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Isolation of Soluble Elastin from Lathyritic Chicks. Comparison to Tropoelastin from Copper Deficient Pigs†

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ABSTRACT: Tropoelastin was isolated from the aortas of chicks rendered lathyritic by treatment with β-aminopropionitrile. The soluble elastin was judged homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis and possessed an estimated molecular weight of 70000. Automated sequential analysis revealed that the N-terminal region of the chick tropoelastin is very homologous to tropoelastin isolated from copper-deficient piglets. N-terminal analysis of a trypsin digest of chick tropoelastin showed that tyrosine frequently is found adjacent to lysine residues. This positioning of tyrosine residues may be significant in terms of a possible regulatory role in elastin cross-link formation.

Significant progress has been made in the study of elastin structure with the recent isolation of a soluble "precursor" of elastin, referred to as tropoelastin. The isolation of this soluble elastin from any source requires inhibition of lysine-derived cross-linkages (Franzblau and Lent, 1969) which are thought to contribute to the extremely insoluble character of mature elastin (Partridge, 1962). The first step in the biosynthesis of elastin cross-links is the oxidative deamination of lysyl residues by the enzyme lysyl oxidase (Pinnell and Martin, 1968). Inhibition of the enzyme by either copper deficiency (Weissman et al., 1963) or β-aminopropionitrile (BAPN)[1] treatment (Sykes and Partridge, 1972) has resulted in the isolation of a non-cross-linked, soluble elastin which is readily amenable to structural studies. The most extensive information currently available on tropoelastin has come from material isolated from copper-deficient piglets. This tropoelastin has a molecular weight of approximately 72000 (Sandberg et al., 1969) and possesses the same amino acid composition as insoluble porcine elastin, with the only difference being that the precursor elastin contains few, if any, cross-linkages but does possess a high content of lysine residues relative to the insoluble elastin. Foster et al. (1973) have published extensive primary sequence data on tryptic peptides derived from porcine tropoelastin which indicate an elastin primary structure distinct from that of collagen and possessing repeating units of tetra-, penta-, and hexapeptides.

Recently, Sykes and Partridge (1974) have reported on the isolation of soluble elastin from the aortas of BAPN-treated chicks. This tropoelastin has a molecular weight of 57000, which differs significantly from that isolated from the porcine source.

Since the isolations of these tropoelastin samples differed in the method of enzyme inhibition, the purification procedure, and animal source, the discrepancy in molecular weights could be attributable to any of these differences. An alternative explanation for the molecular weight difference is suggested by the finding of several laboratories that proteolysis of soluble elastin occurs during isolation procedures. Consequently, various enzyme inhibitors have been employed to protect the tropoelastin from proteolytic attack (Sandberg et al., 1975; Narayanan and Page, 1974).

The present communication reports on the isolation and characterization of tropoelastin from the aortas of BAPN-treated chicks and compares this elastin to that derived from a copper-deficient, porcine source.

Materials and Methods

Preparation of Tropoelastin. The procedure followed was a modification of that described by Sykes and Partridge (1974). The method actually is based on one first reported by Smith et al. (1972). The major modification introduced was the addition of protease inhibitors during all but the final steps of the procedure in order to prevent tropoelastin degradation. One thousand chicks were raised on a diet of commercial starting feed supplemented with 0.1% (w/w) β-aminopropionitrile fumarate (BAPN) beginning on the day of hatching. During the feeding period, there was a mortality of approximately 10%. After 6 days the chicks were sacrificed and their aortas removed and immediately placed in a wash solution containing 20 m*M* disodium ethylenediaminetetraacetate (EDTA), 5 m*M* BAPN, and 5 m*M*

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[1] Abbreviations used are: BAPN, β-aminopropionitrile; EDTA, disodium ethylenediaminetetraacetate; MalNEt, *N*-ethylmaleimide; Pth, phenylthiohydantoin derivatives; TPCK, L-tosylamido-2-phenylethyl chloromethyl ketone.

