Identification of Cyanogen Bromide Peptides Involved in Intermolecular Cross-Linking of Bovine Type III Collagen

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Cyanogen bromide peptides derived from bovine type III collagen and containing reducible intermolecular cross-links were isolated and identified. Two peptides, $\alpha 1(\text{III})\text{CB7}$ and $\alpha 1(\text{III})\text{CB9B}$, from within the helical portion of the molecule were shown to contain the 'amino donor' residues cross-linked to non-helical 'aldehyde donor' residues in the formation of cross-links. This information, in conjunction with previously published data for the order of the cyanogen bromide peptides [Fietzek, Allman, Rauterberg & Wachter (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 84–86], suggests that in type III collagen intermolecular cross-links are located in the end-overlap regions, so as to stabilize a quarter-stagger arrangement of molecules within the fibre in a similar manner to that proposed for type I and type II collagens.

The functional integrity of collagenous connective tissues depends on the formation of covalent intermolecular cross-links. In young animals these cross-links take the form of Schiff bases produced by condensation of aldehydes, derived from oxidative deamination of lysine or hydroxylysine residues, and the ϵ -amino groups of a hydroxylysine or lysine residue (Bailey *et al.*, 1970). Recently it has become apparent that many tissues contain more than one genetically distinct type of collagen (Chung & Miller, 1974; Epstein & Munderloh, 1975), which are intermolecularly cross-linked in an analogous manner to the most ubiquitous form, type I collagen (Bailey & Sims, 1976; Fujii *et al.*, 1976).

Many attempts have been made to determine the exact locus of the intermolecular cross-links between the molecules within a fibre (de Luque *et al.*, 1970; Miller, 1971; Kang, 1972; Zimmerman *et al.*, 1973; Eyre & Glimcher, 1973; Dixit & Bensusan, 1973; Becker *et al.*, 1975; Fujii *et al.*, 1975; Henkel *et al.*, 1976; Scott *et al.*, 1976). Although the interpretation of some must be re-examined in the light of collagen polymorphism, most of the evidence indicates that the cross-links are formed between aldehydes located in terminal non-helical sequences and hydroxylysine residues within the helical portion of an adjacent molecule. Little evidence is available on the location of the intermolecular cross-links in type III collagen, although a peptide derived from type III

collagen has been shown to contain an intramolecular cross-link between *N*-terminal non-helical sequences (Becker *et al.*, 1976). This cross-link has the properties of the intramolecular allysine–aldol found at the *N*-terminal in acid-soluble type I collagen. Partial sequence data have shown similar sequences of amino acids in the triple-helical regions of the type III collagen molecule analogous to the presumptive cross-link site in type I collagen (Fietzek *et al.*, 1977).

In the present paper we describe the isolation and identification of cyanogen bromide peptides that are involved in intermolecular cross-linking of bovine type III collagen.

Materials and Methods

Preparation of reduced tissue

The skin of a 5-month foetal calf was obtained immediately *post mortem* and was washed extensively in 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.5. Subcutaneous tissue was removed by scraping and the skin was diced in a meat grinder cooled with ice. The diced skin was then suspended in 0.2 M- NaH_2PO_4 , pH 4.3, for 2 h at room temperature (20°C) before being resuspended in 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.5, and the skin was reduced with KB^3H_4 (The Radiochemical Centre, Amersham, Bucks., U.K.) (4 mCi/mmol; 1:100 ratio on wet weight of tissue). After 1 h at room temperature the excess borohydride was destroyed by titration of the suspension

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to pH 4 with acetic acid. Finally, the reduction mixture was dialysed exhaustively against cold water, the skin was extracted twice with 1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5, and twice with 0.5 M-acetic acid. The insoluble material was freeze-dried and stored at -20°C .

Pepsin digestion and preparation of type III collagen

Pepsin digestion and fractional precipitation of type III collagen was carried out on both non-reduced and borotritide-reduced calf skins essentially as described by Fujii & Kuhn (1975). Briefly, the insoluble bovine skin was suspended in 0.5 M-acetic acid (2 mg/ml) and digested with pepsin (Worthington, Freehold, NJ, U.S.A.) (enzyme/substrate ratio 1:20) at 20°C for 48 h. The insoluble material was removed by centrifugation (40 000 g for 30 min), and collagen was precipitated from the supernatant by addition of NaCl to 5% (w/v). The precipitate was taken up in 1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5, the pH of the solution was readjusted to 7.5 and the solution was then dialysed against a large volume of 1 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.5. Insoluble material was collected by centrifugation and the supernatant was made 1.7 M with respect to NaCl. The precipitates obtained at 1 M- and 1.7 M-NaCl were assayed for type III content by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Sykes & Bailey, 1971).

