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Elastin Cross-Linking *in vitro*

STUDIES ON FACTORS INFLUENCING THE FORMATION OF DESMOSINES BY LYSYL OXIDASE ACTION ON TROPOELASTIN

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The formation of isodesmosine and desmosine *in vitro* by the action of lysyl oxidase on tropoelastin was studied. The synthesis of desmosines occurred in the absence of additional substances. The formation of desmosines was not affected by removal of molecular O_2 from the reaction medium nor was it affected by the lack of proline hydroxylation in tropoelastin. However, there was virtually no desmosine formation at 15°C, a temperature not conducive to coacervation, indicating that coacervation is an important prerequisite for cross-linking.

Elastin is a polymer of linear polypeptide chains randomly cross-linked into a highly extensible three-dimensional network. Two novel amino acids, isodesmosine and desmosine, are chiefly responsible for cross-linking in elastin (Thomas *et al.*, 1963). The desmosines are each derived from four lysine residues in peptide linkage (Partridge *et al.*, 1964, 1966; Miller *et al.*, 1964; Anwar & Oda, 1966). Cross-linking is initiated by the oxidative deamination of certain lysine residues to α-aminoadipic-δ-semialdehydes (allysines) by the enzyme lysyl oxidase; the allysines then condense with each other or with lysines to form the desmosines and other cross-links (Pinnell & Martin, 1968; Piez, 1968). The reaction of lysyl oxidase is the key step in the cross-linking of elastin, and inhibition of lysyl oxidase activity by copper deficiency or lathyrism results in the accumulation of uncross-linked elastin precursor, termed soluble elastin or tropoelastin (Smith *et al.*, 1968; Sykes & Partridge, 1972).

Several of the stages in the cross-linking of elastin are not fully understood, among them the mode of action of lysyl oxidase on tropoelastin and the relationship between the physicochemical properties of the substrate and the cross-linking process. It is also possible that enzymes other than lysyl oxidase may participate. Answers to these questions have not been forthcoming because of lack of a system for cross-linking tropoelastin *in vitro*. We have devised such a system (Narayanan & Page, 1976) and now report its use to investigate these aspects of cross-linking.

Experimental

Materials

L-[4,5-^{3}H]Lysine and Aquasol were obtained from New England Nuclear, Boston, MA, U.S.A. D-Penicillamine and β-aminopropionitrile were the products of Sigma Chemical Co., St. Louis, MO, U.S.A., and Calbiochem, La Jolla, CA, U.S.A., respectively. Chick tropoelastin, used as standard for protein determinations, was a gift from Dr. R. B. Rucker, University of California, Davis, CA, U.S.A.

Methods

Preparation of tropoelastin. Tropoelastin was purified from pulse-labelled embryonic chick aortas. Eighteen aortas obtained from 17-day-old chick embryos were radioactively labelled for 30 min with L-[4,5-^{3}H]lysine (130 μCi) in 10 ml of Dulbecco–Vogt medium lacking lysine but supplemented with ascorbic acid and β-aminopropionitrile (50 μg/ml each), 100 units of penicillin/ml and 100 μg of streptomycin/ml (Narayanan *et al.*, 1974*a*). The aortas were extracted at 4°C in 0.5 M-acetic acid and the extract was freeze-dried. Tropoelastin was purified from the freeze-dried extract by coacervation followed by extraction in organic solvents (Narayanan & Page, 1976). The preparation so obtained was homogeneous on 5% sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and 6 M-urea/polyacrylamide-gel electrophoresis (Narayanan & Page, 1976) and contained 8×10^4 to 12×10^4 c.p.m./mg of protein.

Preparation of lysyl oxidase. Lysyl oxidase was purified from embryonic chick cartilage by chromatography through collagen coupled to Sepharose 4B followed by DEAE-cellulose chromatography (Narayanan *et al.*, 1974*b*). The enzyme was assayed by quantifying ^{3}H released from tropoelastin as described previously (Narayanan *et al.*, 1974*a*, *b*).