N-ethylmaleimide (MalNEt) in water (pH 7.0) at 4°C. All steps in the purification were carried out at 4°C. After 30 min, the washed tissue was homogenized (Polytron, Brinckmann Instruments) in 10 volumes (v/w) of neutral salt buffer (0.5 *M* NaCl–0.02 *M* sodium phosphate (pH 7.4)) containing the same inhibitor concentrations as the wash. The mixture was extracted overnight with stirring and centrifuged at 25000*g* for 1 hr. The pellet was stored frozen for future isolation of insoluble elastin. The supernatant was filtered through paper to remove insoluble lipid material, and the pH was lowered to 4.0 using 4 *N* acetic acid in order to precipitate the salt-soluble collagen. The mixture was allowed to stand for 2 hr and then centrifuged for 1 hr at 10000*g*. The supernatant was readjusted to neutral pH using 2 *N* NaOH, and NaCl was added to 15% (w/v) to precipitate the tropoelastin. The mixture was allowed to stand overnight and then centrifuged at 13000*g* for 45 min. The precipitate was redissolved in the original extracting buffer and stirred overnight. Undissolved material was removed by centrifugation for 45 min at 13000*g*. The precipitation step with 15% NaCl was repeated twice. The third precipitate was redissolved in 0.01% (v/v) acetic acid, dialyzed against the same solution, and lyophilized. This completed the extraction procedure described by Sykes and Partridge (1974). As judged by amino acid analysis and dodecyl sulfate polyacrylamide gel electrophoresis (see below), however, the lyophilized product still contained large amounts of collagen and other contaminants. Redissolving the lyophilized material in water and centrifuging out the undissolved material removed some collagen and most of the other contaminants. The resulting supernatant was made 0.5 *M* in NaCl and the pH adjusted to 4.0 with 4 *N* acetic acid to precipitate the remaining collagen. The final solution, containing pure tropoelastin, was dialyzed against water and lyophilized.

Preparation of Insoluble Elastin. Insoluble elastin was prepared from the pellet by the Lansing procedure in the following manner (Lansing et al., 1952). The pellet was defatted with ethanol and ether and the dried material was suspended in 0.1 *N* NaOH (50 ml/g of tissue) and kept at 98°C for 45 min with occasional stirring. Insoluble material was washed successively with water, ethanol, and ether, and dried.

Reduction of Elastin. Samples of both insoluble elastin and tropoelastin were reduced with sodium borotritide as described previously (Lent et al., 1969), in order to quantitate cross-links and the cross-link precursor, allysine (Franzblau and Lent, 1969). For insoluble elastin, the dried residue from the neutral salt extraction was suspended in 1 m*M* EDTA (pH 9.0). A mixture of 45 mg of $NaBH_4$ and 5 mCi of NaB^3H_4 (New England Nuclear) was added in a ratio of 1:5, reducing agent–tissue (w/w). Reduction was allowed to proceed for 90 min at room temperature with constant stirring, after which time the pH was lowered to 3 with 50% acetic acid to destroy any excess reducing agent. For tropoelastin, the protein was dissolved in 1 m*M* EDTA and 200 μg of NaB^3H_4 (0.6 mCi) per mg of protein was added. Reduction proceeded for 90 min at 4°C, after which the pH was lowered as above. The sample was then dialyzed against water. Samples of reduced elastin were hydrolyzed both with constant boiling HCl and with 2 *N* NaOH for 22 hr at 108°C. Hydrolysates were chromatographed on a Technicon amino acid analyzer equipped with a stream-splitting device. The gradient of Burns et al. (1965) was used for the base-hydrolyzed elastin. Fractions were assayed for radioactivity by liquid scintillation counting.

Amino Acid Analyses. Samples were taken for amino acid analyses at every stage in the tropoelastin extraction procedure. Hydrolyses were done using constant-boiling HCl in sealed tubes for 22 hr at 108°C. Hydrolysates of unreduced samples were chromatographed on a Beckman 119 automatic amino acid analyzer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The method of Weber and Osborn (1969) was followed for all samples examined. Ten percent gels with a ratio of *N,N*-methylenebisacrylamide to acrylamide of 1:74 were used. Gels were stained with Coomassie Brilliant Blue. Samples of 20–50 μg, based on Lowry determinations (Lowry et al., 1951), were taken for gels at every stage in the tropoelastin extraction. Standards containing rat tail tendon collagen, bovine serum albumin, and actin were run with each set of gels.

