Isolation of tropoelastin a from lathyrctic chick aortae

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Tropoelastin a was isolated from lathyrctic chick aortae by using severe denaturing conditions for the initial extraction. The amino acid composition of this new species of tropoelastin is elastin-like in its high proportion of proline, glycine, alanine and valine. However, it differs from authentic tropoelastin b in containing a higher percentage of polar amino acids and cysteine residues. In addition, the amount of proline hydroxylation is 3 times higher than that found in chick tropoelastin b.

Elastin is an insoluble protein found in the extracellular matrix, whose prime physiological function is to impart elasticity (Rucker & Goettlich-Rieman, 1977; Sandberg *et al.*, 1981). Investigations into the biosynthesis and insolubilization of elastin have demonstrated that elastin is first synthesized as a soluble precursor of approx. 72000 Da, referred to as tropoelastin (Sandberg *et al.*, 1969; Smith *et al.*, 1972; Sykes & Partridge, 1975). Our laboratory has reported on the existence of a second species of tropoelastin which appears to be distinct from previously characterized tropoelastin. This species of tropoelastin was originally identified by the cell-free synthesis of chick aortic and lung mRNA and confirmed in organ culture of both chick and pig aortic and lung tissues (Foster *et al.*, 1980, 1981; Barrineau *et al.*, 1981a). Krawetz *et al.* (1983) have shown that chick tropoelastin a and b contain identical signal-peptide and mature N-terminal sequences. However, to date, there is no direct chemical data differentiating the two species of tropoelastin. Thus far, we know only that the two tropoelastins differ in apparent M_r (Foster *et al.*, 1980), pI (Foster *et al.*, 1980), antigenic determinants (Karr & Foster, 1983) and regulation of their synthesis in chick aortic development (Barrineau *et al.*, 1981b).

The present study was actually undertaken to isolate the microfibrillar components of elastic fibres. During the course of examining various proteins that were extracted from lathyrctic chick aortae in the presence of strong denaturants, we discovered a protein of M_r approx. 73000 which differed from authentic tropoelastin, yet definitely possessed elastin-like properties. The following report describes the characteristics of this protein and details our reasons for believing that it represents tropoelastin a.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Newly hatched chicks (250) were raised on a lathyrctic diet comprising 0.1% α -aminoacetonitrile hydrochloride and 0.05% β -aminohexanoic acid as previously described (Rich & Foster, 1982). After extraction of the aortae overnight in neutral salt buffer to isolate tropoelastin b (Rich & Foster, 1982), the resulting insoluble material was extracted with 2M-CaCl₂ (1 g wet wt./10 ml) for 2 h at 4°C (Keith *et al.*, 1977), then centrifuged at 16000g for 1 h. The resulting pellet was then washed four times with deionized water, followed by centrifugation (16000g) for 15 min, and then autoclaved in deionized water for 20 min. After centrifugation (16000g for 30 min), the pellet was then extracted with 5M-guanidine hydrochloride/0.1% EDTA/0.1M-Tris (pH 8.5) and dithiothreitol (10 mg/ml) overnight at 37°C. The suspension was then centrifuged at 16000g for 1 h, and the supernatant was dialysed against deionized water for 48 h and freeze-dried.

During all of the extraction procedures, 1 ml samples of the supernatants were dialysed against deionized water and examined for reactivity against chick tropoelastin antisera by double immunodiffusion (Foster *et al.*, 1976).

After dialysis and freeze-drying of the guanidine hydrochloride extract, the material was dissolved in 0.05M-Tris (pH 8.8)/0.2M- ϵ -aminohexanoic acid/1 mM-di-isopropyl fluorophosphate by stirring for 1 h at 4°C (1 mg of protein/ml). The sample was applied to a DEAE-cellulose column (Whatman DEAE-52) equilibrated in the same buffer described above. Specific column conditions are described in Fig. 1 legend. Column fractions were monitored both by the A_{230} and by immunodiffusion against chick tropoelastin antiserum (Foster *et al.*, 1976). Protein peaks were pooled, dialysed against deionized water and freeze-dried.

Protein samples were hydrolysed *in vacuo* at 110°C for 22h in constant-boiling HCl. Amino acid analyses were performed on a Beckman model 119C automated amino acid analyser. Cysteine was analysed as cysteic acid in separate samples oxidized with performic acid (Hirs, 1967).

SDS/polyacrylamide vertical-slab-gel electrophoresis was performed by the method of Laemmli (1970), with an 8% acrylamide running gel. We have previously demonstrated that tropoelastins a and b are not well resolved by SDS/polyacrylamide-gel electrophoresis (Foster *et al.*, 1980). If, however, rabbit reticulocyte lysate is added to samples containing both tropoelastins, the separation of tropoelastins a and b is accentuated by the presence of a prominent 70000-Da protein within the lysate (Foster *et al.*, 1980). To take advantage of this fact, the samples derived from the ion-exchange column were radiolabelled by reduction with NaB^3H_4 (Foster *et al.*, 1975). For comparison, authentic tropoelastin b was also reduced with the same reagent. Radiolabelled samples were separated on SDS/polyacrylamide-gel electrophoresis in the presence of lysate proteins, and fluorography of the resulting gel was performed as described by Bonner & Laskey (1974).

