counted in 10 ml of liquid scintillation fluid. Scintillation counting is a method of determining radioactivity by counting the number of radioactive decay events that occur in a sample. In this experiment, [14C]valine was used as a tracer to label the proteins of interest. The labeled proteins were then subjected to electrophoresis to separate them based on their molecular weights and mobilities. After electrophoresis, the proteins were sliced into 1-mm sections and digested with a mixture of enzymes and oxidants to release the radioactivity. The released radioactivity was then measured using liquid scintillation counting. The ratio of the amount of radioactivity released to the total amount of protein was then calculated to determine the amount of radioactivity per protein unit.

Fig. 1. Preparation of labeled soluble elastin.

- Separation of Soluble Collagen—Crude salt extract was adjusted to 1 m NaCl with 3.5 m NaCl, and pepstatin was added at 5 μg per g wet weight of original tissue. The solution was titrated to pH 4.0 with 4 N acetic acid, and collagen was allowed to precipitate for 2 hours. It was then separated by centrifugation at 90,000 × g for 45 min. The collagen-free supernatant was saved.
- Preparation of Soluble Collagen—Crude salt extract was adjusted to 1 M NaCl with 3.5 M NaCl, and pepstatin was added at 5 μg per g wet weight of original tissue. The solution was titrated to pH 4.0 with 4 N acetic acid, and collagen was allowed to precipitate for 2 hours. It was then separated by centrifugation at 90,000 × g for 45 min. The collagen-free supernatant was saved.
- Preparation of Soluble Elastin—The collagen-free supernatant was passed over a Sephadex G-10 column (32.5 × 2.5 cm) which was eluted with 1 m NaCl, buffered with 0.02 m phosphate, pH 8.0, containing 0.5% 2-mercaptoethanol at 4°. The void volume was collected and warmed to 37° for 30 min. The concave that formed was collected by centrifugation at 48,200 × g for 30 min at 32°. The supernatant, containing other proteins, was stored at 4°.
- The pellet was redissolved in 0.02 m NaCl in 0.02 m sodium acetate, pH 4.0, and dialyzed overnight against 1 m NaCl in 0.02 m phosphate buffer, pH 8.0, containing 0.5% 2-mercaptoethanol. The concave was repeated, and the pellet of purified soluble elastin was redissolved in 0.02 m sodium acetate, pH 4.0, and dialyzed against distilled water saturated with toluene at 4° overnight. It was stored at 4°.

1 Pepstatin was used as a specific inhibitor of cathepsin (7).
2 A unit of pepstatin had an activity of 2.28 pmoles of tyrosine per mg of protein per min at 37°. The pepsin used as substrate was a solution of porcine pepsin (10 μg per ml) with 0.02 m KCl-HCl buffer (pH 2.0), and 0.1 ml of the same buffer with or without pepstatin was mixed and incubated for 3 min. Then, 0.1 ml of a solution of porcine pepsin (10 μg per ml) was added. After incubation at 37° for 25 min, 2.0 ml of 1.7 m perchloric acid were added and the mixture kept for 1 hour at room temperature. It was then centrifuged and the absorbance of the acid-soluble fraction was read at 280 nm.
3 The cathepsin activity of the aorta was taken from Kirk (9) and was assumed to be 0.00376 mmole of tyrosine per g wet weight per hour.

The chromogenicity of α-elastin, prepared from insoluble aortic pig elastin according to Partridge et al. (10), is the same as that of soluble elastin as determined by amino acid analysis.
tillation counting was performed in a Packard Tri-Carb liquid scintillation spectrophotometer, model 3320.

**Gel Filtration**—Gel filtration was performed with a column (32.5 × 2.54 cm) containing Sephadex G-10 at 4°C using 1 M NaCl, buffered with 0.02 M sodium phosphate at pH 8.0 and containing 0.5% 2-mercaptoethanol, as the eluting buffer. A flow rate of 30 ml per hour was used. The void and included volumes were determined with blue dextran (Pharmacia) and ammonium sulfate, respectively. The elution position of [14C]valine was also determined.

