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A Structural Model for Desmosine Cross-Linked Peptides

By ROBERT P. MECHAM* and JUDITH A. FOSTER
Department of Biochemistry, Interdepartmental Medical Biochemistry Section, Boston University School of Medicine, Boston, MA 02118, *U.S.A.*

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Desmosine-enriched peptides were isolated from a thermolysin digest of bovine ligamentum nuchae elastin and a partial sequence was determined. A 'two-cross-link' model is proposed in which a second cross-link, perhaps lysinonorleucine, joins two peptide chains approx. 35 amino acid residues removed from the desmosine. Implied in this model is a certain asymmetry or directionality which places restrictions on the 'sense' of the peptide chains (either always parallel or anti-parallel) in order to align the cross-linking sites. Imposing such restrictions raises the possibility of specific alignment of elastin precursor molecules by microfibrillar proteins and/or aligning peptides on the precursor molecules themselves.

The lysine-derived cross-links in elastin (Franzblau, 1971) in large measure contribute to the protein's elastic mechanical properties. Their formation involves enzymic oxidation of certain lysine ε-amino groups, forming α-aminoadipic acid δ-semialdehyde (allysine) (Lent *et al.*, 1969). Once formed, these reactive aldehyde groups are thought to condense spontaneously with another allysine molecule by an aldol condensation forming allysine aldol, or with the ε-amino group from an unoxidized lysine residue via a Schiff-base reaction forming dehydrolysinonorleucine.

The most complex of the elastin cross-links are desmosine and isodesmosine (Partridge *et al.*, 1963; Thomas *et al.*, 1963; Pax *et al.*, 1974). At present, the biosynthetic route for these isomers is not clearly defined, although synthetic processes involving condensation of dehydrolysinonorleucine with allysine aldol (Piez, 1968; Gray *et al.*, 1973; Foster *et al.*, 1974) and a route involving dehydromerodesmosine as an intermediate (Francis *et al.*, 1973) have been proposed.

Elastin cross-links are found in alanine-enriched areas of the molecule and are formed from lysine molecules separated by two or three alanine residues (Sandberg *et al.*, 1971, 1972; Foster *et al.*, 1974). It has been proposed by Gray *et al.* (1973) that this clustering of alanine and lysine residues favours an α-helix conformation with the lysine side chains appositely positioned on one side of the helix, allowing for easy condensation of the juxtaposed cross-link precursors after enzymic oxidation. Circular-dichroism studies suggest that desmosine-enriched peptides, although enriched in alanine, are in an extended helix rather than an α-helix (Foster *et al.*, 1976). Although the extended helix differs slightly from an α-helix, the separation of the lysine residues does not seem too distant for condensation of cross-link intermediates (Wender *et al.*, 1974).

The present study was undertaken to determine the primary sequence around elastin cross-linking sites and to relate this information to possible structural 'signals' which might modulate or otherwise direct cross-link formation. Since cross-link formation appears to be an irreversible step in the formation of mature elastin from soluble precursor molecules, information of the type obtained in this study is vital to our understanding of elastin fibrogenesis.

Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

* To whom reprint requests should be addressed. Present address: Pulmonary Division, Department of Medicine, Washington University School of Medicine at the Jewish Hospital of St. Louis, St. Louis, MO 63110, U.S.A.

Experimental

Materials

Chemicals and enzymes were obtained as follows: NaB^3H_4, New England Nuclear, Boston, MA, U.S.A.; thermolysin (three times crystallized; A grade), Calbiochem, La Jolla, CA, U.S.A.; Aminex 50W-X4 ion-exchange resin, Bio-Rad Laboratories, Richmond, CA, U.S.A.; type P Chromobeads, Technicon Corp., Elmhurst, IL, U.S.A.; *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride and 2-aminonaphthalene-1,5-disulphonic acid, Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Preparation of elastin

Elastin from bovine ligamentum nuchae was prepared by the autoclave method of Partridge &

Davis (1955). The amino acid analysis (as described below) of the purified product is given in Table 1.

Reduction with NaB^3H_4

Ligamentum nuchae elastin (5g) was reduced by suspending the protein in 250ml of 0.001M-EDTA (pH9) and adding a mixture of 182mg of $NaBH_4$ and 27mg of NaB^3H_4 (100mCi). The pH was maintained at 9 with 0.01M-HCl. After 2h, excess of NaB^3H_4 was destroyed by adjusting the pH to 3 with 50% (v/v) acetic acid. The protein was filtered and washed thoroughly with 0.01M-HCl, water, ethanol and ether, and allowed to dry.

Thermolysin digestion

Reduced elastin (5g) was suspended in 100ml of 0.1M-ammonium acetate (pH7.5) and 50mg of thermolysin added. The mixture was allowed to digest for 2–3h at 37°C, whereupon another 50mg of thermolysin was added and the mixture left overnight (37°C). The elastin had been completely solubilized by the thermolysin at this point. Digestion was stopped by adjusting the pH to 3 with acetic acid, at which point a white flocculent precipitate appeared, which was removed by centrifugation (10000*g*, 20min, 4°C).