Incubations. Tropoelastin containing 4×10^4 to 6×10^4 c.p.m. in 0.02 M-NaH_2PO_4 buffer containing 0.15 M-NaCl, pH 7.7, was preincubated at 37°C for

1h and then 50–100μg of lysyl oxidase was added. The final volume was made up to 4.0ml with the buffer and the incubation continued for 24h. Control incubations were carried out in the presence of 100μg of β-aminopropionitrile/ml, a specific inhibitor of lysyl oxidase. A residue fraction of unlabelled aortas from centrifugation at 20000g that were homogenized in 0.25ml of incubation buffer per aorta, was added to some incubations to supplement enzymes or other substances that may be required for the synthesis of desmosines and that may have been lost during purification. For experiments described in Tables 2 and 5, tropoelastin containing allysyl residues was obtained by incubation with lysyl oxidase in the absence of the non-radioactive aorta fraction but in the presence of 2mM-D-penicillamine (at this concentration D-penicillamine inhibits lysyl oxidase activity by 70%). The latter was added to bind with the allysyl residues formed by lysyl oxidase action and thereby prevent their cross-linking (Pinnell *et al.*, 1968). Before subsequent incubation, the penicillamine was removed by dialysis at 4°C against 0.5M-acetic acid and then against the incubation buffer.

To study the effect of removal of molecular O_2 on the formation of desmosines, the tropoelastin with allysines was evacuated for 20min at 4°C and then flushed for 20–30min with N_2 from which traces of O_2 had been removed by passage through two flasks containing Fieser's solution (a solution of 2g of sodium anthraquinone-β-sulphonate and 15g of $Na_2S_2O_4$ in 20% KOH) and one saturated lead acetate (Fieser & Fieser, 1967). After the incubation, any 1,2-dihydropyridines formed were destroyed by adding a solution of 0.1M-$NaIO_4$ containing 40μmol of OsO_4 and stirring overnight in the dark (Davis, 1978); the latter treatment was included to prevent the oxidation of dihydropyridines to desmosines during the subsequent analytical procedures.

Amino acid analysis of desmosines and prolines. Proteins were hydrolysed in 6M-HCl for 48h at 108°C. Radioactive desmosines were then separated on a Beckman model 120C automatic amino acid analyser with UR-30 resin (Narayanan & Page, 1976). Elution was achieved at 62°C with 0.35M-sodium citrate buffer. Amino acids from non-radioactive proteins (elastin or soluble elastin) served as internal markers and carriers. The effluent was collected in fractions and 1.0ml portions were counted for radioactivity in Aquasol (Narayanan & Page, 1976).

Protein. This was determined by the method of Lowry *et al.* (1951). For tropoelastin determinations, chick tropoelastin was used as the standard.

Results

We have shown previously that isodesmosine and desmosine are formed in a homogeneous preparation of tropoelastin by incubation with purified chick cartilage lysyl oxidase in the presence of an unlabelled aorta residue fraction (Narayanan & Page, 1976; Narayanan *et al.*, 1977). In this system, desmosine formation is dependent on tropoelastin concentration and is linear with incubation time (Narayanan *et al.*, 1977). To determine whether enzymes in addition to lysyl oxidase are needed for the formation of desmosines we incubated soluble elastin with and without the unlabelled aorta residue fraction or with heat-inactivated aorta residue. From Table 1 it can be seen that desmosines were formed in the absence of the aorta residue fraction. However, addition of the unlabelled aorta residue enhanced the quantity of desmosines formed by 64% (57–107% in several experiments). However, the enhancement also occurred in the presence of heat-inactivated aorta residue, although to a somewhat lesser extent (30%). Similar results were obtained with the extracts of unlabelled aortas (Table 1).

In the formation of isodesmosine and desmosine, the final step is the condensation of an allysine with

Table 1. *Effect of aorta fractions on the formation of desmosines*

Unlabelled chick aortas were extracted twice by homogenizing each aorta in 0.25ml of 0.1M-NaH_2PO_4, pH7.7, containing 0.15M-NaCl. The extract and pellet fractions were separated by centrifugation at 20000g and the pellet was then suspended by homogenizing in buffer (0.25ml/aorta). Incubations and analysis were carried out as described under 'Methods'. Each incubation contained 6.2×10^4 c.p.m. of tropoelastin.

		$10^{-2}\times$ Radioactivity (c.p.m.)		
Addition to incubations	$10^{-3}\times$ Lysyl oxidase activity (c.p.m.)	Isodesmosine	Desmosine	Isodesmosine + desmosine
None	6.8	3.6	2.8	6.4
Pellet suspension (0.5ml)	7.4	6.3	4.2	10.5
Heated pellet suspension* (0.5ml)	4.9	5.3	3.0	8.3
Pellet+β-aminopropionitrile†, (400μg)	0.8	0.2	0.6	0.8
Extract (0.5ml)	7.4	4.2	3.8	8.0
Heated extract* (0.5ml)	7.3	4.5	3.4	7.9

* Heated at 100°C for 15min.