CM-cellulose chromatography

The denatured collagen chains were chromatographed on CM-cellulose essentially as described by Chung & Miller (1974), except that the column was equilibrated with 1 M-urea/0.05 M-sodium acetate buffer, pH 4.8. The column effluent was monitored at 230 nm by using a Cecil CE212 u.v. spectrophotometer.

Cyanogen bromide cleavage

Type III collagen (10 mg/ml) in 70% (w/v) formic acid was digested with an equal weight of CNBr under N_2 for 4 h at 30°C and then diluted approximately 10-fold. Excess reagents were removed firstly by rotary evaporation *in vacuo* at $25-30^{\circ}\text{C}$ and then by freeze-drying.

Chromatography of the cyanogen bromide peptides

Cyanogen bromide peptides were chromatographed on a CM-cellulose column (1.8 cm $\times$ 10 cm) equilibrated with 0.02 M-NaCl/0.02 M-sodium formate, pH 3.6, and eluted at 42°C with a linear gradient of NaCl (0.02–0.17 M) over a total volume of 1 litre.

Molecular-sieve chromatography of cyanogen bromide peptides was carried out either on a Bio-Gel A0.5m (200–400 mesh) column (2.5 cm $\times$ 120 cm) equilibrated at room temperature with 1 M-

CaCl_2 /0.05 M-Tris/HCl buffer, pH 7.5 (Piez, 1968), or on a Bio-Gel P-10 (200–400 mesh) column (2.6 cm $\times$ 95 cm) equilibrated at room temperature with 0.1 M-acetic column.

When necessary, peptides were desalted on columns of Bio-Gel P-2 equilibrated with 0.1 M-acetic acid.

Reduction and carboxymethylation of cystine residues

Disulphide-bond reduction and carboxymethylation of cysteine in either peptides or chains was carried out as described by Crestfield *et al.* (1963). Briefly, the peptides were dissolved in 8 M-urea/0.2% EDTA/4.8% Tris/HCl buffer, pH 8.5, and reduced under N_2 atmosphere with 2-mercaptoethanol for 4 h at room temperature. Carboxymethylation was achieved by adding 1 equiv. (based on mercaptoethanol) of iodoacetic acid dissolved in 1 M-NaOH. Excess reagents were removed by gel filtration on Bio-Gel P-2.

Amino acid analyses

Amino acid compositions of isolated peptides were determined with a Jeol JLC-6AH analyser after hydrolysis under N_2 with 6 M-HCl at 110°C for 24 h.

Cross-link analysis was carried out on [^3H]borohydride-reduced samples after hydrolysis with 6 M-HCl at 110°C for 24 h on a column (1 cm $\times$ 60 cm) of Zeo-Carb 225 the pyridine/formate buffers (Bailey *et al.*, 1970). The identity of individual cross-links isolated from this column was confirmed by re-chromatography on the long basic column of a Beckman or Jeol analyser with 0.35 M-sodium citrate buffer, pH 5.28.

Results

The borotritide-reduced calf skin was found to contain approximately equal amounts of dihydroxylysinoxonorleucine and hydroxylysinoxonor-

Fig. 1. Elution profile of radioactively labelled cross-links from foetal calf skin on a column of Zeo-Carb 225 resin

Experimental details are given in the text. Abbreviations: Lys(OH)-Nle, hydroxylysinoxonorleucine; Lys(OH) $_2$ -Nle, dihydroxylysinoxonorleucine.

leucine (Fig. 1), but no histidinohydroxymerodesmosine, as a result of the acid phosphate treatment.

Pepsin digestion of both reduced and non-reduced calf tissues resulted in 85–90% of total collagen being rendered soluble in 0.5M-acetic acid; of this about 15–20% was precipitated at or below 1.7M-NaCl, and these precipitates were shown to contain pure type III collagen by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

CM-cellulose chromatography (not shown) of this material after heat denaturation showed the major peak to be eluted in the position expected for type III collagen, between type I $\alpha 1$ - and $\alpha 2$ -chains. However, the recovery of applied collagen was often below 50%, although the material desorbed by high-salt washes was also shown to contain type III chains. The recovery of material from CM-cellulose could be greatly increased by reduction and carboxymethylation before chromatography; under these conditions the position of elution of the major component was slightly earlier in the chromatogram. This property has been previously observed for both human and bovine type III collagens (Chung & Miller, 1974; Fujii & Kuhn, 1975). In view of this poor recovery it was decided to eliminate this step from the purification of the [^3H]borohydride-reduced material in some instances, in which case the whole type III precipitate from reduced skin was digested with CNBr.