Amino acid analysis

Samples for analysis were hydrolysed under vacuum for 16–22h at 110°C with constant-boiling-point HCl. Amino acid analyses were routinely performed by using a JLC 6AH automatic amino acid analyser with a modified program to include a 0.4M-sodium citrate buffer, pH5.3, for the resolution of the elastin cross-linking amino acids desmosine, isodesmosine, merodesmosine and lysinonorleucine. Isodesmosine, reduced isodesmosine and reduced desmosine all co-chromatograph in this system. Since all cross-link-containing peptides reported in this work were derived from reduced elastin, all values for isodesmosine represent contributions from any or all of these three species. The values reported therefore refer to all three species as 'desmosine', collectively.

The cross-linking amino acids in elastin originate from two to four lysine residues. Therefore, their ninhydrin colour yield must be calculated accordingly. In this study, allysine aldol, lysinonorleucine and dehydrolysinonorleucine are expressed as leucine equivalents divided by 2, merodesmosine and dehydromerodesmosine as leucine equivalents divided by 3, and desmosine and isodesmosine as leucine equivalents divided by 4.

Alkaline hydrolysis was used to detect certain cross-links or cross-link precursor amino acids that are destroyed by acid hydrolysis. The samples were hydrolysed in alkali-resistant glass tubes with 0.2ml of 2M-NaOH at 110°C for 16–22h, after which the sample was diluted with water so that the Na^+ concentration was 0.2M. The pH was adjusted to 2 with concentrated HCl and the sample applied to an ion-exchange column (0.6cm×150cm) packed with Chromobeads type A (Technicon Corp.). The column was equipped with a stream-splitting device which allowed one half of the sample to be diverted to a fraction collector where 1ml samples were counted for radioactivity in a Packard model 3310 liquid-scintillation counter, with Aquasol (New England Nuclear) as the scintillation fluid. The remaining sample was analysed with ninhydrin. The column was eluted with a sodium citrate gradient described by Burns *et al.* (1965).

Peptide fractionation by ion-exchange chromatography

Aminex 50W-X4 resin (20–30 micron, H^+ form), or type-P Chromobeads, was washed in a Buchner funnel successively with 5 or 6vol. each of 1M-NaOH, water, 2M-HCl and water. Before use, the resin was converted into the pyridinium form by washing with several volumes of 2M-pyridine followed by a final equilibration with 0.02M-pyridine/acetate, pH2.7. A 0.9cm×60cm column was packed under a nitrogen pressure of 75.7–113.6N/m² (20–30lb/in²) at 55°C. Samples were applied to the column in starting buffer and eluted with a continuous six-buffer pyridine/acetate gradient (Schroeder, 1967). The column pressure was 75.7–113.6N/m² with a flow rate of approx. 60ml/h; 5min fractions were collected. At the end of the run, the column was washed with 8.5M-pyridine/acetate, pH5.6.

The strong u.v. absorption by the pyridine/acetate buffer at wavelengths commonly used to detect proteins or peptides made it impossible continuously to monitor the column effluent. Therefore peptides were detected by ninhydrin after alkaline hydrolysis as described by Foster *et al.* (1974).

Gel filtration

Gel-filtration chromatography on columns (1.5cm ×100cm) of Sephadex G-50 or G-25 (Pharmacia, Uppsala, Sweden) or Bio-Gel P6 or P2 (Bio-Rad Laboratories) eluted with 0.1M-acetic acid was used to desalt and provide further separation of peptide components after initial fractionation by ion-exchange chromatography. A column (1.5cm× 60cm) of Sephadex LH-20 was also used with an elution buffer of 50% ethanol containing 0.5% benzene.

Purity of isolated peptides

Several methods were utilized to monitor the purity of peptides throughout the isolation and purification

procedures. One of the methods was *N*-terminal determination by dansyl chloride (Gray, 1972). Dansylated end groups were analysed by t.l.c. on polyamide layers.

SDS/polyacrylamide-gel electrophoresis was also used to monitor peptide purification. The method of Weber & Osborn (1969) was followed, except that the gel buffer contained 0.5M-urea (Goldberg *et al.*, 1972). Gels contained 10% acrylamide. Electrophoresis was carried out at 2–3mA/gel overnight. Gels were stained with Coomassie Brilliant Blue and destained in 10% (v/v) methanol/7% (v/v) acetic acid.

A peptide-'mapping' procedure was also used which involved dansylating the peptide mixture by the method described above for dansyl-end-group determination and chromatographing the dansylated peptides on polyamide thin-layer plates. After development, the thin-layer plate was sectioned into grids and the polyamide layer in each grid scraped from the plate and counted for radioactivity in Aquasol.

