The Chemistry of the Collagen Cross-Links

PURIFICATION AND CHARACTERIZATION OF CROSS-LINKED POLYMERIC PEPTIDE MATERIAL FROM MATURE COLLAGEN CONTAINING UNKNOWN AMINO ACIDS

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A polymeric form of the α_1 -chain C-terminal peptide α_1 CB6 (poly- α_1 CB6) was purified from CNBr digests of insoluble bovine tendon type-I collagen by gel filtration and ion-exchange chromatography. The purified material had a molecular weight of 1.5×10^6 – 5×10^6 on gel filtration and an amino acid content virtually identical with that of monomeric peptide α_1 CB6. The material could be adsorbed on affinity gels containing immobilized anti-(α_1 CB6-peptide non-helical region) antibodies and was an inhibitor of haemagglutination by the same antibodies of α_1 CB6-peptide-coated sheep erythrocytes. Periodate treatment of the material had no effect. Alkali hydrolysates were shown to contain two unknown amino acids, which were purified by gel filtration and ion-exchange chromatography in volatile buffers and are believed to be components of the mature cross-link of collagen.

In a recent paper we postulated that bovine tendon collagen (type I) is cross-linked in mature tissue by stable cross-links laid down in the C-terminal peptide α_1 CB6 (Light & Bailey, 1979). The maturation process resulted in the disappearance of monomeric α_1 CB6 peptide from CNBr digests of the insoluble collagen and the accumulation of high-molecular-weight material that appeared to contain predominantly α_1 CB6 peptide. Further studies of mature type-I collagen from a variety of sources confirmed this report, and showed that monomeric α_1 CB6 peptide could be observed in low-temperature pepsin digests of the insoluble collagen with a concomitant disappearance of the high-molecular-weight material (Light, 1979). This evidence indicated that the polymeric material (poly- α_1 CB6) was cross-linked only by interactions involving the C-terminal non-helical region of α_1 CB6 peptide. We speculated that this mechanism was achieved by the non-staggered lateral alignment of penta-fibrils within the collagen fibre.

In the present paper we report a method for the purification of poly- α_1 CB6 and confirm that it consists predominantly of α_1 CB6 peptide. We also present evidence for the presence of two unknown amino acids in alkali hydrolysates of this material that are believed to be components of the stable cross-link.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and Methods

KB^3H_4 (113 mCi/mmol) and carrier-free Na^{125}I were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Galactose oxidase was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and pepsin was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Sephadex and Sepharose products were supplied by Pharmacia (Uppsala, Sweden).

Preparation of tissue and CNBr digestion

Bovine achilles tendons were obtained from mature animals and were finely chopped after dissecting away excess fatty tissue. The tendon was then washed sequentially in 4 M-urea containing 0.5 M-acetic acid and then water before being freeze-dried. Samples were then digested with CNBr as previously described (Light & Bailey, 1979) and freeze-dried. In one experiment a portion of the dry peptide material was labelled through [^{125}I]iodination of tyrosine by the method of Glazer & Sanger (1964) before being freeze-dried and added back to the bulk.

Purification of poly- α_1 CB6

Polymeric cross-linked peptide material (poly- α_1 CB6) was separated from other CNBr-cleavage peptides by gel filtration in 1 M- CaCl_2 in 50 mM-Tris/HCl buffer, pH 7.5, on A-1.5 m agarose (Bio-

Rad Laboratories, Watford, Herts., U.K.). The material migrating at the void volume was pooled and dialysed exhaustively against water, and then 0.2 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4, containing 2 M-urea. The material was applied to a column of DEAE-Sephacrose CL-6B equilibrated in the same buffer. Unbound material was collected, dialysed exhaustively against water and freeze-dried. Non-collagenous material was eluted from the ion-exchange gel with 0.5 M-HCl.

A portion of the collagenous material eluted from the DEAE-Sephacrose CL-6B was dissolved in 1 M-CaCl₂ in 50 mM-Tris/HCl buffer, pH 7.5, and separated on a column of A-5m agarose (Bio-Rad Laboratories) in the same buffer.