† This treatment represents the background value.

dehydromerodesmosine to form 1,2-dihydropyridines, the latter are subsequently oxidized to desmosines. The oxidation may occur spontaneously in the presence of molecular O_2 (Davis & Anwar, 1970) or by the simultaneous formation of 1 mol of lysinonorleucine (Piez, 1968). We investigated the possibility of involvement of molecular O_2 by incubation of allysine-containing tropoelastin in the absence of O_2. Allysine-containing tropoelastin, prepared as described under 'Methods', was incubated after evacuation for 20min to remove air from the incubation mixture followed by flushing with N_2 from which traces of O_2 had been removed by passing through Fieser's solution. This procedure appeared to have effectively removed O_2 from the reaction mixture, since the activity of lysyl oxidase, an enzyme requiring O_2 (Narayanan *et al.*, 1974*b*), was completely inhibited. Thus, in a separate experiment, in the unevacuated incubation of lysyl oxidase plus substrate, the lysyl oxidase activity (measured by c.p.m. due to ^3H release) was 713 c.p.m., whereas the activity in samples that were evacuated, evacuated plus N_2 and evacuated plus O_2 were respectively 0, 0 and 556 c.p.m. In addition, incubations were carried out in the presence of O_2 (positive control) or 5mM-D-penicillamine (negative control). After incubation, a solution of 0.1M-$NaIO_4$ containing 40μmol of OsO_4 was added to the treated samples and stirred in the dark overnight. This was carried out to destroy any 1,2-dihydropyridines that may be oxidized to desmosines during subsequent procedures. Thus if O_2 is a requirement for the formation of desmosines, no or less desmosines will be expected in the treated samples than in the positive control. As shown in Table 2, the removal of O_2 did not significantly inhibit the formation of desmosines, since the O_2-depleted incubation mixtures contained 64 and 70% as much desmosines as the positive control. Formation of desmosines was considerably inhibited by the presence of D-penicillamine.

In tropoelastin (and elastin) approx. 10% of the proline residues are hydroxylated, but the role of hydroxyproline has not been elucidated. In collagen, hydroxyproline residues are necessary to stabilize the triple helix at 37°C and therefore for its secretion (Jimenez *et al.*, 1973; Rosenbloom *et al.*, 1973). We have previously shown that hydroxyproline is not necessary for the secretion of tropoelastin or for oxidative deamination (Narayanan *et al.*, 1977), although the influence of hydroxylation on cross-link formation was not evaluated. To study this question, tropoelastin was prepared from aortas incubated in the presence of 0.5mM-$\alpha\alpha'$-bipyridyl, an inhibitor of proline and lysine hydroxylation (Rosenbloom *et al.*, 1973). The tropoelastin so obtained did not contain significant amounts of hydroxyproline (Table 3). However, on incubation of this substrate with lysyl oxidase, the enzyme activity as well as quantity of desmosines formed were comparable with those of the preparation containing normal amounts of hydroxyproline (Table 4).

Because of the abundance of hydrophobic amino acids, both collagen and elastin become insoluble under physiological conditions. Lysyl oxidase does not act on soluble collagen molecules; enzyme action and subsequent cross-link formation occurs only after collagen fibril formation (Siegel, 1974). Thus one biological role of insolubilization and fibril

Table 2. *Effect of removal of molecular O_2 on the formation of desmosines from tropoelastin*

Tropoelastin was incubated for 24h with lysyl oxidase in the presence of 2mM-D-penicillamine, but without the unlabelled aorta residue. After removing the penicillamine by dialysis at 4°C against 0.5M-acetic acid and then against the incubation buffer, the allysine-containing soluble elastin was divided into equal portions of 6.6×10^4 c.p.m. (Expt. 1) and 8.6×10^4 c.p.m. (Expt. 2) and incubated as follows. Lysyl oxidase activities were 5.4×10^3 c.p.m. and 7.4×10^3 c.p.m. of ^3H released respectively.

Expt. no.	Treatment	Addition to incubation	$10^{-2}\times$Radioactivity (c.p.m.) Isodesmosine	Desmosine	Isodesmosine+ desmosine	Radioactivity (%)
1	$+O_2$	None	1.2	1.0	2.2	100
	$-O_2$	None	0.6	0.8	1.4	64
	$+O_2$	D-Penicillamine*	0.3	0.1	0.4	18
2	$+O_2$	None	2.9	2.1	5.0	100
	$-O_2$	None	2.0	1.5	3.5	70
	$+O_2$	D-Penicillamine*	0.6	0.3	0.9	18
3	Elastin, untreated†		3	5	8	
	Elastin, treated†‡		3	3	6	

* 5mM.