Tryptic Digestion. Lyophilized protein was dissolved in 0.2 *M* NH_4HCO_3 (pH 8.0) at a concentration of 10 mg/ml. Freshly prepared TPCK-treated trypsin, 1 mg/ml in 0.05 *M* Tris (pH 8.0), was added 1:100, enzyme–substrate and digestion allowed to proceed at 37°C with gentle mixing. After 2 hr an identical amount of new enzyme was added, and digestion was continued for an additional 2 hr. The sample was then lyophilized and resuspended in water at a concentration of 20 mg/ml. An aliquot of the digest was subjected to one cycle of the automated sequenator for quantitation of N-terminal amino acid residues.

Sequential Analyses. Automated sequence analysis was performed on a Beckman Model 890C sequencer according to Edman and Begg (1967). The extreme hydrophobic nature of the tropoelastin led us to use the procedure of Braunitzer et al. (1970) for rendering the protein less extractable. However, instead of performing the derivatization prior to the application of the protein to the sequenator, the reaction was performed directly in the sequenator cup. Tropoelastin was dissolved in 0.8 ml of 50% ethanol and dried to a film in the sequenator cup using the standard Beckman application program (02772). The cup was then opened and 2–3 mg of sulfophenyl isothiocyanate in 0.8 ml of dimethylallylamine buffer (DMAA) and 80 μl of tributylamine were added. The cup was spun at a low speed for 15 min with several nitrogen flushes. The reaction mixture was then dried again using the application program. The protein sequencing program (071472), using the quadrol buffer system, was then initiated at the coupling step to ensure complete reaction of the N-terminal amino acid. Pth-norleucine was added to all tubes in the sequenator and served as an internal standard. The fractions were converted to Pth derivatives in the usual manner and extracted in ethyl acetate (Edman and Begg, 1967). Gas chromatographic analyses were performed on a Beckman Model 65 gas chromatograph according to the method of Pisano and Bronzert (1969) on 10% of each residue. The remaining aqueous and ethyl acetate layers were separately hydrolyzed with hydriodic acid (Smithies et al., 1971) to regenerate the free amino acid, and amino acid analyses were performed.

Results

Preparation of Tropoelastin. A flow diagram illustrating the isolation of tropoelastin is given in Figure 1 with each of the various purification steps designated alphabetically. Polyacrylamide gel electrophoretic patterns of several of these fractions are given in Figure 2. As stated above the method of isolation followed was essentially according to

chick aortas	extraction in neutral salt buffer 0.02 *M* sodium phosphate–0.5 *M* NaCl, 5 m*M* BAPN–5 m*M* MalNEt–20 m*M* EDTA at 4°C overnight centrifuge at 25000*g* 1 hr
pellet A	supernatant B filter acidify to pH 4.0 with 4 *N* HOAC centrifuge 10000*g* 1 hr
precipitate C	supernatant D adjust pH to 7.2 with 2 *N* NaOH add NaCl to 15% centrifuge 13000*g* 45 min
precipitate redissolved in original buffer centrifuge 13000 *g* 45 min	supernatant E
insoluble material F	supernatant G repeat precipitation
precipitate redissolve	supernatant H
insoluble I	supernatant J repeat precipitation
precipitate K redissolve in 0.01% HOAC dialyze, lyophilize redissolve in H_2O centrifuge 20000*g* 40 min	supernatant L
precipitate M	supernatant N NaCl to 0.5 *M* pH to 4.0
precipitate O	supernatant P (tropoelastin)

FIGURE 1: Flow diagram summarizing the isolation and purification of tropoelastin from aortas of lathyritic chicks. Each of the various purification steps is labeled alphabetically.