## RESULTS

The procedure described (Fig. 1) was the best of several modifications tried. Mixing the labeled and the unlabeled copper-deficient pig aorta before extraction minimized loss of the labeled products in mechanical manipulations. The initial washing with buffer saline at room temperature removed unidentified proteins with most of the free radioactive valine, but the protein had very low radioactivity and disc electrophoresis of the combined supernatants showed no collagen or elastin in it. Precipitation of the collagen in the coacervate. The collagen before coacervation of the soluble elastin avoided co-

**radioactivity eluted near the included volume (147 ml) of the gel filtration column. The small size of the free valine peak indicates that most of the nonincorporated valine was removed by the initial washing of the incubated tissue.**

The slight overlap of the protein and valine peaks in gel filtration required the sacrifice of a small fraction of the protein peak. The first five tubes of the excluded peak were used for the preparation of soluble elastin. The procedure with slight modifications was carried out with six separate newborn pig aortas. The specific activity of the purified elastin ranged from 2.8 × 10^4 to 5.4 × 10^4 dpm per mg. The same differences occurred in total incorporation into protein and probably reflect variations in the viability of the tissue during incubation.

The distribution of radioactivity among the several fractions in the last experiment is given in Table I. It shows a stepwise increase in specific activity of the protein during an approximately 3-fold purification of soluble elastin. No change in specific activity occurred during reincorporation of the isolated soluble elastin. The soluble collagen had only a small fraction (≈ 3%) of the specific activity of the soluble elastin. The specific activity of the insoluble elastin was nearly as high as that of the soluble. The gelatin fraction had an almost negligible activity.

Disc electrophoresis of crude salt extracts showed several bands when stained with Coomassie blue (Fig. 3a). The crude extract contains α1 and α2 collagen and soluble elastin which are identified by their migration rates, and other proteins moving at faster migration rates. It was discovered that the soluble elastin can be reversibly coacervated in the polyacrylamide gel, using the same conditions (1 M NaCl, pH 8.0, 37°C) that are used for coacervation of free solutions of the protein. Disc electrophoresis of the purified soluble elastin (Fig. 3b) showed a major band with a relative mobility (Kp) of 0.006 ± 0.009. A densitometric scan of the Coomassie blue-stained band was symmetrical, indicating homogeneity of this protein. A faint diffuse band with a migration rate much lower than that of collagen sometimes appeared.

### Table I

Incorporated radioactivity of labeled proteins extracted from pig aortas

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg/g wet tissue)</th>
<th>Specific activity (dpm/mg)</th>
<th>Total incorporated activity (dpm × 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude salt extract</td>
<td>60.3</td>
<td>214</td>
<td>106</td>
</tr>
<tr>
<td>Collagen-free extract</td>
<td>32.1</td>
<td>326</td>
<td>105</td>
</tr>
<tr>
<td>Soluble elastin (first coacervate)</td>
<td>15.1</td>
<td>544</td>
<td>82.1</td>
</tr>
<tr>
<td>Soluble elastin (second coacervate)</td>
<td>14.9</td>
<td>543</td>
<td>80.0</td>
</tr>
<tr>
<td>Insoluble elastin</td>
<td>154</td>
<td>396</td>
<td>610</td>
</tr>
<tr>
<td>Soluble collagen</td>
<td>18.2</td>
<td>17.2</td>
<td>3.13</td>
</tr>
<tr>
<td>Gelatin</td>
<td>92.3</td>
<td>2.89</td>
<td>2.67</td>
</tr>
<tr>
<td>Other soluble proteins</td>
<td>16.4</td>
<td>135</td>
<td>22.1</td>
</tr>
</tbody>
</table>

* Values for crude salt extract calculated as the sum of values for soluble collagen and collagen-free extract.

**Fig. 2.** Sephadex G-10 gel filtration of collagen-free salt extract. The two radioactive peaks (74 ml and 101 ml) match those of blue dextran (75 ml) and free valine (101 ml).
in both the crude extract and the purified soluble elastin. Since this band also showed reversible coacervation, it is presumed to be an aggregated soluble elastin.