† Residues per 1000 residues. This experiment was a control for the influence of oxidation on desmosines.

‡ With 0.1M-$NaIO_4$ containing 40μmol of OsO_4 as described under 'Methods'.

formation appears to be the apposition of molecules in a conformation permitting lysyl oxidase action and cross-linking. In two separate experiments, we explored the possibility that coacervation of tropoelastin serves a role similar to fibril formation in collagen. In the first experiment, we compared the lysyl oxidase activity and the amount of desmosines formed at 15°C, a temperature not conducive to coacervation, with the amount formed at 37°C, at which temperature the substrate will coacervate (Sykes & Partridge, 1974). As a control, these parameters were measured at 37°C in the presence of β-aminopropionitrile. From Expt. 1 of Table 5 it can be seen that at 37°C the amount of desmosines formed was 9 times that formed in the presence of β-aminopropionitrile and that even though 60% of the lysyl oxidase activity observed at 37°C was present at 15°C, the quantity of desmosines formed

Table 3. *Content of radioactive hydroxyproline in tropoelastin preparations from aortas incubated with and without αα′-bipyridyl*

Aortas (18) were labelled with 100μCi of L-[G-^{3}H]-proline in the presence or absence of 0.5mM-αα′-bipyridyl. Tropoelastin was purified and subjected to amino acid analysis as described under 'Methods'.

	10^{-2} × Radioactivity (c.p.m.)		
Incubation	Hydroxyproline	Proline	Hydroxyproline/proline*
Without αα′-bipyridyl	2.7	9.1	0.3
With αα′-bipyridyl (0.5mM)	0	20.9	0

* Hydroxyproline/proline ratio of chick soluble elastin 0.1 (Rucker & Goettlich-Riemann, 1972).

Table 4. *Synthesis of desmosines from tropoelastin preparations with and without hydroxyproline*

The tropoelastin preparations were obtained as described in Table 3. Incubations with lysyl oxidase were carried out with or without 100μg of β-aminopropionitrile/ml essentially as described in 'Methods'.

			10^{-2} × Radioactivity (c.p.m.)		
Tropoelastin	Addition	10^{-3} × Lysyl oxidase activity (c.p.m.)	Isodesmosine	Desmosine	Isodesmosine +desmosine
−Hydroxyproline	None	2.6	2.1	1.9	4.0
−Hydroxyproline	β-Aminopropionitrile	0.7	0.3	0.3	0.6
+Hydroxyproline	None	2.6	2.3	1.2	3.5
+Hydroxyproline	β-Aminopropionitrile	0.8	0.2	0.3	0.5

Table 5. *Effect of incubation temperature on the formation of desmosines from tropoelastin*

In Expt. 1, 4.4×10^{4}c.p.m. of tropoelastin were incubated with lysyl oxidase and unlabelled aorta fraction at the temperatures indicated and desmosine production was measured. For Expt. 2, radioactive tropoelastin was incubated with lysyl oxidase for 24h in the presence of 2mM-D-penicillamine, but without unlabelled aorta residue. The penicillamine was removed by dialysis at 4°C against 0.5M-acetic acid and then against the incubation buffer. The tropoelastin so prepared, and presumably containing allysine groups, was divided into three equal portions of 4.7×10^4 c.p.m. and incubated at the temperatures specified.

				10^{-2} × Radioactivity (c.p.m.)		
Expt. no.	Incubation temp. (°C)	Addition	10^{-3} × Lysyl oxidase activity (c.p.m.)	Isodesmosine	Desmosine	Isodesmosine +desmosine
1	37	None	4.6	3.2	2.5	5.7
	15	None	2.7	0	0.7	0.7
	37	β-Aminopropionitrile	0.9	0.3	0.3	0.6
2	37	—	—	1.9	1.8	3.7
	15	—	—	0.5	1.0	1.5
	−20	—	—	0.5	0.8	1.3

at 15°C was not significantly greater than that of the β-aminopropionitrile-treated control. In the second experiment, tropoelastin was incubated with lysyl oxidase at 37°C in the presence of D-penicillamine; the inhibitor was then removed by dialysis at 4°C against 0.5M-acetic acid followed by the incubation buffer. The allysine-containing tropoelastin so prepared was then incubated at 15 or 37°C and the amounts of desmosines formed at both temperatures were compared with those of the unincubated control. As seen from Table 5 (Expt. 2), the quantity of desmosines formed at 37°C was 3 times that of the unincubated control, whereas at 15°C it was only slightly greater.