Sequencing procedure

Automated sequence analysis was carried out with the Beckman model 890C sequencer by the procedure of Edman & Begg (1967). The resultant amino acid thiazolinone derivatives were converted into the more-stable amino acid phenylthiohydantoins by incubation in hot 1.0M-HCl and extracted into ethyl acetate. A portion (usually 10%) of each sequencer fraction in ethyl acetate was analysed by using a Beckman model 65 gas–liquid chromatograph by the method of Pisano & Bronzert (1969). In most cases, samples from the ethyl acetate and aqueous phases were also counted for radioactivity in Aquasol. The remaining sample from both phases was then transferred to acid-cleaned hydrolysis tubes and hydrolysed to free amino acids by using the hydroiodic acid method of Smithies *et al.* (1971). The samples were then analysed with the amino acid analyser.

To minimize the extreme losses of the hydrophobic elastic peptides during sequencing extraction procedures (Braunitzer *et al.*, 1970), the film-forming properties and polarity of the peptides being sequenced were increased by the attachment of 2-aminonaphthalene-1,5-disulphonic acid to the *C*-terminal residue via a water-soluble carbodi-imide by the method of Foster *et al.* (1973).

Results

Characterization of the thermolysin digest

N-Terminal analysis of the thermolysin digest of elastin with dansyl chloride revealed a multiplicity of amino groups, with glycine, leucine, alanine and valine being the most prevalent. Present in lower but nevertheless significant amounts were phenylalanine and isoleucine.

SDS/polyacrylamide-gel electrophoresis of the digest indicated a mol.wt. of less than 15000 for most of the peptides. Gel-filtration chromatography on Sephadex G-50 showed peptide material being eluted over the entire fractionation range of the gel (30000–1000 mol.wt. for peptides and globular proteins), with most of the material eluted near the region corresponding to mol.wt. of 7000 and below.

Acidic peptide precipitate

When the thermolysin digest was terminated by adjusting the pH to 3 with acetic acid, a white flocculent precipitate appeared. The amino acid analysis of this precipitate is given in Table 1. Quantitatively, the acidic precipitate comprised less than 5% of the total elastin digest. SDS/polyacrylamide-gel electrophoresis of the precipitate showed that most of the material had a mol.wt. less than 15000. There were, however, two sharp bands of 48500 and 41000 mol.wt.

Table 1. *Amino acid analyses of purified ligament elastin before and after thermolysin digestion*

Abbreviations: N.D., not determined; Hyp, hydroxyproline; Des, desmosine; LNL, lysinonorleucine.

	Amino acid content (residues/1000 residues)		
	Reduced ligament elastin before digestion	Thermolysin-digested elastin after acidification and removal of the precipitate	Acidic precipitate
Lys	5	4	32
His	0	1	23
Arg	7	6	34
Hyp	9	8	0
Asp	8	8	171
Thr	10	9	74
Ser	10	9	73
Glu	17	16	80
Pro	119	114	25
Gly	329	333	110
Ala	221	223	89
Cys	Trace	Trace	12
Val	139	142	58
Met	0	0	5
Ile	26	26	50
Leu	62	64	51
Tyr	5	5	83
Phe	28	28	31
Trp	N.D.	N.D.	N.D.
Des	1.4	1.4	0
LNL	0.7	0.8	0

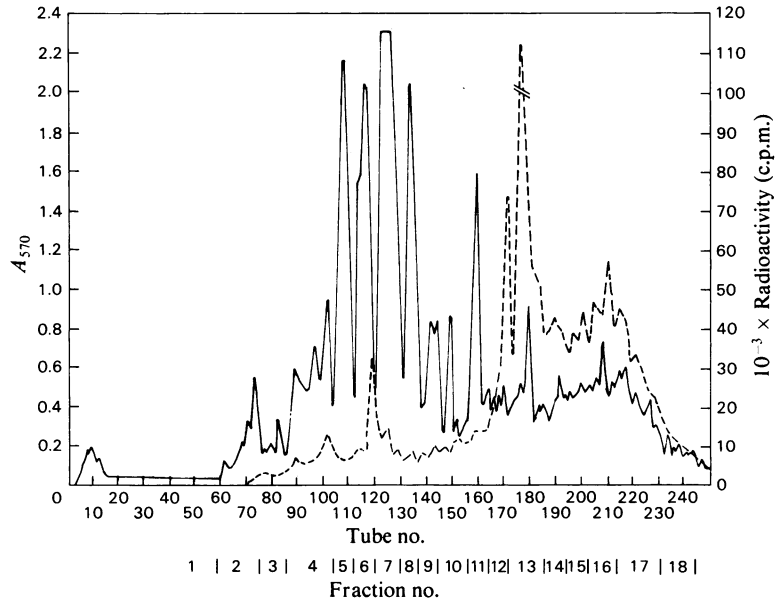

Fig. 1. *Elution profile of thermolysin-digested elastin from an Aminex 50W-X4 column* (0.9 cm × 60 cm)
The column was eluted at 55°C with a continuous pyridine/acetate gradient (Schroeder, 1967) at a flow rate of 60 ml/h followed by a column wash with 8.5 M-pyridine/acetate (pH 5.6). Peptide material was detected by allowing a sample from each tube to react with ninhydrin after alkaline hydrolysis, and measuring the A_{570} (——). Radioactivity (----) was determined by liquid-scintillation counting. Peptide fractions were pooled as indicated, i.e. 1–18. Fraction 19 (not shown on the Figure) is the material eluted with the column wash.

