Radioimmunological Identification of Tropoelastin

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Antiserum was prepared in sheep against insoluble elastin isolated from embryonic-chick aortae. In an indirect immunoprecipitation test, the antiserum reacted quantitatively with small amounts of radioactively labelled purified tropoelastin prepared from embryonic-chick aortae. The antiserum did not cross-react with chick procollagen, and the antiserum was used to identify radioactively labelled tropoelastin secreted by chick aorta cells in suspension culture.

The protein elastin, which imparts an elastic, rubber-like, quality to major blood vessels, is very inert and insoluble, owing to extensive cross-linking of the polypeptide chains by desmosines and other cross-linkages (Partridge, 1962; Franzblau *et al.*, 1965) derived from the oxidation of lysine residues by a copper-requiring enzyme (Miller *et al.*, 1965; Partridge *et al.*, 1966; Pinnell & Martin, 1968). Sandberg *et al.* (1969) prepared a soluble protein called tropoelastin from the aortae of copper-deficient pigs, and this protein consisted of a single polypeptide chain with an estimated mol.wt. of about 70000 and an amino acid composition which closely resembled that of insoluble elastin, except for the absence of the desmosines. A significant portion of the tropoelastin molecule has been sequenced (Foster *et al.*, 1973), and a model for cross-linked elastin has been proposed on the basis of these data (Gray *et al.*, 1973). A similar protein has been isolated from the aortae of copper-deficient or lathyrotic chicks (Rucker *et al.*, 1973; Foster *et al.*, 1975).

Isolated aortae incubated *in vitro* synthesize a soluble protein which shows many of the properties of tropoelastin (Murphy *et al.*, 1972; Smith & Carnes, 1973). Cells isolated from the media of pig aortae can synthesize elastin after they have been in long-term culture (Pathrapamkel *et al.*, 1974). We have shown that cells isolated from embryonic-chick aortae can synthesize and secrete procollagen and tropoelastin into the surrounding medium when incubated in suspension culture under the appropriate conditions (Rosenbloom & Cywinski, 1976).

The unequivocal identification of tropoelastin during the biosynthesis of insoluble elastin is difficult, since the soluble protein does not possess the unique chemical characteristics of the insoluble form. Sykes & Chidlow (1974) have found that antiserum prepared against insoluble chick elastin reacted in agar-gel diffusion and immunoelectrophoresis experiments

with tropoelastin prepared from lathyrotic chicks. In the present paper we report an indirect immunoprecipitation method capable of identifying small amounts of radioactively labelled tropoelastin.

Materials and Methods

The procedure of Sykes & Chidlow (1974) was followed to prepare antiserum to elastin. Fifty chick aortae were extracted four times with 100 ml of 0.1 M-NaOH at 98°C for 10 min. The residue was washed successively with water, ethanol and then acetone. After the material was air-dried, it was pulverized in a liquid-N₂ mill. Then 10–20 mg of the product was suspended in 50% Freund's complete adjuvant and injected into the back of two sheep, every 2 weeks, one intradermally and one subcutaneously. Each animal received a total of five injections and they were bled 2 weeks after each injection. The intradermally injected sheep produced a higher titre of antibody. The bleeding after the fifth injection of the intradermally injected sheep was used in the work described below.

Tropoelastin labelled with [³H]valine was prepared by incubating thoracic aortae from 17-day embryonic chicks with 10 μ Ci of [³H]valine/ml (New England Nuclear Corp., Boston, MA, U.S.A.) for 2 h at 37°C (Murphy *et al.*, 1972). The aortae were then homogenized in 5 M-guanidine hydrochloride, and the solubilized material was dialysed against 5.5 M-urea. Tropoelastin was separated from contaminating collagen and other proteins by minor modification of the procedure of Sandberg *et al.* (1969). The labelled proteins were applied to a column (2 cm $\times$ 0.9 cm) of Amberlite IRF-97M. The column was eluted with a 40 ml linear gradient (0.01–0.4 M) of sodium phosphate, pH 7.0, containing 5.5 M-urea, and the labelled protein eluted in the position characteristic of tropoelastin was collected.