Separation of the cyanogen bromide peptides from non-reduced type III collagen on CM-cellulose (Fig. 2) gave a pattern similar to that observed by Rauterberg *et al.* (1976). The pools from the CM-cellulose column were analysed by polyacrylamide-gel electrophoresis (Furthmayer & Timpl, 1971), and those found to contain more than one peptide were further purified by molecular-sieve chromatography. The material desorbed by 1M-NaCl at the

Fig. 2. Separation of cyanogen bromide peptides of pepsin-solubilized type III collagen on CM-cellulose. Experimental details are given in the text. The arrow marked s is where sample was applied, and the arrow marked g is where gradient was initiated. The peak identification uses the nomenclature of Rauterberg *et al.* (1976) and is based on the identification of the peptides present based on further purification and amino acid compositions.

Table 1. Amino acid compositions of some cyanogen bromide peptides isolated from pepsin-digested type III collagen. Experimental details are given in the text. Nomenclature of cyanogen bromide peptides follows that of Rauterberg *et al.* (1976).

Amino acid	CNBr peptide ...	Amino acid composition (mol of residue/mol)						
		3A	4	5	6	7	1,8,2	9A
4Hyp		12	19	34	14	4.0	25	14
Asp		2.0	7.9	11	4.3	2.8	10	8.9
Thr		1.1	4.5	2.4	1.1	0.8	2.4	2.5
Ser		2.7	4.0	11	5.3	0.9	5.9	5.4
Hse		0.4	0.6	0.7	0.6	0.8	0.6	0.7
Glu		5.1	12	16	6.6	3.3	13	11
Pro		13	24	34	11	3.2	21	18
Gly		26	52	81	31	12	59	41
Ala		7.6	11	22	10	2.8	16	12
Cys		—	—	—	—	—	—	—
Val		0.8	—	3.0	1.4	0.8	3.4	1.3
Met		—	—	—	—	—	—	—
Ile		1.2	1.1	3.7	—	—	3.4	1.2
Leu		—	2.8	2.8	1.2	0.9	4.9	3.3
Tyr		1.1	—	—	—	—	—	—
Phe		0.8	1.1	2.0	0.9	0.9	1.2	—
Hyl		—	0.3	0.7	0.4	1.2	0.4	0.9
Lys		1.4	6.7	7.8	2.0	0.8	5.5	3.7
His		0.6	—	—	0.8	0.5	—	0.8
Arg		2.6	3.2	9.2	5.7	1.8	12	8.4
Total		80	150	242	95	39	183	133

end of the normal gradient when examined by polyacrylamide-gel electrophoresis was found to contain two major peptides (mol.wts. approx. 40000 and 30000), which on reduction with 2-mercaptoethanol before electrophoresis migrated with mol.wts. 22000 and 10000, with the latter predominating. This susceptibility to mercaptoethanol is characteristic of peptide $\alpha 1(\text{III})\text{CB9B}$ (Rauterberg *et al.*, 1976). The higher-molecular-weight material probably represents a partial cleavage product containing the sequence CB9A-9B. The recovery of these peptides from the CM-cellulose column was always very poor. The peptide $\alpha 1(\text{III})\text{CB7}$ was isolated directly from cyanogen bromide digests of $\alpha 1(\text{III})_3$ by chromatography on a Bio-Gel P-10 column. The amino acid compositions of the peptides isolated, shown in Table 1, are similar to those previously reported (Rauterberg *et al.*, 1976).