Galactose oxidase treatment

Carbohydrate in poly- α_1 CB6 was labelled with ³H by the following method. A 50 mg portion of dry pure poly- α_1 CB6 was suspended in 10 ml of 0.15 M-NaCl in 0.1 M-sodium phosphate buffer, pH 5.0, and reduced for 1 h at room temperature with 5 mg of KBH₄. The solution was then acidified to pH 3.0 with acetic acid and dialysed at 4°C overnight against 0.15 M-NaCl in 20 mM-sodium phosphate buffer, pH 7.4. After equilibration to room temperature, 1 mg of galactose oxidase was added to the solution, followed by 3 mg of KBH₄. The reaction was stopped after 1 h by acidification to pH 3.0 with acetic acid, and the labelled poly- α_1 CB6 was dialysed against 0.5 M-acetic acid, freeze-dried and added back to the bulk of the preparation.

Periodate degradation and pepsin treatment of poly- α_1 CB6

A 5 mg portion of poly- α_1 CB6 was treated with an equal weight of NaIO₄ in 0.1 M-sodium citrate buffer, pH 5.5, as described by Robins & Bailey (1973a). The products were reduced with excess of KBH₄ after adjustment of the pH of the solution to 7.4 with NaOH. The products of the reaction were investigated by SDS/polyacrylamide-gel electrophoresis and by gel filtration on Sephadex G-50.

Pepsin digestion of poly- α_1 CB6 was carried out at 37°C in 0.5 M-acetic acid, at protein concentrations of 2.5–5 mg/ml. Pepsin was added [1:20 (w/w) enzyme/substrate ratio] and samples were taken at time intervals of 15, 30, 60 and 120 min and 20 h. The resulting peptide mixture was resolved by gel filtration in water on Sephadex G-25 or by SDS/polyacrylamide-gel electrophoresis.

Borohydride reduction

Bovine achilles tendon obtained from a 3-year-old animal was exhaustively washed in 0.15 M-NaCl in 20 mM-sodium phosphate buffer, pH 7.4, at 4°C. The tendon was equilibrated in 0.1 M-sodium phosphate buffer, pH 5.0, and KBH₄ was added

(collagen/KBH₄ ratio 30:1, w/w). The reaction was allowed to proceed at room temperature (approx. 19°C) for 1 h, at which time acetic acid was added to adjust the pH to 3.0. The collagen was dialysed against 0.5 M-acetic acid, and poly- α_1 CB6 was prepared as described above. The ³H-labelled polymeric material was hydrolysed in 6 M-HCl and analysed for cross-links as described by Robins & Bailey (1973a).

Antibodies to α_1 CB6 peptide and haemagglutination assays

Antibodies to purified bovine α_1 CB6 peptide from neutral-salt-soluble type-I collagen were raised in rabbits and purified by adsorption on pepsin-treated bovine type-I-collagen-liganded and α_1 CB6-peptide-liganded Sepharose 4B. The methodology and rationale have been described by Duance *et al.* (1977). Anti-(α_1 CB6 peptide) antibodies were then used either as sera or as purified antibodies in haemagglutination assays. Poly- α_1 CB6 was used in haemagglutination inhibition tests with α_1 CB6-peptide-coated sheep erythrocytes and anti-(α_1 CB6 peptide) antibodies, and was tested as an antigen by direct haemagglutination of poly- α_1 CB6-coated sheep erythrocytes by anti-(α_1 CB6 peptide) antibodies. Poly- α_1 CB6 was also shown to bind to anti-(α_1 CB6 peptide) antibodies coupled to Sepharose 4B.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis analysis of purified poly- α_1 CB6, periodate-treated poly- α_1 CB6 and pepsin-treated poly- α_1 CB6 was carried out in 10% (w/v) acrylamide gels as previously described (Light & Bailey, 1979). Peptides were stained with Coomassie Brilliant Blue and scans were obtained by using a Joyce-Loebl Chromoscan instrument with a filter at 520–595 nm.

Hydrolysis procedures and amino acid analysis

Purified galactose oxidase-³H-labelled poly- α_1 CB6 was hydrolysed with 6 M-HCl or 2 M-NaOH as described by Robins & Bailey (1973a), except that alkali hydrolyses were carried out for 48 h. Routine amino acid analysis was carried out on a Locarte amino acid analyser in 0.2 M-citrate buffers. Unusual compounds were identified by using the pyridine/formic acid system described by Bailey *et al.* (1970).