Ion-exchange chromatography

To effect better separation of peptides than that obtained with gel filtration, 50 mg of the reduced elastin digest was chromatographed on an Aminex 50W-X4 ion-exchange column. In later experiments, the resin was changed to Technicon type-P Chromobeads, which afforded slightly better resolution and lower column pressures. Fractions from the Aminex column were designated with the prefix 'TA' followed by the fraction number. Fractions from the Technicon column were designated 'Th,t'. Fig. 1 shows the typical elution profile of a sample of the elastin digest chromatographed on the ion-exchange column. Table 2 gives the amino acid analyses of the pooled fractions. Amino acid analysis of the starting material plus summation of the total nmol from each fraction indicated 90–95% recovery from the ion-exchange column. It is noteworthy that most of the radioactivity, and hence cross-links (fractions TA-11–TA-19 inclusive), were separated quite well from the fractions that did not contain cross-links (TA-1–TA-10). To obtain enough peptide in each fraction for purification and subsequent analysis, many runs of 50–100 mg of the digest were separated on the ion-exchange column and similar fractions between runs pooled.

Desmosine- and lysinonorleucine-enriched peptides

Peptides enriched in desmosine, isodesmosine, merodesmosine and lysinonorleucine were eluted from the ion-exchange column over a relatively broad range at the high-salt end of the pyridine/acetate gradient (fractions TA-16–TA-19 and Th,t-12–Th,t-14). The radioactivity profile showed minor broad peaks which served as guides for pooling fractions, which were then freeze-dried and chromatographed on Sephadex G-50, G-25, LH-20, or Bio-Gel P6 or P2. Fractions containing the highest content of desmosine were pooled and rechromatographed separately. The compositions of the desmosine-enriched peptides are given in Table 3.

Sequence analysis of desmosine-enriched peptides

Peptide TA-17,G-50-II and a sample from peptide TA-18,G-50-IV,LH20-II were sequenced without modification, but yielded no reliable results owing to peptide washout. The results of sequencing the modified peptide Th,t-11,P6-1,P2-1 is shown in Table 4. An indication of the purity of this peptide was ascertained by dansylation and subsequent chromatography as described in the Experimental section, which yielded only one radioactive and fluorescent

Table 2. *Amino acid analysis of pooled fractions from the Aminex ion-exchange column*

Values for isodesmosine (Iso) and desmosine (Des) are leucine equivalents divided by 4, lysinonorleucine (LNL) and allysine aldol (Aldol) are leucine equivalents divided by 2. Merodesmosine (Mero) was only found in trace amounts. Values in parentheses were determined after alkaline hydrolysis.

Fraction (see Fig. 1) ...	Amino acid content (residues/1000 residues)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Lys	—	—	—	—	—	—	—	2.1	—	—	8.6	14.3	12.7	15.3	23.7	48.2	14.8	14.6	16.2
His	—	—	11.0	1.9	—	—	—	1.5	—	—	—	8.8	9.3	16.1	14.3	7.6	12.6	7.5	17.0
Arg	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11.4	8.5	28.3	59.9	101.2
Hyp	—	—	23.5	63.2	13.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Asx	25.5	45.6	30.6	24.3	6.0	2.7	1.4	5.4	4.2	15.1	10.9	14.4	12.7	11.9	16.9	9.9	8.6	8.4	12.6
Thr	17.2	11.8	36.3	12.4	20.3	2.3	3.8	12.2	4.1	14.6	10.6	12.1	10.3	11.8	14.7	11.4	13.4	11.1	10.7
Ser	20.4	9.4	6.7	7.3	13.2	24.8	4.8	5.9	5.6	23.8	21.5	22.0	21.0	21.7	29.8	22.7	27.7	20.3	21.3
Glx	43.7	32.3	20.5	19.4	49.8	15.1	7.4	21.7	6.2	19.2	13.5	18.8	19.9	21.4	26.0	19.3	20.1	14.6	17.3
Pro	179.9	132.7	184.0	126.9	176.2	146.7	68.7	131.6	176.6	125.3	90.9	108.5	115.0	187.6	153.2	147.7	136.5	121.0	159.2
Cys	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gly	301.1	352.1	280.9	358.9	342.0	383.6	324.5	398.9	417.2	282.2	334.2	205.1	187.1	235.7	211.7	191.2	180.9	217.1	177.9
Ala	135.6	265.0	154.3	143.8	131.1	267.8	385.2	74.0	124.0	225.2	194.5	405.4	452.5	317.8	380.5	380.7	409.8	340.2	235.2
Val	101.0	98.8	156.7	177.4	192.6	78.9	157.4	43.5	34.0	40.7	36.9	42.7	49.8	32.6	40.4	47.7	30.4	37.4	29.0
Ile	27.8	31.0	43.5	30.0	15.4	21.2	7.0	65.7	7.0	13.5	8.2	5.4	5.7	6.1	7.5	9.1	8.2	12.2	12.9
Leu	78.7	21.3	52.0	24.1	21.2	40.5	22.0	213.9	72.1	113.6	59.9	41.7	41.1	38.4	26.6	34.8	40.3	46.9	31.5
Tyr	21.0	—	—	4.1	4.9	—	7.1	3.3	16.0	30.8	58.4	18.1	19.2	17.1	21.1	18.4	27.7	19.7	24.3
Phe	48.1	—	—	1.3	13.7	16.3	10.4	20.3	132.9	95.9	151.4	82.7	43.8	66.4	19.4	31.6	23.8	51.2	112.8
Iso	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	5.1	7.5	7.4	4.5
Des	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.8	4.6	5.5	4.0	2.2
LNL	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.5	4.0	6.1	15.6
Mero	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Trace	Trace	Trace
Aldol	—	—	—	—	—	—	—	—	—	—	(5)	(15)	(40)	(3)	—	—	—	—	—