CM-cellulose chromatography was not found useful for separating the peptides from reduced skin because much of the radioactivity was incompletely eluted as a very broad peak towards the end of the normal gradient (chromatogram not shown). However, on Bio-Gel A0.5m the peptide mixture gave a pattern (Fig. 3) that was clearly quite distinct from those of $\alpha 1$ - and $\alpha 2$ -chain cyanogen bromide peptides. A major peak of radioactivity was closely associated with the low-molecular-weight peptide identified as peptide $\alpha 1(\text{III})\text{CB7}$, but the remainder was distributed among several small peaks associated with peptides of higher molecular weight. To facilitate isolation and characterization of the low-molecular-weight peptide, initial separation of the CNBr digest was carried out on Bio-Gel P-10 (Fig. 4). In this manner the cross-link-associated radioactivity was resolved into two major peaks, each containing about half of the total radioactivity but with the second pool having a much higher specific radioactivity. Pool P10-3 appeared to contain only a single peptide, whereas pool P10-1 contained several higher-molecular-weight peptides excluded from the column. To resolve the peptides of pool P10-1 of the

Fig. 3. Separation of cyanogen bromide peptides from borohydride-reduced type III collagen on a Bio-Gel A0.5m column (2.5 cm $\times$ 120 cm) eluted with 1M- CaCl_2 /0.05M-Tris/HCl buffer, pH 7.5

Experimental details are given in the text. —, A_{230} ; ----, ^3H radioactivity per 0.2 ml of column effluent.

chromatogram shown in Fig. 4, they were chromatographed on a Bio-Gel A0.5m column. This resolved the mixture into several absorbance peaks (Fig. 5a), but resulted in only poorly resolved peaks of radioactivity. The pools 10Ag1-3 and 10Ag4

Fig. 4. Separation of cyanogen bromide peptides from borohydride-reduced type III collagen on Bio-Gel P-10 column (2.6 cm $\times$ 95 cm) eluted with 0.1M-acetic acid. Experimental details are given in the text. —, A_{230} ; ----, ^3H radioactivity per 0.2 ml of column effluent.

Fig. 5. Separation of (a) pool P10-1 from Fig. 4 on Bio-Gel A0.5m as described in Fig. 3, (b) pool 10Ag1-3 from Fig. 5(a) on Bio-Gel A0.5m after reduction and carboxymethylation, and (c) pool 10Ag4 from Fig. 5(a) on Bio-Gel A0.5m after reduction and carboxymethylation. Experimental details are given in the text. —, A_{230} or A_{228} ; ----, ^3H radioactivity in a sample of the column effluent.

Table 2. *Amino acid compositions of peptides containing reducible cross-links isolated from type III collagen*
Experimental details are given in the text.

Amino acid	CNBr peptide	Amino acid composition (mol of residue/mol)	
		P10-3	10Ag5
4Hyp		3.2	11.4
Asp		2.1	4.1
Thr		0.8	0.4
Ser		0.5	5.2
Hse		0.7	—
Glu		3.7	4.7
Pro		2.3	14
Gly		11.3	31
Ala		2.9	7.7
Cys		—	1.1
Val		0.9	3.1
Ile		—	1.7
Leu		1.0	0.3
Tyr		0.4	0.1
Phe		1.3	0.9
Hyl		1.4	0.6
Lys		0.6	1.3
His		0.9	2.3
Arg		1.7	4.8
Lys(OH)-Nle*		Trace	0.1
Lys(OH) ₂ -Nle†		0.4	0.05

* Hydroxylysinoxonorleucine, based on leucine equivalents.

† Dihydroxylysinoxonorleucine, based on leucine equivalents.

from several chromatograms were combined and individually reduced with mercaptoethanol and carboxymethylated. They were then rechromatographed on the same column (Figs. 5b and 5c); this showed that from each pool reduction of the disulphide bonds dissociated a lower-molecular-weight peptide carrying with it cross-link-associated radioactivity. The amino acid compositions of these cross-link-containing peptides are shown in Table 2.

The size and amino acid composition of peptide P10-3 is very similar to that of peptide $\alpha 1(\text{III})\text{CB7}$ in Table 1 and reported by Rauterberg *et al.* (1976). We can therefore identify it as such. The size and amino acid composition of peptide 10Ag5 is almost identical with that of peptide $\alpha 1(\text{III})\text{CB9B}$ isolated by Rauterberg *et al.* (1976), and the behaviour of this peptide on treatment with mercaptoethanol confirms its identity. It is not known why the cross-linked peptides with disulphide bonds intact chromatographed as a series of such poorly resolved peaks in Fig. 5(a), but it could be due to the

association of peptide CB9B with partially cleaved peptides, for example peptide CB9A-9B, and perhaps also to a partial cleavage of the *N*-terminal non-helical peptide leaving some intact peptide $\alpha 1(\text{III})\text{CB9B} \times \text{CB3A}$, which would appear in this pool. Evidence for this is found in Figs. 5(b) and 5(c), where there is a small peak of radioactivity eluted just before peptide 10Ag5 in the position that could be expected for a peptide of the size of peptide CB9A-9B or peptide CB9B $\times$ CB3A.