Preparation of labelled components from alkali hydrolysates

A 700 mg portion of pure galactose oxidase-³H-labelled poly- α_1 CB6 was hydrolysed in 2 M-NaOH as described above. After hydrolysis the

solution was neutralized with HCl and desalted in batches on a Sephadex G-10 column (60 cm $\times$ 5 cm) equilibrated in water. Two labelled fractions were collected (peaks 1 and 2; Fig. 7a). Peak 1 was then resolved on a Sephadex G-10 column (90 cm $\times$ 2.5 cm) equilibrated in water, and two major peaks of radioactivity were pooled (peaks 1a and 1b). The labelled material in the higher-molecular-weight peak (fraction 1a) was further purified by gel filtration in water on Sephadex G-25 (87 cm $\times$ 2.5 cm) and by ion-exchange chromatography at 56°C in 7% (v/v) formic acid on the short column (9 cm) of a Locarte amino acid analyser adapted for preparative procedures (Fig. 8).

The second major peak from Sephadex G-10 gel filtration (peak 1b) was also purified by this latter procedure. Fraction 1a was tightly bound to the column and was only eluted with 2 M-NH₃, whereas fraction 1b was eluted with the unbound material. Peak 2, collected after desalting of the hydrolysate, was chromatographed on Sephadex G-10 (90 cm $\times$ 2.5 cm) in water, and one labelled peak (2a) was collected.

Finally, fractions 1a, 1b and 2a were further purified by gel filtration in 4 M-urea on a Sephadex G-10 column (85 cm $\times$ 1.6 cm). The column was calibrated with respect to molecular weight with oxytocin (mol.wt. 1007), streptomycin (mol.wt. 581), lactose (mol.wt. 360), lysine (mol.wt. 146) and glycine (mol.wt. 75). The effluent from the gel-filtration procedures was monitored in each case at 230 nm.

Results

Purification of poly- α_1 CB6

Fig. 1(a) shows a typical elution profile of a CNBr digest of insoluble tendon after separation on A-1.5m agarose. Polymeric peptide material moving at the void volume accounted for approx. $12 \pm 2.4\%$ (four determinations) of the total material and was associated with the major peak of ¹²⁵I radioactivity. A proportion of the radioactivity co-migrated with the peak containing the α_2 -chain C-terminal peptide α_2 CB3,5, which, like α_1 CB6 peptide, also contains tyrosine.

The A-1.5m agarose void-volume material was partially included in A-5m agarose under the same conditions of chromatography and migrated as a broad symmetrical peak (Fig. 1b). The apparent molecular weight of the moiety is therefore in the range 1.5×10^6 – 5×10^6 . The collagenous material did not bind to DEAE-Sepharose CL-6B, and a small amount of non-collagenous contamination, accounting for less than 7% dry weight, was eluted with 0.5 M-HCl. The purified poly- α_1 CB6 did not enter the matrix of 10% (w/v) SDS/polyacrylamide gels (Fig. 2).

Fig. 1. (a) Separation of CNBr-cleavage peptides from mature insoluble collagen on an A-1.5m column and (b) resolution of void-volume material from the A-1.5m gel filtration on an A-5m column

Experimental details are given in the text. (a) —, A_{230} ; ----, radioactivity. (b) —, A_{230} ; ----, relative elution volumes of void-volume (V) and included-volume markers (Blue Dextran and glycine respectively).

Periodate and pepsin degradation

Periodate treatment of poly- α_1 CB6 had no effect in that no fragments in the mol.wt. range 10000–500000 could be observed on SDS/polyacrylamide-gel electrophoresis of the treated material (Fig. 2). Similarly, no low-molecular-weight fragments (mol.wt. 1500–30000) were observed when the treated material was chromatographed in Sephadex G-50 (Figs. 3 and 4).

Pepsin treatment for 20 h at 37°C, however, completely digested poly- α_1 CB6 to low-molecular-weight fragments (mol.wt. <10000; see Fig. 2). This process was shown to be time-dependent, and a low-molecular-weight peptide could be prepared from a 15 min digest at 37°C (indicated by bar in Fig. 5a). This peptide contained ³H previously incorporated by the galactose oxidase method. After 30 min and 1 h the major labelled fractions were associated with much-lower-molecular-weight material (Figs. 5b and 5c).