Disc electrophoresis of the crude salt extract gave a single radioactive peak (Fig. 4a). Disc electrophoresis of the purified soluble elastin gave a single peak with the same migration rate. When the gels containing these fractions were coacervated, the peak of radioactivity was found to correspond exactly to the coacervated bands (Fig. 4b).

**DISCUSSION**

The establishment of soluble elastin as a physiological precursor of elastin requires the demonstration that it is an intermediate in normal elastin biosynthesis. Repeated efforts in our laboratory to isolate this protein from normal pig aorta have been unsuccessful. The failure to detect it might be due to its short life-span. Resort was had, therefore, to radioactive labeling of the protein. Murphy et al. (14) showed that the 14C-labeled amino acids (glycine, proline, and alanine), that occur in high proportion in elastin and collagen, are incorporated into two sodium dodecyl sulfate-soluble proteins of molecular weights about 68,000 and 100,000 by embryonic chick aorta. These were presumed to be soluble elastin and collagen, respectively, since their molecular weights agreed within ±10% of the weights reported for those proteins (2, 3, 15, 16). 14CValine incorporation was detected only in the 68,000 molecular weight peak by gel electrophoresis. This peak also contained a small but significant amount of hydroxyproline. Pulse-chase experiments indicated rapid conversion of the soluble to an insoluble fraction which they interpreted to be the result of cross-linkage. These results were consistent with the synthesis of a soluble precursor of elastin, but the quantity of the labeled products was not sufficient to permit their further characterization.

The rationale of our approach has been the use of carrier proteins from pig aorta to provide quantities that could be isolated and characterized. The copper-deficient pig aorta fulfills this requirement in providing large quantities of soluble collagen and soluble elastin as well as the insoluble proteins. The assumption is made that the minute amounts of newly synthesized, labeled proteins will be sequestered with the pool of nonlabeled proteins during their isolation and purification. 14CValine has been used as the label because of the high proportion of valine in elastin compared to collagen (17).

The standard method for preparation of salt-soluble elastin (3) was employed, based upon its solubility in high salt concentration in the cold and its reversible coacervation when warmed. The applicability of this property to the protein in acrylamide gels provided an additional and independent method of identification of the protein band after gel electrophoresis. The separations of soluble collagen (18) and insoluble elastin (5) were also carried out by standard methods.

The high specific activity of the purified soluble elastin (Table I) indicates a significant synthesis of this protein by the newborn pig aorta during a 2-hour incubation period. Unexpectedly, the specific activity of insoluble elastin was almost as high. Since there was some 10 times as much insoluble as soluble elastin, the proportion of the total activity in the insoluble elastin was far higher than that in the soluble. This indicates that the soluble elastin may be a short-lived precursor of insoluble elastin, which would explain the lack of a detectable quantity of the protein under normal circumstances. The nature of the inferred cross-linkages is unknown at present. In view of the slow formation of desmosines (19, 20), it is unlikely that these account for them. Other possible cross-links have been reviewed by Gallop et al. (21).

The presence of a minute quantity of a soluble aggregate of elastin is indicated by the variable faint band of slower migration rate observed by dark field illumination in the coacervated gels.
This band has not been observed in previous preparations of soluble elastin from copper-deficient pig aorta. Its presence in these experiments suggests the formation of soluble oligomers, perhaps as a result of aldol condensation, since the normal aorta in the incubation mixture contains lysyl oxidase activity which is lacking in the copper-deficient aorta. The amount of this higher molecular weight fraction was too small to give a separate peak of radioactivity, and its migration rate has not been determined accurately but it has a higher molecular weight than collagen and procollagen α chains.

The far lower specific activity of the soluble collagen compared to the elastin can be due only in part to its lower content of valine or its cross-linkage. In view of the low activity in the gelatin, it signifies a comparatively slow synthesis of collagen.

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REFERENCES

Biosynthesis of Soluble Elastin by Pig Aortic Tissue in Vitro
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