Table 3. *Amino acid compositions of desmosine-enriched peptides*
Values for desmosine (Des) are leucine equivalents divided by 4, lysinonorleucine (LNL) values are leucine equivalents divided by 2. For peptide nomenclature, see the text.

	Amino acid content (residues/peptide, assuming Des = 1)				
	TA-17,G-50-II	TA-18,G-50-4, LH20-II	Th,t-11,P6-1, P2-1	Th,t-12,P2-1, G-50-II	Th,t-13,P2-1
Lys	0.7	0.4	1.1	0.6	1.7
Arg	0.6	0.3	0.5	0.3	0.7
Ser	0.1	0.8	0.7	0.6	2.6
Glu	0.4	0.4	1.0	0.5	1.6
Pro	2.9	2.7	3.0	4.0	8.1
Gly	5.3	3.7	8.3	7.1	14.3
Ala	19.7	12.9	20.2	21.0	28.6
Val	1.0	1.0	1.5	1.6	3.7
Leu	1.2	1.3	2.4	1.8	2.9
Tyr	0.8	0.9	1.2	1.0	1.8
Phe	0.4	0.5	0.3	0.5	1.2
LNL	1.0	0.4	0.8	1.0	0.9
Des	1.0	1.0	1.0	1.0	1.0

Table 4. *Sequential degradation of desmosine-enriched peptide Th,t-11,P6-1,P2-1*
Desmosine was determined qualitatively by the appearance of radioactivity at step 6. Quantification of the phenylthiohydantoin derivative of reduced desmosine(*) proved to be a difficult problem (see the Results section). Total desmosine in the starting material was 360nmol.

Sequencer step no.	Amino acid released (nmol)	
1	Alanine	690
	Leucine	320
2	Alanine	402
	Glycine	258
3	Alanine	350
4	Alanine	300
	Glycine	140
5	Alanine	160
	Glycine	78
6	Alanine	40
	Desmosine	*
7	Tyrosine	30
	Alanine	22

spot. Each sequencer fraction was counted for radioactivity (both the ethyl acetate and acid phases), analysed by g.l.c. and by using the amino acid analyser after back-hydrolysis to free amino acids with hydroiodic acid. In those fractions where cross-link amino acids were indicated by an increase in radioactivity, the effluent from the amino acid analyser was collected and counted for radioactivity so that one could relate the counts to the desmosine peak on the chromatogram.

Non-cross-linked peptides

Most of the ninhydrin-positive material was eluted from the ion-exchange column as sharp peaks in the early stages of the pyridine/acetate gradient (see Fig. 1). Amino acid analysis of these fractions (TA-4–TA-10 and Th,t-2–Th,t-7) indicated high values for glycine, alanine, valine, proline and, in some cases, leucine. There was very little radioactivity in this part of the effluent, except for a radioactive peak primarily in fractions TA-6 and TA-7, which was analysed and found to contain allysine. Sequence analysis of one of the peptides in TA-4 indicated a partial sequence of Val-Gly-Val-X-Pro/Hyp-Gly . . ., where X signifies an unknown residue and Pro/Hyp indicates a partial hydroxylation of proline residues.

The size of these non-cross-linked peptides is small, since most of the material ran with the tracking dye in polyacrylamide-gel electrophoresis (10% gel). Gel-filtration chromatography indicated that most of the peptide material had mol.wts. less than 5000.