Discussion

The process of cross-linking of tropoelastin into elastin involves the oxidative deamination of the lysines to allysines and, subsequently, the condensation of three allysine molecules and a lysine into 1,2-dihydropyridines through intermediate steps of allysine aldol, dehydrolysinonorleucine and dehydromerodesmosines. Finally, the 1,2-dihydropyridines are oxidized to isodesmosine or desmosine (Piez, 1968; Davis & Anwar, 1970). Lysyl oxidase, a copper-dependent enzyme, accomplishes the oxidative deamination (Pinnell & Martin, 1968). We have attempted to answer several questions about the cross-linking process.

The intermediates identified in elastin cross-linking indicate that additional enzymes may be involved, especially at the final oxidation step. However, this appears unlikely because incubation of purified tropoelastin in the presence of purified lysyl oxidase without added cofactors results in the formation of desmosines (Tables 1, 2 and 5). Our conclusion is supported by the observation of Barnes *et al.* (1969), who extracted guinea-pig aorta elastin with boiling oxalic acid and obtained α-elastin; the α-elastin preparation so obtained gave rise to desmosines upon incubation. In the experiments described in Table 1, the enhancement of desmosines produced by unlabelled aorta fractions does not appear to result from the presence of enzymes within this fraction, since the addition of fractions heated to 100°C is also effective. It may be that previously formed allysines in the fractions condense with lysines of the radioactive soluble elastin.

The formation of desmosines is not significantly affected by efforts to remove molecular O_2. The lack of effect does not appear to be due to incomplete removal of O_2, because similar treatment results in total inhibition of lysyl oxidase activity. This experiment indicates that there is no absolute requirement of O_2 for the formation of desmosines. The final oxidation step, which appears to be non-enzymic and spontaneous, may be accompanied by the simultaneous formation of 1 mol.prop. of lysinonorleucine from dehydrolysinonorleucine, as postulated by Piez (1968). Indeed lysinonorleucine has been identified in non-reduced elastin samples (Franzblau *et al.*, 1965, 1969). However, the fact that the number of lysinonorleucine residues (1 per 1000 residues) is less than those of desmosines (2–5 per 1000 residues) indicates that the oxidation may be achieved *in vivo* both by O_2 and dehydrolysinonorleucine.

The physiological role of hydroxyproline in collagen appears to increase the denaturation temperature and hence the stability of the collagen triple helix at physiological temperatures; therefore the hydroxyproline residues are necessary for the secretion of native molecules (Jimenez *et al.*, 1973; Rosenbloom *et al.*, 1973). However, in soluble elastin proline hydroxylation is not a prerequisite for the secretion (Narayanan *et al.*, 1977; Uitto *et al.*, 1976). The data in the present paper show that lysyl oxidase activity and formation of the desmosines are not influenced by the lack of hydroxylation of prolines. Therefore, since the cells making elastin also synthesize collagen and therefore contain prolyl hydroxylase, tropoelastin may be inadvertently hydroxylated because of amino acid sequences around proline residues that favour hydroxylation.

No desmosines are formed at 15°C, a temperature not conducive to coacervation. It has recently been shown that coacervation results in the alignment of the tropoelastin molecules into a fibrillar structure (Cox *et al.*, 1974); therefore this process may play a role in the apposition of the lysine residues in a proper conformation so that after lysyl oxidase action they can readily condense into cross-links. Another possibility is that the uncoacervated tropoelastin is a more effective substrate to lysyl oxidase, so that after the enzyme action the loss of positive charges due to the conversion of ε-amino groups into aldehyde groups favours coacervation; in this case the reactants brought into apposition will be three allysine residues and one lysine residue. Alternatively, the effect of coacervation might be simply one of concentrating the tropoelastin molecules into a localized space. In support of the latter possibility, we have observed that heating tropoelastin at 100°C for 15min, a process that is likely to destroy the fibrillar structure, does not affect the lysyl oxidase activity or the quantity of the desmosines formed. (In a typical experiment, 3.9×10^4 c.p.m. of soluble elastin gave rise to 733c.p.m. of isodesmosine plus desmosine. After heating at 100°C for 15min, the same preparation gave rise to 558 c.p.m. of desmosines.)

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