Haemagglutination and affinity chromatography

Haemagglutination and haemagglutination inhibition assays were carried out with α_1 CB6 peptide, poly- α_1 CB6 and pepsin-treated type-I collagen being used as antigens against anti-(α_1 CB6 peptide) antibodies. Table 1 lists the results obtained and shows that poly- α_1 CB6-coated erythrocytes were agglu-

Fig. 2. *Densitometry of SDS/polyacrylamide gels*. Samples were electrophoresed in 10% (w/v) acrylamide gels and stained with Coomassie Brilliant Blue as described in the text. (a) Neutral-salt-soluble type-I collagen CNBr-cleavage peptides; (b) 10-year bovine tendon type-I collagen CNBr-cleavage peptides; (c) purified poly- α_1 CB6; (d) periodate-treated poly- α_1 CB6; (e) pepsin-treated poly- α_1 CB6 (2h digest).

Fig. 3. *Gel filtration on Sephadex G-50 of periodate-treated poly- α_1 CB6*. Experimental details are given in the text. —, A_{230} ; ----, conductivity. Analysis of the minor included peak revealed no peptide material.

Fig. 4. *Affinity chromatography on anti-(α_1 CB6 peptide)-antibody-liganded Sepharose 4B*

(a) Affinity adsorption of crude α_1 CB6 peptide; (b) affinity adsorption of pure poly- α_1 CB6; (c) affinity adsorption of pepsin-treated type-I collagen. In each case peak P represents unbound material eluting in the running buffer (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4). Continuous arrows indicate elution with 0.2 M-glycine, pH 2.2, and broken arrows indicate return to running buffer. Note that no pepsin-treated type-I collagen was bound, demonstrating the specificity of the anti-(α_1 CB6 peptide) antibodies against the C-terminal non-helical region.

tinated by anti-(α_1 CB6 peptide) and that poly- α_1 CB6 was an inhibitor of agglutination of α_1 CB6-peptide-coated erythrocytes by the same antibodies. As expected, pepsin-treated type-I-collagen-coated erythrocytes were not agglutinated, as the antibodies were directed specifically against the C-terminal non-helical region. As a corollary to these findings poly- α_1 CB6 could be adsorbed on agarose gels containing immobilized anti-(α_1 CB6 peptide) antibodies (Fig. 4).

Reducible components of poly- α_1 CB6

When whole neutral-salt-washed tendon was reduced with KB^3H_4 a small proportion of the radioactivity was associated with poly- α_1 CB6. Hydrolysis and subsequent analysis of the labelled amino acids on pyridine/formate-buffered columns showed

Fig. 5. Gel filtration on Sephadex G-25 of pepsin-treated poly- α_1 CB6

Experimental details are given in the text. (a) 15 min digest; (b) 30 min digest; (c) 120 min digest. —, A_{230} ; ----, ^3H radioactivity. Bar indicates reproducibly prepared labelled peptide.

Table 1. Haemagglutination assays of anti-(α_1 CB6 peptide) antibodies for activity against poly- α_1 CB6

Experimental details are given in the text. The titre is expressed as the reciprocal of the highest serum dilution to give agglutination.

Antigen	Poly- α_1 CB6 added as inhibitor ($\mu\text{g/ml}$, final concentration)	Titre
Pepsin-treated type-I collagen	—	0
Neutral-salt-soluble type-I collagen	—	1/256
α_1 CB6 peptide	—	1/64
Poly- α_1 CB6	—	1/32
α_1 CB6 peptide	50	1/4
α_1 CB6 peptide	150	0

that only one component accounted for the greater proportion of the radioactivity incorporated (Fig. 6a). No significant amounts of radioactivity were

Fig. 6. Ion-exchange chromatography in pyridine/formate buffers of amino acids from acid and alkali hydrolysates of poly- α_1 CB6

Experimental details are given in the text. (a) Acid hydrolysate of poly- α_1 CB6 prepared from KB^3H_4 -reduced bovine tendon; (b) and (c) peak 1b and peak 1a respectively, partially purified components from an alkali hydrolysate of galactose oxidase- ^3H -labelled poly- α_1 CB6 (see Fig. 7). Broken arrow indicates elution with 2M-pyridine. Abbreviations: DHLNL, dihydroxylysineonorleucine; HLNL, hydroxylysineonorleucine; Hyl, hydroxylysine.

found in the positions characteristic of hydroxylysineonorleucine or dihydroxylysineonorleucine, although a small amount of label was associated with hexosyl-lysine residues (Robins & Bailey, 1973b).