Results and discussion

DEAE-cellulose chromatography of the guanidine hydrochloride extract of the lathyrotic chick aortae is shown in Fig. 1. One of the protein fractions (peak 1) was eluted directly after the initiation of the salt gradient, and a second very broad protein fraction appeared near the middle of the gradient (peak 2). Also included on the chromatogram is the position of authentic tropoelastin b, as determined by applying purified tropoelastin b to the same column under identical conditions. When peaks 1 and 2 were examined by double immunodiffusion, only peak 1 showed reactivity toward antibody directed against chick tropoelastin. SDS/polyacrylamide-gel electrophoresis revealed peak 1 to be homogeneous, with an apparent M_r of approx. 70000–73000, whereas peak 2 was heterogeneous, containing at least four major proteins, varying in M_r from 30000 to 75000 (see Fig. 2). The amino acid compositions of peak 1, peak 2 and authentic tropoelastin b are given in Table 1. The composition of peak 2, which comprises at least four proteins, is quite distinct from that of elastin. The amino acid composition of peak-1 protein is very similar to authentic tropoelastin because of its high content of glycine, proline, alanine and valine residues. Together these amino acids comprise 76% of peak 1 and 84% of tropoelastin b. Peak 1 differs from tropoelastin, since it possesses more polar amino acids (argin-

Fig. 1. DEAE-cellulose chromatogram of guanidine hydrochloride extract

Chromatography was performed in 0.05M-Tris (pH 8.8) with a linear gradient from 0 to 1M-NaCl in the Tris buffer. The column dimensions were 1.5cm × 16cm, with a flow rate of 0.5ml/min at room temperature; 2.5ml fractions were collected.

Table 1. Amino acid compositions of tropoelastin b and DEAE-cellulose column fractions

Compositions are expressed as residues per 1000 amino acid residues.

Amino acid	Peak 1	Peak 2	Tropoelastin
Lysine	33	38	28
Histidine	2	15	1
Arginine	14	40	5
Hydroxyproline	55	17	15
Aspartic acid	20	116	4
Threonine	17	54	13
Serine	21	74	8
Glutamic acid	37	130	13
Proline	132	77	152
Glycine	307	145	347
Alanine	150	77	172
Half-cysteine*	5–8	21	1
Valine	117	49	153
Methionine	–	8	–
Isoleucine	17	30	16
Leucine	45	62	43
Tyrosine	10	21	13
Phenylalanine	18	26	17

* Determined as cysteic acid (see the text for details).

ine, aspartic and glutamic acids) and serine. Because peak 1 behaves as a basic protein on the ion-exchange column, this implies that many of the aspartic and glutamic acid residues exist as amides. Peak 1 also contains five to eight cysteine residues, as compared with one residue found in authentic tropoelastin.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of peak 1 (a) and peak 2 (b) eluted from the DEAE-cellulose column. Gels were stained with 0.2% Coomassie Blue.

Significantly, 29% of the proline residues in peak 1 are hydroxylated, whereas the average hydroxylation of proline residues in tropoelastin b is approx. 10%. As seen in SDS/polyacrylamide-gel-electrophoretic analysis of peak 1 (Fig. 2), there is no evidence of any contaminating collagen, which would contribute to increased hydroxyproline residues.

Since peak 1 resembles tropoelastin, we decided to examine the electrophoretic mobility of peak 1 in the presence of reticulocyte lysate protein as mentioned above. Fig. 3 gives a fluorogram of radiolabelled peak 1 and tropoelastin run on SDS/polyacrylamide-gel electrophoresis under these conditions. Peak 1 migrates slower than tropoelastin b and in the position similar to that reported for tropoelastin a (Foster *et al.*, 1980). This fluorogram also demonstrates that peak 1 does not contain any tropoelastin b.

All of the data obtained on peak 1 agrees with the characteristics detailed for tropoelastin a. As mentioned in the introduction, tropoelastin a has heretofore been described only in cell-free and organ-culture systems. Peak 1 possesses the following characteristics reported for tropoelastin a: (1)

Fig. 3. Fluorogram of SDS/polyacrylamide-gel electrophoresis of radiolabelled peak 1 (a) and tropoelastin b (b) performed in the presence of unlabelled reticulocyte lysate proteins

similar migration to tropoelastin a in SDS/polyacrylamide-gel electrophoresis with reticulocyte lysate present; (2) a high content of valine and proline residues; (3) reactivity with antisera directed against chick tropoelastin; (4) presence of cysteine residues; (5) necessity for reducing and denaturing conditions for isolation; and (6) an amino acid composition which is elastin-like, yet distinct from that of tropoelastin b.

In conclusion, although the overall goal of this study was to isolate microfibrillar components of elastic fibres, the strong denaturing conditions utilized have resulted in the isolation of a chemically unique tropoelastin molecule which has the characteristics of tropoelastin a. It should be pointed out that the recovery of this protein from the lathyrctic chick aortae was very low (1 mg) as compared with tropoelastin b (50 mg). However, we have previously shown that the amount of tropoelastin a present in chick aortae tissue diminishes greatly with age and represents a small proportion of the amount of tropoelastin synthesized in 1-day-old chicks (Barrineau *et al.*, 1981b).

The significance of the high proline hydroxylation of tropoelastin a is unknown; however, it may imply primary structural differences in the tropoelastins, especially in regard to the immediate environment of proline residues.

It is also noteworthy that the amino acid composition determined for peak 1 is similar to

amino acid compositions reported for insoluble elastins isolated from very young developing tissues or aged tissues (Keeley & Partridge, 1974; Faris *et al.*, 1976).

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