Procollagen and tropoelastin labelled with [^{14}C]proline were prepared by incubating cells isolated from the thoracic aortae of 17-day chick embryos by trypsin/collagenase digestion of the tissue (Rosenbloom & Cywinski, 1976). The cells ($2 \times 10^6/\text{ml}$) were incubated in suspension culture in Krebs medium A (Krebs, 1950), supplemented with $25 \mu\text{g}$ of ascorbate/ml, 2% foetal calf serum (Flow Laboratories, Rockville, MD, U.S.A.), $3 \mu\text{Ci}$ of [^{14}C]proline (New England Nuclear Corp.)/ml and $200 \mu\text{g}$ of pancreatic trypsin inhibitor (Worthington, Freehold, NJ, U.S.A.)/ml, which was necessary to inactivate small amounts of residual trypsin remaining from the cell-isolation procedure. After 2 h of incubation, the cell suspension was centrifuged at $1200g$ for 5 min, and the medium containing the secreted labelled procollagen and tropoelastin dialysed against 0.5M-acetic acid. The labelled proteins were stored in 0.5M-acetic acid, since storage at neutral pH led to slow degradation of the tropoelastin.

The radioactively labelled antigens were dialysed against 50 mM-sodium phosphate (pH 7.2)/150 mM-NaCl for 2–3 h just before use in the immunoprecipitation test. Batches of the sheep antiserum (0.1–5 μl) were mixed with 6000–10000 c.p.m. of the antigen in a final reaction volume of 200 μl of 50 mM-sodium phosphate (pH 7.2)/150 mM-NaCl containing 0.1% Triton X-100. In reaction tubes containing less than 5 μl of specific antiserum, normal sheep serum was added so that the total amount of sheep serum was maintained at 5 μl . The tubes were maintained at 16°C for 24 h and then the appropriate quantity of rabbit anti-(sheep globulin) was added to precipitate the sheep globulin quantitatively. The samples were allowed to react for 24 h at 16°C and then centrifuged at $1500g$ for 10 min; the precipitates were washed and dissolved in 350 μl of 0.5 M-acetic acid. It was necessary to carry out the reaction below 20°C to minimize non-specific precipitation of the tropoelastin, probably due to coacervation of this highly hydrophobic protein. Sodium dodecyl sulphate (1%, w/v, final concn.) and mercaptoethanol (1%, v/v, final concn.) were added to the dissolved precipitates and supernatants, and the samples placed in a boiling-water bath for 2 min. They were then dialysed against 0.1% sodium dodecyl sulphate/0.01 M-sodium phosphate, pH 7.4, and the proteins subjected to polyacrylamide-disc-gel electrophoresis in sodium dodecyl sulphate as previously described (Murphy *et al.*, 1972).

Results and Discussion

The ability of the sheep antiserum prepared against insoluble chick aorta elastin to react with the [^3H]-valine-labelled tropoelastin preparation was tested by using various amounts of antiserum. The precipitates were dissolved in 0.5 M-acetic acid and then they, as well as the labelled proteins remaining in the super-

Fig. 1. Polyacrylamide-gel electrophoresis of labelled tropoelastin and procollagen after reaction with sheep antiserum raised against insoluble chick aorta elastin

(a) [^3H]Valine-labelled tropoelastin was allowed to react with 3 μl of the sheep antiserum, and then rabbit anti-(sheep globulin) was added as described in the Materials and Methods section. The precipitates were dissolved in 0.5 M-acetic acid. Samples of the labelled proteins in the dissolved precipitate and supernatant remaining after immunoprecipitation were subjected to electrophoresis in sodium dodecyl sulphate. (b) A solution containing [^{14}C]proline-labelled procollagen and tropoelastin synthesized by isolated aorta cells was allowed to react with antiserum to elastin as in (a) above. Samples of the precipitated and soluble proteins were subjected to electrophoresis and the gels cut into 1.5 mm slices. Precipitate (●); supernatant (○). Marker β chains (190 000 mol.wt.) of rat tail collagen were located at fraction 8, α chains (95 000 mol.wt.) of rat tail collagen at fraction 13 and bovine serum albumin (68 000 mol.wt.) at fractions 22–23.