Amino acid analysis

The amino acid content of acid hydrolysates of poly- α_1 CB6 is shown in Table 2 and compared with the known amino acid content of α_1 CB6 peptide. There is a very close correlation between the two.

When alkali hydrolysates of ^3H -labelled poly- α_1 CB6 (labelled by the galactose oxidase method) were subjected to repeated separations on Sephadex G-10 and G-25, three major peaks of radioactivity were obtained (fractions 1a, 1b and 2a; see Figs. 7 and 8). No radioactive peaks were obtained on chromatographing these preparations by ion-exchange chromatography in non-volatile citrate buffers, but fractions 1a and 1b each gave a peak of radio-

Table 2. *Amino acid compositions of various fractions obtained from mature bovine tendon collagen*
 Experimental details are given in the text.
 Amino acid composition (mol/1000 mol of total residues)

Amino acid	α_1 CB6 peptide*	Poly- α_1 CB6†	Poly- α_1 CB6‡	Type-I collagen§
Hyp	74.4	74 ± 4	77	87
Asp	46.5	45 ± 3.2	47	46
Thr	18.6	22 ± 2.1	17	16
Ser	41.9	44 ± 4.5	39	34
Hse	—	0.8 ± 0.04	0.7	—
Glu	69.8	75 ± 3.2	74	78
Pro	158.1	138 ± 8.0	139	137
Gly	316.0	305 ± 6.3	312	331
Ala	97.6	125 ± 7.1	118	121
Cys	—	—	—	—
Val	14.0	19 ± 2.1	19	18
Met	—	—	—	—
Ile	13.9	15 ± 1.4	15	9
Leu	27.9	28 ± 3.3	29	21
Tyr	13.9	11 ± 2.1	10	4
Phe	9.3	10 ± 1.2	11	13
Hyl	9.3	10 ± 1.2	10	5
Lys	18.6	19 ± 3.0	21	32
His	9.3	7 ± 2.2	8	3
Arg	55.8	51 ± 4.7	53	50

* Based on sequence analysis data (Gallop & Paz, 1975).

† Based on three determinations.

‡ Eluted from anti-(α_1 CB6 peptide) antibody affinity gel with 0.2 M-glycine, pH 2.2.

§ Taken from Rauterberg & Kuhn (1971)

Fig. 7. Preparation of galactose oxidase- ^3H -labelled compounds from an alkali hydrolysate of poly- α_1 CB6. Experimental details are given in the text. (a) Desalting of hydrolysate on a Sephadex G-10 column (60 cm $\times$ 5 cm); (b) gel filtration on peak 1 from (a) on a Sephadex G-10 column (90 cm $\times$ 2.5 cm); (c) gel filtration of peak 1a from (b) on a Sephadex G-25 column (87 cm $\times$ 2.5 cm); (d) gel filtration of peak 2 from (a) on a Sephadex G-10 column (90 cm $\times$ 2.5 cm). —, A_{230} ; ----, radioactivity; - · - · -, conductivity.

Fig. 8. Ion-exchange chromatography in pyridine/formate buffers of partially purified components 1a and 1b (see Fig. 7)

Experimental details are given in the text. —, Elution profile of fraction 1a; ----, elution profile of fraction 1b.