Sykes and Partridge (1974). However, the latter authors had suggested the application of DEAE-cellulose chromatography after precipitation of salt-soluble collagen by acidification with acetic acid. This procedure was used for removal of acidic proteins which were found by the latter investigators to lower their yields of tropoelastin. We have omitted this step in our procedure and proceeded directly to three NaCl precipitations. Fraction K (see Figure 1) which resulted from the third salt precipitation was not homogeneous as judged by gel electrophoresis (see Figure 2). When fraction K was redissolved in H_2O a residue remained which was separated by centrifugation and found to be high in acidic amino acids. The supernatant contained both collagen and tropoelastin as judged by electrophoretic mobilities, and the remaining collagen was removed by acid precipitation (fraction O). Based on a starting wet weight of 55 g of whole chick aortas, 35 mg of purified tropoelastin was recovered. We have subsequently repeated this isolation procedure on 2000 chicks and have recovered 65 mg of tropoelastin. The amino acid composition of the purified tropoelastin (fraction P) is given in Table I. Also included in Table I are the amino acid analyses of aortic insoluble elastins isolated from normal and lathyritic chicks. All analyses incorporate data from reduced elastin samples which were examined by both acid and alkaline hydrolyses for cross-link quantitation. The molecular weight of the purified tropoelastin was estimated to be approximately 70000–72000 by dodecyl sulfate gel electrophoresis. The tropoelastin sample was found to be 90–95% pure by N-terminal analysis on the automated sequencer.

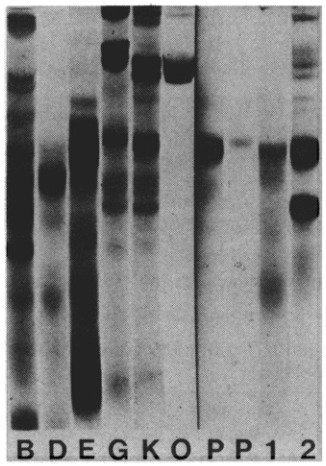

FIGURE 2: Polyacrylamide gel electrophoretic patterns of: (1) several fractions (B,D,E,G,K,O) obtained during the tropoelastin isolation procedure (see Figure 1 for designation); (2) purified tropoelastin (P) at loadings of approximately 10 and 50 μg; (3) standard bovine serum albumin (1) and rat tail tendon collagen, bovine serum albumin, and actin (2).

Table I: Amino Acid Compositions of Chick Soluble and Insoluble Elastin.[a]

Amino Acid	Tropoelastin Fraction *P*	Lathyritic Insoluble Elastin	Control Insoluble Elastin
Lysine	38.5	13.2	3.6
Histidine			
Arginine	8.0	5.9	4.5
Hydroxyproline	10.4	17.8	22.0
Aspartic acid	3.9	3.3	1.9
Threonine	10.7	7.8	3.1
Serine	6.8	5.4	5.1
Glutamic acid	13.5	13.3	12.0
Proline	124.3	141.2	128.0
Glycine	337.6	340.1	352.1
Alanine	178.4	168.4	176.2
Valine	174.9	165.6	175.0
Isoleucine	17.4	20.7	18.8
Leucine	54.1	54.4	47.4
Tyrosine	12.3	13.1	12.0
Phenylalanine	19.5	21.1	23.5
Allysine[b]	2.4	0.5	6.0
A.C.P.[c]	2.8	10.9	12.0
Lysinonorleucine		1.7	1.1
Isodesmosine		3.0	2.8
Desmosine		3.1	3.2
Merodesmosine			1.8

[a] Compositions are expressed as residues per 1000 amino acid residues. [b] Allysine, determined as ε-hydroxynorleucine the reduced derivative. [c] A.C.P. is the aldol condensation product of two allysine residues determined as the reduced derivative. Cross-links are expressed as lysine equivalents.

Primary Sequence Studies. The first 42 amino acid residues in the N-terminal region of the tropoelastin molecule were determined by automated sequential analysis. The sequence obtained is illustrated in Figure 3.