Discussion

One of the major obstacles to a thorough study of elastin has been the protein's extreme insolubility and relative resistance to solubilization by enzymes with limited specificities, such as chymotrypsin and trypsin. Relatively harsh chemical methods or non-specific enzymes have of necessity been used to effect solubilization.

In this study, ligamentum nuchae elastin was completely solubilized by thermolysin, an enzyme having activity directed towards the *N*-terminal peptide bond of amino acids with hydrophobic side chains, such as leucine, isoleucine, valine, phenyl-

alanine and alanine. Most of the elastin peptides generated by thermolysin are small, however, having mol.wts. less than 7000.

An important question about the primary structure of desmosine-cross-linked peptides is the relative position of the two isomers desmosine and isodesmosine in the sequence. Foster *et al.* (1974) showed that both desmosine and isodesmosine can occur in the same sequence position. Davril & Hahn (1974), however, suggest that they occur at separate sites close together on the peptide chains. Data in the present paper do not help discern a correct answer, since the cross-linked peptides being studied have been reduced, and, as mentioned above, reduced desmosine and isodesmosine co-chromatograph on the amino acid analyser, making positive identification of the isomers impossible.

Sequencing cross-linked peptides

A major difficulty encountered in sequencing elastin peptides is the drastic loss of peptide material from the automated sequencer reaction cup during the various sequencer extraction steps. Such washout is a common occurrence for hydrophobic peptides, which are somewhat soluble in the organic solvents used in the sequencer (Braunitzer *et al.*, 1970). In a previous report, washout reported when sequencing non-reduced desmosine-enriched peptides was not as great as that encountered in the present study (Foster *et al.*, 1974). It appears that reduction of the pyridinium nucleus of the desmosine cross-link, and hence neutralization of the positive charge on the ring, increases the hydrophobicity of the peptide.

In addition to the peptide losses caused by washout, resulting in very low repetitive yields, sequencing cross-linked peptides presents other difficulties. When two or more chains covalently linked in one or more locations are sequenced simultaneously, the cross-linking loci may not be detectable as a phenylthiohydantoin derivative until the final attachment site is cleaved. Also, if the cross-link is linking two or more chains, the interpretation of the sequencing data will be complicated by two or more amino acids being sequenced per step, compounded by the degree of carry-over and the fact that different peptides will be released from the cross-link at different times. It appears that peptide losses through the extraction cycles are increased once a peptide segment is released from the cross-link. For a discussion of the problems and strategies involved in sequencing cross-linked peptides, see Foster *et al.* (1974).

Desmosine-enriched peptides

Since desmosine and isodesmosine originate from four lysine residues, it is possible for them to link four, three or two polypeptide chains. Sequence data indicate, however, that desmosines links only two chains, not three or four (Foster *et al.*, 1974; Gerber & Anwar, 1974, 1975).

Table 3 summarizes the amino acid compositions

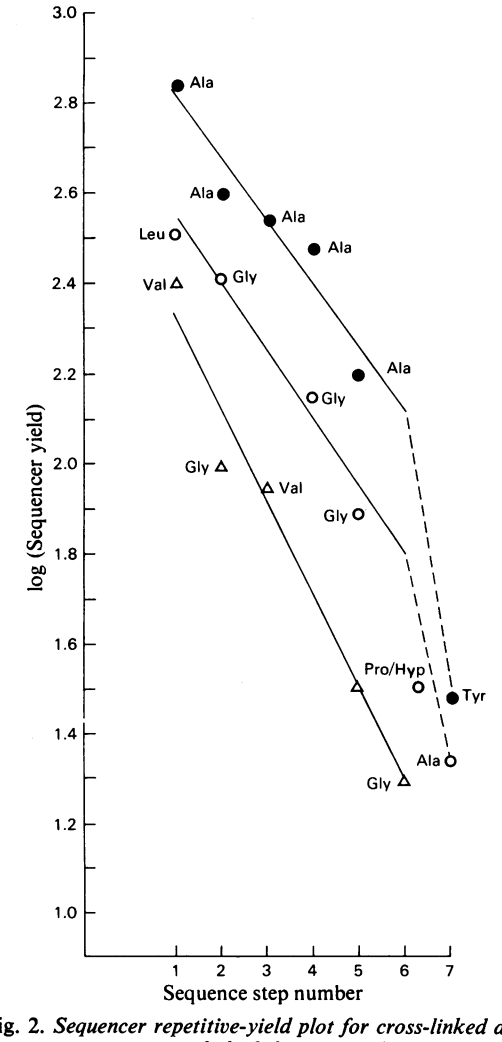

Fig. 2. *Sequencer repetitive-yield plot for cross-linked and non-cross-linked elastin peptides*

The repetitive yield, calculated from the slope of the line generated in a plot of the log of the sequencer recovery at step *n* against *n*, for the two arms of the cross-linked peptide Th,t-11,P6-1,P2-1 is 83% (●) and 84% (○). The coefficient of determination for the least-squares linear regression fit (r^2) is 0.89 and 0.98 for the two peptide chains respectively. For the non-cross-linked peptide TA-4 (△), the repetitive yield is 80% and $r^2 = 0.95$. The segmented line shows the fall in yield after the desmosine cross-link is released from the peptide chain. Pro/Hyp represents proline and hydroxyproline occurring at the same sequence position.