Fig. 2. Precipitation curve for $[^3\text{H}]$ valine-labelled tropoelastin

$[^3\text{H}]$ Valine-labelled tropoelastin (6000 c.p.m.) was allowed to react with 0.1–3.0 μl of antiserum as described in the Materials and Methods section. Portions of the labelled protein in the precipitates and supernatants were subjected to polyacrylamide-gel electrophoresis as described in Fig. 1. By summation of the counts in fractions 19–24, an estimate of the tropoelastin in the precipitates and supernatants was made. The fraction of the tropoelastin which was precipitated, expressed as a percentage of the total, was then plotted as a function of the quantity of antiserum used in the test.

natants, were treated with sodium dodecyl sulphate and mercaptoethanol and subjected to electrophoresis on polyacrylamide gels. The results obtained with one test sample in which a maximum of the radioactivity was precipitated are illustrated in Fig. 1(a). A large fraction of the radioactivity in the precipitate is located in a peak corresponding to mol.wt. 70000, a value previously reported for tropoelastin. In addition, a small but significant fraction was found in a lower-molecular-weight region (50000–55000). We believe that the latter polypeptides are derived from the cleavage of the 70000-mol.wt. tropoelastin. Similar cleavage products have been observed previously (Foster *et al.*, 1975). Most of the proteins which were non-reactive and found in the supernatant were less than 70000 mol.wt. To quantify the tropoelastin which was precipitated as a function of the amount of antiserum used, the radioactivity in the gel fractions containing the 70000-mol.wt. peak were summed. This quantity was calculated for both the precipitate and supernatant, and the fraction precipitated is plotted in Fig. 2, as a function of the

quantity of specific antiserum. A smooth curve was obtained, and greater than 90% of the undegraded tropoelastin was precipitated by as little as 1 μl of the specific antiserum.

One of the most likely proteins to cross-react with the elastin antibodies is procollagen. Therefore samples of both procollagen and tropoelastin labelled with $[^{14}\text{C}]$ proline were prepared by incubating isolated aorta cells with $[^{14}\text{C}]$ proline and collecting the medium containing the secreted procollagen and tropoelastin. After reaction of the labelled proteins with the antiserum, the precipitated and soluble proteins were subjected to electrophoresis as above. The results of a typical reaction are illustrated in Fig. 1(b). The precipitate contained about 85% of the labelled proteins migrating in the 70000-mol.wt. region, as well as a much smaller amount of lower-molecular-weight material. The protein in the peak around fraction 10 has been shown previously to be largely procollagen (Murphy *et al.*, 1972; Rosenbloom & Cywinski, 1976). Significantly, essentially all of it remained in the supernatant after immunoprecipitation.

The indirect precipitation method described here can be used to identify soluble elastin in biological systems in which other methods of characterization are difficult or impossible. The use of gel electrophoresis in sodium dodecyl sulphate enables one to estimate the molecular weight of the precipitated proteins, and, if $[^{14}\text{C}]$ proline is used as a label, hydroxy $[^{14}\text{C}]$ proline analyses can be performed on the labelled proteins in different regions of the gel (Murphy *et al.*, 1972; Rosenbloom & Cywinski, 1976). Sykes & Chidlow (1974) reported that the lower limit of sensitivity of their assay for identifying tropoelastin was in the low microgram range. Although we have not rigorously quantified the weight of tropoelastin in our labelled preparations, preliminary estimates set an upper limit of 50 ng for the amounts used in Fig. 1. Since the labelled tropoelastin preparations can be readily prepared, the method may be adaptable to an immunodisplacement assay in which unlabelled tropoelastin or elastin fragments may be identified and quantified.

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