In order to examine the amino acid residues positioned

N-Terminal Sequence of Chick Tropoelastin

```
          5                  10                  15
Gly-Gly-Val-Pro-Gly-Ala-Ile-Pro-Gly-Gly-Gly-Val-Pro-Ala-Gly-Val-
          20                 25                  30
Phe-Phe-Pro-Gly-Ala-Gly-Val-Gly-Gly-Leu-Gly-Ala-Gly-Leu-Gly-Ala-
     35                 40
Gly-Leu-Pro-Ala-Gly-Val-(Val)-Pro-Leu-Gly
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Tentative assignments are given in separate parentheses, i.e., -(Val)- means a valine residue is suspected at this position, but not definitely proven.

FIGURE 3: Automated sequential analyses of chick tropoelastin.

Homologies between N-Terminal Regions of Porcine and Chicken Tropoelastin

```
          5          10         15         20         25
Pig   GGVPG AVPGG VPGGV FFPGA GLGGL G
Chick GGVPG AIPGGGVPAGV FFPGA GVGGL G
```

FIGURE 4: A comparison of the N-terminal regions of chick and porcine tropoelastins. G = glycine, A = alanine, V = valine, P = proline, F = phenylalanine, L = leucine, and I = isoleucine.

adjacent to the α-carboxyl group of lysine residues, tropoelastin was digested with trypsin and an aliquot of the digest examined for N-terminals by one cycle of the automated sequenator. The results are given in Table II.

Table II: N-Terminal Analysis of Tryptic Digest of Tropoelastin.

	Pth-Amino Acid					
	Glycine	Alanine	Valine	Leucine	Phenyl-alanine	Tyrosine
nmoles	7.6	37.84	4.48	4.16	4.00	17.12
%	10	50	6	6	5	23

Discussion

The isolation of a homogeneous tropoelastin preparation possessing a molecular weight of approximately 70000 clearly demonstrates a similarity in the effect of copper deficiency and BAPN-induced lathyrism on aortic elastin of young animals. That the N-terminal areas of both the porcine and chick tropoelastins are very homologous is illustrated in Figure 4. With the exception of a glycine insertion in residue 11, the only differences between the two soluble elastins are conservative substitutions attributable to one base change in the coding triplet. It should be noted here that reduction followed by base hydrolysis revealed that tropoelastin contained 2 allysine residues and 1–2 aldol condensation products of 2 allysine residues per 1000 amino acid residues. This suggests that BAPN inhibition of the enzyme lysyl oxidase was not complete. Since reduction studies have not been performed on porcine tropoelastin, we cannot yet compare these results to the copper-deficient material.

Sequencing work performed on porcine tropoelastin has revealed that tyrosine frequently occupies a position adjacent to the α-carboxyl of lysyl residues (Foster et al., 1973). More recently, we have reported on the sequence of several desmosine cross-linked peptides from insoluble elastin and found that a tyrosine residue is located next to the pyridinium nucleus of the desmosine (Foster et al., 1974). We have postulated that tyrosine may play a regulatory role in cross-link formation. Since one of the four lysine residues involved in the synthesis of the pyridinium ring must retain its ϵ-amino group, a tyrosine residue may inhibit the deamination of an adjacent lysyl either sterically or through hydrogen bonding. In the present study, N-terminal analysis of a tryptic digest of chick tropoelastin resulted in the recovery of 7.6 nmol of glycine, 37.84 nmol of alanine, 4.48 nmol of valine, 4.16 nmol of leucine, 4.00 nmol of phenylalanine, and 17.12 nmol of tryosine (see Table II). Assuming that valine, leucine, and phenylalanine are all equal to one amino acid residue then at a minimum, there would be four tyrosine N-terminal residues. This is approximately the same number of tyrosine present after tryptic digestion of porcine tropoelastin (Foster et al., 1973).

Sykes and Partridge (1972, 1974) had reported on the isolation of chick tropoelastin possessing a molecular weight of 57000. We had previously repeated this work without the addition of any proteolytic inhibitors and isolated two soluble elastin fractions, the more prominent one having a molecular weight of 55000–60000 and the second one possessing a molecular weight of approximately 70000. Purification of these fractions by gel filtration revealed a similar amino acid composition. However, the N-terminal sequence of the lower molecular weight material was Ala-Ala-Gly-Leu, whereas that of the higher molecular weight material was Gly-Gly-Val-Pro. In the present study, the addition of enzyme inhibitors prevented the appearance of the lower molecular weight material which strongly suggests that it represents a degradation product of the 70000 component. The apparent specificity of this degradation which occurs especially in tropoelastin isolated from BAPN-treated chicks should be noted and investigations are currently underway to determine if such a cleavage is biologically significant and, possibly, analogous to the procollagen to collagen conversion. This apparent specific proteolysis is not observed with copper-deficient piglets (Sandberg et al., 1969) or chicks (Rucker and Goettlick-Riemann, 1972) since the 72000 molecular species is dominant even in the absence of inhibitors.