Fig. 9. Gel filtration on Sephadex G-10 in 4M-urea of fractions 1a, 1b and 2a prepared from an alkali hydrolysate of poly- α_1 CB6

Experimental details are given in the text. (a) Fraction 1b (see Fig. 8); (b) fraction 1a (see Fig. 8); (c) fraction 2a (see Fig. 7d). —, A_{230} ; ----, ^3H radioactivity. The elution volumes of molecular-weight markers are indicated by arrows in (a): Ox, oxytocin (mol.wt. 1007); Strep, streptomycin (mol.wt. 581); Lac, lactose (mol.wt. 360); Lys, lysine (mol.wt. 146); Gly, glycine (mol.wt. 75).

activity, coincident with a ninhydrin-positive peak, on ion-exchange chromatography in pyridine/formate buffers. Neither of these components co-chromatographed with any known amino acids (Figs. 6b and 6c).

One of these three components (1b) is probably not significant with respect to cross-linking, as it has a molecular weight of only 150–200 (Fig. 9a) and is uncharged (Fig. 6b). A second compound (1a), also labelled by the galactose oxidase method, had a molecular weight of 750–800 (Fig. 9b). Another possible cross-link compound (2a) was partially purified and had a similar molecular weight (Fig. 9c).

Amino acid analysis in non-volatile buffers of both acid and alkali hydrolysates showed no significant novel compounds. Acid hydrolysates were not subjected to pyridine/formate-buffered ion-exchange chromatography, in part because of the lack of resolution of the acid residues, but, more importantly, because labelled galactose was cleaved from cross-links by this method.

Discussion

We have previously shown (Light & Bailey, 1979) that during the course of ageing to maturity the C-terminal region of the α_1 -chain of bovine tendon collagen apparently becomes involved in the formation of high-molecular-weight polymeric material. This results in the disappearance of α_1 CB6 peptide from SDS/polyacrylamide-gel analyses of CNBr digests of insoluble tendon from mature animals and the concomitant appearance of tyrosine-containing (labelling with ^{125}I) high-molecular-weight material (Light & Bailey, 1979), indicating that it is a polymeric form of α_1 CB6 peptide.

We have shown in this present paper that the polymeric component could be prepared by A-1.5m-agarose-gel filtration (Fig. 1), and subsequently separated from non-collagenous contaminants by ion-exchange chromatography. This material was present in amounts equivalent to the theoretical amount of α_1 CB6 peptide (i.e. 13%) and was the major tyrosine-containing component.

The polymeric material runs as a broad symmetrical peak on A-5m agarose, indicating apparent heterogeneity. The molecular weight of the complex peptide estimated (1.5×10^6 – 5×10^6) may be too high owing to the branched rather than linear polymeric nature of the material. The component corresponds to the high-molecular-weight material previously observed on 10% (w/v) polyacrylamide gels of CNBr digests of mature collagen (Light & Bailey, 1979; Light, 1979) and does not enter the gel matrix (Fig. 2). These data led to the conclusion that poly- α_1 CB6 must be derived from interfibrillar bonds linking α_1 CB6 peptides from different molecules.

Periodate treatment of poly- α_1 CB6 has no effect, demonstrating the absence of any known reducible cross-links (Figs. 2 and 3). This evidence is further confirmed by the amino acid analysis of poly- α_1 CB6 derived from KB³H₄-reduced tendon (Fig. 6a). Neither of the reduced aldimine or ketoimine bonds was present in acid hydrolysates of this material. These data support the previous findings (Robins *et al.*, 1973) showing a disappearance of reducible cross-links during maturation, and is consistent with the hypothesis that the reducible cross-link forms part of the stable cross-link.

Pepsin treatment of poly- α_1 CB6 at 37°C results in very rapid degradation to small peptides (Fig. 5). However, a discrete peptide could be prepared from the 15 min digest, and was shown to be associated with radioactivity when derived from poly- α_1 CB6 that had been labelled with ³H by the galactose oxidase method. These data argue that the polymeric material is specifically cross-linked and is not likely to be merely randomly aggregated by non-covalent bonds, particularly as the preparation of the small labelled peptide was found to be reproducible. This supports the previous suggestion (Light, 1979) that poly- α_1 CB6 is specifically cross-linked through the C-terminal non-helical-region, as pepsin treatment of mature collagen at 15°C leads to the disappearance of poly- α_1 CB6 and liberates α_1 CB6 peptide.