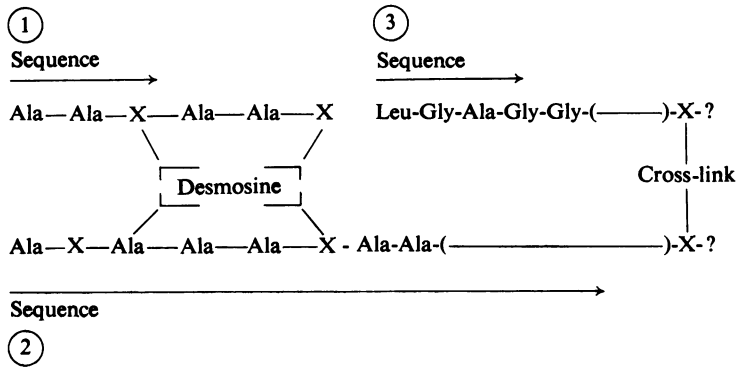

Fig. 3. *Proposed model for desmosine peptide in which a second cross-link joins the two peptide chains approx. 35 amino acid residues removed from the desmosine*

The second cross-link need not be desmosine and in fact could be lysinonorleucine, which was present on a molar basis equal to desmosine in the peptide that was sequenced. The second cross-link may serve to hold and align the peptide chains, so that desmosine formation can take place at the appropriate site. Three sequencing sites are indicated, which correspond to the peptide sequences described in Table 4. X is a modified lysine residue which constitutes an arm of the cross-link.

of the desmosine-enriched peptides isolated in the present study. Of note is the presence of lysinonorleucine in quantities approximately equal to the desmosine content in four of the five peptides.

Table 4 contains the sequence data for peptide Th,t-11,P6-1,P2-1. Both the acid and ethyl acetate phases from the sequencer conversion and extraction steps were analysed on the amino acid analyser after back-hydrolysis to free amino acids. Quantification of desmosine after this procedure proved to be a difficult problem in that values from the analyser were much lower than expected. Relative desmosine concentration per sequencer fraction was best determined by radioactivity before hydrolysis.

The first step of the Edman degradation should yield all amino acids with a free *N*-terminal in an amount approximately equal to the total amount of desmosine. As shown in Table 4, the amount of *N*-terminal leucine in step 1 is approximately equal to the desmosine concentration, whereas alanine is present in twice the amount. It appears therefore that three chains are being sequenced, two chains with *N*-terminal alanine, and one with *N*-terminal leucine. The sequence data agree with those reported by Foster *et al.* (1974) for similar desmosine-enriched peptides, except for the presence of the third sequence with leucine as the *N*-terminal residue.

Gerber & Anwar (1974) have described the partial sequences of seven peptides *C*-terminal to desmosine from bovine ligamentum nuchae and five peptides from pig aortic elastin. The sequence Leu-Gly-Ala-Gly-Gly . . ., which was detected in sequencer steps 1–5 in the present study, corresponds to one of these peptides. Its presence as a free contaminating peptide seems unlikely, since it was evident in three of the desmosine-enriched peptides, all of which were purified differently. It appears therefore that the peptide is bound to the desmosine peptide being sequenced. This is further evident when repetitive yield (average yield at each step) is considered. Repetitive yield is calculated from the slope of the line in a plot of log recovery at step *n* against *n*. From the data in Table 4, the repetitive yield for the putative third chain with *N*-terminal leucine (see Fig. 2) is approximately equal to the repetitive yield of the alanine chains (84%, cf. 83%). If the peptides were separate, it is very unlikely that their repetitive yields would be equal, owing to differing degrees of peptide washout. A second factor to be considered is the drastic loss of peptide material from both chains when the desmosine residue is removed by sequential degradation at step 6 (see Fig. 2). Such a loss was not evident with the non-cross-linked peptide TA-4.

Fig. 3 depicts our proposed model for the type of desmosine cross-link peptide sequenced in this study. The Leu-Gly-Ala-Gly-Gly . . . peptide is placed *C*-terminal to the desmosine cross-link, which fits the data presented for similar peptides by Foster *et al.* (1974) and Gerber & Anwar (1974). This therefore necessitates a second cross-link, not necessarily desmosine, relatively close to the first linking the two chains. The *N*-terminal leucine is generated by thermolysin cleaving one of the intermediate chains on the *N*-terminal side of leucine directly *C*-terminal to desmosine.