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Equilibrium Perturbation by Isotope Substitution†

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ABSTRACT: When malic enzyme is added to a mixture of malate-*2-d,* TPN, CO_2, pyruvate, and TPNH at concentrations calculated to be at equilibrium, the TPNH level first drops and then increases slowly to its original level. This equilibrium perturbation is caused by slower cleavage of C–D than C–H bonds during hydride transfer as malate-*2-d* and TPNH are partly converted into TPND and malate-*2-h* in the process of establishing isotopic equilibrium. With malate-*2-d,* isotope effects for malic enzyme at pH 7.1 and malate dehydrogenase at pH 9.3 of 1.45 and 1.70–2.16 (depending on oxaloacetate level) were determined with this method, while the corresponding isotope effects on V/K_{malate} and V for the chemical reactions were 1.5–1.8 and 1.0, and 1.9 and 1.5 for the two enzymes. The advantage of this method is its extreme sensitivity, and the lack of interference from various artifacts. The sensitivity is sufficient to permit determination of ^{13}C and ^{15}N isotope effects in favorable cases, and values of 1.031 for malic enzyme with $^{13}CO_2$, and 1.047 for glutamate dehydrogenase with $^{15}NH_4^+$ have been determined. In the course of this work it was discovered that the equilibrium constants for oxidation by DPN, and oxidative decarboxylation by TPN are lower for malate-*2-d* than for malate-*2-h* by a factor of 0.76–0.82. Changes in K_{eq} upon deuterium substitution, which are predicted by the calculations of Hartshorn and Shiner (1972), should be observed for many other reactions as well.

It is well established that cleavage of C–D bonds is slower than cleavage of C–H bonds, and thus that dehydrogenases and other enzymes where C–H bond cleavage is involved show deuterium isotope effects (v_H/v_D) varying from 1 to as high as 15 (Bright and Gibson, 1967). The study of isotope effects has been given considerable impetus by the recent discovery of Northrop (1975) that comparison of the apparent deuterium and tritium isotope effects on V/K will permit calculation of the true isotope effects on the bond-breaking step, regardless of how many other steps precede or follow the bond breaking one, and thus a determination of how rate limiting the bond-breaking step really is. The usual method of determining deuterium isotope effects is to vary both deuterated and nondeuterated substrates, and compare the slopes (V/K effect) and intercepts (V effect) of the resulting reciprocal plots. This method requires concentrations to be accurately known (for comparison of V/K values), and that neither substrate contains any inhibitors (for comparison of V values), and is only capable of determining effects greater than about 1.1. Tritium isotope effects are determined by comparing the specific activity of the substrate with that of the first portion of tritiated product formed (which measures the V/K effect only), and realistically only values above 1.05 are meaningful.

While some enzymes show very nice isotope effects when the above methods are applied, others do not. Thus at pH 7 with malic enzyme the deuterium effects on V/K and V are 1.5–1.8 and 1.0, although the V effect does approach 3 at high and low pH; the V/K effect is nearly pH independent (Schimerlik and Cleland, 1975). With glutamic dehydrogenase we have seen in unpublished experiments no effect on either V or V/K with glutamate-*2-d* as substrate, although Fisher et al. (1970) have seen an effect of 1.5–1.8 on the pre-steady-state burst of TPNH production on the enzyme (norvaline-*2-d,* on the other hand, gives effects of 6 and 3.2 on V and V/K). These low values for enzymes where there certainly should be effects on the bond-breaking steps are caused by the rate-limiting step at high (V) or low (V/K) substrate concentrations being some other step than the bond-breaking one. It would thus be of considerable value to have a method for determining isotope effects lower than 1.1, or for measuring them under conditions where they were more fully expressed than in the usual experiments where initial velocities of the chemical reaction are determined.

When reaction mixtures are made up at concentrations calculated to be near equilibrium, and enzyme is then

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