Poly- α_1 CB6 has a very similar amino acid composition to intact α_1 CB6 peptide (Table 2) and exhibits the characteristically high proportion of tyrosine and histidine expected in α_1 CB6 peptide. No tryptophan or cysteine was observed, indicating the absence of any non-collagenous glycoprotein components. A small proportion of homoserine in the amino acid analysis indicates a low content of other CNBr-cleavage peptides, as expected, since α_1 CB6 peptide is known to cross-link to α_1 CB5 peptide (Henkel *et al.*, 1976) in immature tissue. None of the major CNBr-cleavage peptides is involved in cross-linking in poly- α_1 CB6 (Light, 1979), thus eliminating all regions of the molecule except the very small peptides α_1 CB1, α_1 CB5, α_2 CB1 and α_1 CB4 from participation in the formation of poly- α_1 CB6. The other large tyrosine-containing peptide α_2 CB3,5 is not involved in the high-molecular-weight component, as shown by its presence on gel filtration (Fig. 1) and electrophoresis (Fig. 2) of mature collagen CNBr-cleavage peptides. These data argue strongly in favour of the polymeric material consisting predominantly of α_1 CB6 peptide. Haemagglutination and immunoadsorbent studies of poly- α_1 CB6 also confirm its α_1 CB6 peptide content, as the polymeric material cross-reacted with anti-(α_1 CB6 peptide) antibodies, was an inhibitor of agglutination of α_1 CB6-peptide-coated erythrocytes by the same antibodies and could be bound to immobilized anti-(α_1 CB6 peptide) antibodies (Table 1).

We have suggested (Light & Bailey, 1979) that poly- α_1 CB6 is formed by means of C-terminal interactions across the collagen fibre. Conclusive evidence has shown that the reducible cross-links are formed only from interactions between the N- and C-terminals and helical regions in other molecules, consistent with the quarter-staggered end-overlap model (Miller & Robertson, 1973; Henkel *et al.*, 1976; Light, 1979). Therefore the interpentafibrillar C-terminal cross-links shown to occur in this present study can only form if the pentafibrils are aligned in a non-staggered fashion, i.e. with the C-terminals of adjacent pentafibrils in register. This hypothesis has been recently supported by new X-ray-diffraction evidence showing that the pentafibrils in tendon fibres are arranged in a non-staggered fashion (Frazer *et al.*, 1979). A molecular weight of 1.5×10^6 – 5×10^6 therefore would be consistent with the interaction of 38–125 aligned molecules. Extrapolating from this, by using a mean molecular diameter of 1.5 nm, would give rise to fibres of diameter 57–188 nm, assuming that the polymerization process occurs across the complete width of the fibre. This value is in good agreement with tendon collagen fibre diameters measured by electron-microscopic methods (Parry *et al.*, 1978). Although only an approximate estimation, this good fit with known fibre diameters further supports our contention that the interaction of C-terminal residues within the fibre giving rise to a meshwork of cross-links is the mechanism of stabilization of type-I collagen during maturation.

When alkali hydrolysates of galactose oxidase-³H-labelled poly- α_1 CB6 were analysed for radioactivity after separation in non-volatile buffers, it was shown that the radioactivity was eluted as a very broad peak. When subsequent analyses were carried out in pyridine/formate buffers, two previously unknown ninhydrin-positive components were observed and were associated with all the radioactivity. One of these compounds corresponded to the reducible component of poly- α_1 CB6 (Fig. 6a). Three components were purified by repeated gel filtration on Sephadex G-10 and G-25 and ion-exchange chromatography in 7% formic acid.

Molecular-weight determinations of these labelled components by gel filtration in 4 M-urea showed that one of the preparations contained only very-low-molecular-weight material (1b) migrating coincident with lysine (Fig. 9a). However, both of the other preparations (1a and 2a), as well as containing low-molecular-weight material, contained labelled compounds (A and B) with mol.wts. between 750 and 800 (Figs. 9b and 9c). Although possibly heterogeneous, these peaks contain labelled components that are in the molecular-weight range that we might expect for the stable cross-link if it is derived, as previously suggested, from the reducible cross-link (i.e.

hydroxylysine plus galactose and glucose, lysine aldehyde and a third unknown amino acid from another penta-fibril). Although conclusions drawn from these preliminary findings must remain tentative, we believe that these components are derived from or actually represent the stable cross-link(s) of mature type-I collagen.

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