As mentioned above, the second cross-link in this model need not be desmosine. In fact, it may be

lysinonorleucine, which was present on a molar basis equal to desmosine in the peptide that was sequenced. The function of the second cross-link may be to hold and align the chains so that desmosine formation can take place at the appropriate site. Dehydrolysinonorleucine or allysine aldol could easily serve such a function, owing to the simple condensation reactions involved in their synthesis.

The distance between the two cross-links can be estimated from sequence work done in this laboratory on copper-deficient insoluble elastin. We were able to sequence a peptide homologous to the tropoelastin tryptic peptide T9c reported by Foster *et al.* (1973), demonstrating an alanine-enriched region on the *C*-terminus (Mecham, 1976). Torres *et al.* (1977), studying peptides homologous to T9c from a a thrombin digest of tropoelastin, found a similar *C*-terminal alanine enrichment. The significance of this is that two possible cross-linking sites (alanine-enriched areas) are indicated in peptide T9c separated by approx. 35 amino acid residues. It is therefore proposed that the second cross-link (lysinonorleucine?) is separated from the desmosine by approx. 35 amino acid residues. Assuming two intermittent peptides of this length, the total molecular weight of the desmosine peptide being sequenced should be approx. 7000, which agrees with that of the peptide determined by gel filtration.

The implications of this model are important. Having to form two cross-links relatively close together implies a certain asymmetry or directionality and places restrictions on the 'sense' of the peptide chains (either always parallel or always anti-parallel) in order to align the cross-linking sites. Imposing such a restriction raises the possibility of specific alignment by microfibrillar proteins and/or specific peptide regions on the elastin precursor molecules. The acidic peptide detected on initial acidification of the thermolysin digest may serve such a function.

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References

Braunitzer, G., Schrank, B. & Ruhfus, A. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1589–1590

Burns, J. A., Curtis, C. F. & Kacser, J. H. (1965) *J. Chromatogr.* **20**, 310–318

Davril, M. & Hahn, K. (1974) *FEBS Lett.* **43**, 331–336

Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91

Foster, J. A., Bruenger, E., Hu, C. L., Albertson, K. & Franzblau, C. (1973) *Biochem. Biophys. Res. Commun.* **53**, 70–74

Foster, J. A., Rubin, L., Kagan, H. M. & Franzblau, C. (1974) *J. Biol. Chem.* **249**, 6191–6196

Foster, J. A., Bruenger, E., Rubin, L., Imbermin, M., Kagan, H., Mecham, R. & Franzblau, C. (1976) *Biopolymers* **15**, 833–841

Francis, G., John. R. & Thomas, J. (1973) *Biochem. J.* **136**, 45–55

Franzblau, C. (1971) *Compr. Biochem.* **26C**, 659–711

Gerber, G. E. & Anwar, R. A. (1974) *J. Biol. Chem.* **249**, 5200–5207

Gerber, G. E. & Anwar, R. A. (1975) *Biochem. J.* **149**, 685–695

Goldberg, B., Epstein, E. H., Jr. & Sherr, C. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3655–3659

Gray, W. R. (1972) *Methods Enzymol.* **25B**, 121–138

Gray, W. R., Sandberg, L. B. & Foster, J. A. (1973) *Nature (London)* **246**, 461–466

Lent, R. W., Smith, B., Salcedo, L. L., Faris, B. & Franzblau, C. (1969) *Biochemistry* **8**, 2837–2845

Mecham, R. P. (1976) Ph.D. Dissertation, Boston University

Partridge, S. M. & Davis, H. F. (1955) *Biochem. J.* **61**, 11–21

Partridge, S. M., Elsden, D. F. & Thomas, J. (1963) *Nature (London)* **197**, 1297–1298

Pax, M. A., Pereyra, B., Gallop, P. M. & Seifter, S. (1974) *J. Mechanochem. Cell Motil.* **2**, 231–239

Piez, K. A. (1968) *Annu. Rev. Biochem.* **37**, 547–570

Pisano, J. J. & Bronzert, T. J. (1969) *J. Biol. Chem.* **244**, 5597–5607

Sandberg, L. B., Weissman, N. & Gray, W. R. (1971) *Biochemistry* **10**, 52–56

Sandberg, L. B., Gray, W. R. & Bruenger, E. (1972) *Biochim. Biophys. Acta* **285**, 453–458

Schroeder, W. A. (1967) *Methods Enzymol.* **11**, 351–361

Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Bilman, J. C. & Ballantyne, D. L. (1971) *Biochemistry* **10**, 4912–4921

Thomas, J., Elsden, D. F. & Partridge, S. M. (1963) *Nature (London)* **200**, 651–652

Torres, A. R., Alvarez, V. L., Sandberg, L. B. & Gray, W. R. (1977) *Adv. Exp. Med. Biol.* **79**, 267–276

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Wender, D. B., Treiber, L. R., Bensusan, H. B. & Walton, A. G. (1974) *Biopolymers* **13**, 1929–1941