The non-integral values observed for cross-link residues in the amino acid compositions is probably the result of two factors: firstly that not all of the peptides $\alpha 1(\text{III})\text{CB7}$ and $\alpha 1(\text{III})\text{CB9B}$ are involved in intermolecular cross-linking and we have isolated a mixture of cross-linked and uncross-linked peptides, and secondly because the colour constants for the cross-link residues are not known and we have had to approximate by using leucine equivalents.

Discussion

The results presented above clearly demonstrate that type III collagen isolated from foetal calf skin is intermolecularly cross-linked by the reducible cross-links hydroxylysinoxonorleucine and dihydroxylysinoxonorleucine, thus confirming observations made previously (Bailey & Sims, 1976). From the analysis it would appear that there is a higher concentration of cross-link involving the *C*-terminal non-helical region, i.e. peptide CB9B, and a difference in ratio of the two types of cross-link between the *N*- and *C*-terminals. This suggests that the *C*-terminal lysine residue is hydroxylated to a greater extent than is the *N*-terminal lysine aldehyde. The studies by Fujii *et al.* (1976), in which they reported that the major cross-link was lysinoxonorleucine, were carried out on the small proportion of neutral-salt-extractable collagen, which is clearly non-representative of the cross-linking of the total intact tissue.

We have identified two major loci for the sites of intermolecular cross-links residing in cyanogen bromide peptide $\alpha(\text{III})\text{CB7}$ and the *C*-terminal disulphide-bonded peptide $\alpha 1(\text{III})\text{CB9B}$. This provides experimental evidence for the conclusion that these peptides might be involved in cross-linking, which was previously based solely on the order of the cyanogen bromide peptides with the $\alpha 1(\text{III})$ chain and the remarkable similarity between some sequences in these peptides and those in the presumptive cross-link region of type I collagen (Fietzek *et al.*, 1977).

We have, of necessity, used pepsin digestion to enable us to solubilize and purify the type III collagen, since it is only a minority component even of this foetal skin. We are aware that, by doing so, we have restricted the information obtained to the identification of the helical 'amino donor' of the cross-link

Scheme 1. Schematic representation of the mode of attachment of peptides containing intermolecular cross-links described in the present paper

without any direct evidence as to the nature of the 'aldehyde donor'. However, indirect information is available from these experiments. The fact that the tissue was, initially, totally insoluble and was subsequently solubilized by limited proteolysis with pepsin indicates that one partner in the cross-link must be located in the non-helical sequences of the molecule accessible to pepsin. These regions have been shown to exist at both the *N*- and *C*-terminals of the molecule in salt-soluble type III collagen (Timpl *et al.*, 1975). Secondly, unless the quarter-stagger theory for collagen is totally incorrect for type III collagen, given the order of the cyanogen bromide peptides 3A-3B-3C-7-6-1,8,2-4-5-9A-9B (Fietzek *et al.*, 1977), it would require that a peptide in the position of peptide $\alpha 1(\text{III})\text{CB}7$ could only cross-link with an aldehyde at the *C*-terminus and one in the position of a peptide $\alpha 1(\text{III})\text{CB}9\text{B}$ could only cross-link with an aldehyde at the *N*-terminus. We can therefore confidently propose that the peptides we have isolated represent pepsin digestion products of peptide $\alpha 1(\text{III})\text{CB}7 \times \alpha 1(\text{III})\text{CB}9\text{B}$ and peptide $\alpha 1(\text{III})\text{CB}3\text{A} \times \alpha 1(\text{III})\text{CB}9\text{B}$ (where $\times$ represents a cross-link) and the manner in which they arise is outlined in Scheme 1. Reprecipitated type III collagen obtained from pepsin digests of tissue produces fibres with the typical collagen axial periodicity, and the fact that we have found that the peptides involved in intermolecular cross-linking are those from the helical region, i.e. peptides $\alpha 1(\text{III})\text{CB}7$ and $\alpha 1(\text{III})\text{CB}9\text{B}$, which would be predicted by the quarter-stagger theory, strongly suggests that type III fibres exist *in vivo* with a similar end-overlap to the other fibrous collagens, and hence probably form the normal 70nm axial periodicity. Of course, the correctness of the assignments of aldehyde-donor peptide, and definitive proof of the quarter-stagger theory for type III collagen, would require the isola-

tion of a cross-linked peptide without prior pepsin digestion in which both partners can be readily identified.

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