COLLAGEN CROSSLINKING: ISOLATION OF A DIMERIC CROSSLINKED PEPTIDE OF α1-CB6 FROM BOVINE CORNEAL AND SCLERAL COLLAGENS

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1. Introduction

The pattern of CNBr fragments of various collagens on SDS–polyacrylamide electrophoresis gels includes a major band with mobility intermediate between α2-CB(3–5) and α2-CB4 which has been called α1-CB(3–7) and α1-CB(5–8–3) on the basis of molecular weights from the gels [1,2]. We have noted a similar component which we will call component X, in gel patterns of CNBr fragments from unreduced corneal collagen [3], reduced corneal collagen and reduced scleral collagen. Our CNBr digestion conditions are harsher than those most used and we find no methionine in the digest; also we have not isolated any uncleaved fragments [3], so its seems unlikely that this major component could be an uncleaved peptide as has been suggested. Furthermore α1-CB3, part of both the proposed uncleaved peptides, is readily isolated in high yield (≤60%) from CNBr digests of both corneal and scleral collagen (J.J.H., N. A. Panjwani, M.J.C.C., unpublished results). In the present work we have isolated component X from CNBr digests of corneal and scleral collagen and identified it as a dimer of α1-CB6.

2. Methods

Amino acid analysis, cyanogen bromide digestion and SDS–polyacrylamide gel electrophoresis were by the methods in [3]. Preparation of reduced labelled corneal and scleral collagens and measurement of radioactivity were as in [4,5]. CM-cellulose chromatography was as in [5]. Amino acid analysis and determination of radioactivity of peptides separated by SDS–gel electrophoresis was as in [5].

3. Results

3.1. *Component X from SDS–polyacrylamide gel electrophoresis of bovine scleral digests*

The staining profile from a typical gel after SDS electrophoresis of a CNBr digest of reduced scleral collagen (fig.1) is very similar to that for type I collagen from bovine cornea [3], with no evidence of significant amounts of collagen types II or III.

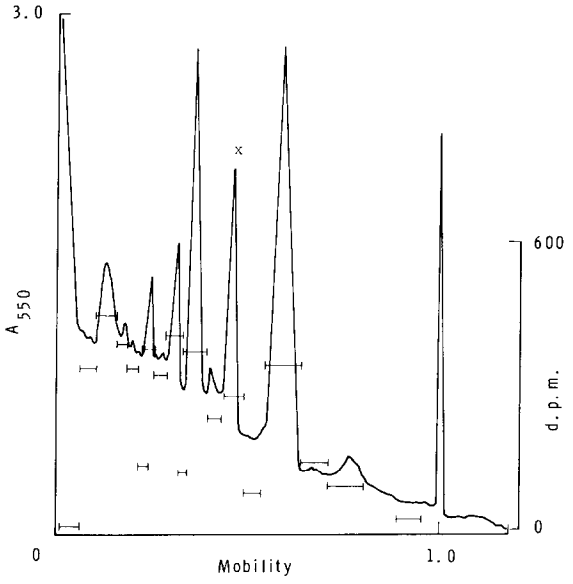

Fig.1. SDS–polyacrylamide gel electrophoresis of a radioactively labelled CNBr digest of bovine scleral collagen. The continuous line shows the staining profile with Coomassie blue, under conditions described in the text. The short horizontal bars indicate the radioactivity in disintegrations per minute of portions of the gel treated as described in the text.

Table 1
Amino acid compositions of component X from bovine sclera, both excised from SDS–gels and isolated from a CNBr digest by column chromatography, compared with compositions for α1-CB6, α1-CB(3–7) and α1-CB(5–8–3) from bovine skin [8]

Amino acid	Component X isolated by column chromatography[a]	Component X excised from SDS gels[a]	Fragment α1-CB6	Fragment α1-CB(3–7)	Fragment α1-CB(5–8–3)
Hydroxyproline	99.4	91.0	106	89.6	106.2
Aspartic acid	41.3	43.3	45	44.8	43.1
Threonine	18.3	17.8	19.5	12.1	11.5
Serine	35.3	29.0	38.0	24.7	28.2
Homoserine	0	0	0	4.8	6.6
Glutamic acid	65.7	68.0	67	80	84.4
Proline	126.9	137.0	133.0	128.3	102.6
Glycine	325.0	333.0	328.0	344.3	338.5
Alanine	124.0	131.0	113.0	135.1	135.5
Valine	23.0	20.0	16.4	20.6	18.6
Methionine	0	0	0	1.0	2.0
Isoleucine	11.5	11.0	10.7	6.5	3.7
Leucine	21	19.0	20.5	16.7	15.5
Tyrosine	0	0	0	0	0
Phenylalanine	14.0	11.5	9.7	14.3	14.9
Hydroxylysine	7.0	5.7	5.6	2.9	5.1
Histidine	4.0	1.3	3.6	0	2.0
Lysine	27.0	26.0	28.1	32.9	33.8
Arginine	55.0	53.0	56.4	46.0	48.8

[a] Mean of 2 analyses

Compositions of α1-CB(3–7) and α1-CB(5–8–3) are calculated from the sum of single peptide fragments in [8]

Results are expressed as residues/1000 residues

Amino acid analysis of peak X from SDS gels (fig.1) gave a composition (table 1) which is similar to that of α1-CB6. It was unlike that of α1-CB(3–7) or α1-CB(5–8–3), identities suggested for the corresponding component from skin [1,2]. Component X from sclera was determined as 38 ± 1.05 kdaltons (10 determinations) from SDS–gel electrophoresis using CNBr peptides of corneal collagen as markers. This is ~2 times that calculated for α1-CB6, and clearly different from that of α1-CB(5–8–3) which is 42 007 daltons [2]. The absence of homoserine and methionine from component X, apparent even after gross overloading of the amino acid analyser showed that it cannot be either α1-CB(3–7) or α1-CB(5–8–3). Component X must be a dimer of α1-CB6. The radioactive profile of the gel (fig.1) indicates that component X is radioactively labelled suggesting that the crosslink in the dimer is reducible.

3.2. *Component X from bovine corneal collagen on SDS–gels*

A CNBr digest of unreduced corneal collagen was subjected to a second digestion with CNBr before examination by SDS–gel electrophoresis. The pattern was unchanged from that shown [3] and component X appeared undiminished, confirming that it is not an uncleaved peptide. Component X, determined by SDS–gel electrophoresis on 5% gels using the collagen α1 chain and its CNBr fragments as markers, was 37 ± 1.2 kdaltons for corneal collagen, similar to the value for sclera (see above). These values are significantly less than 42 007 daltons [2] for the uncleaved peptide α1-CB(5–8–3).

Treatment of a CNBr digest of reduced corneal collagen with periodate for 5 min [6] gave no breakdown of component X assessed by SDS–gel electrophoresis.

3.3. *Isolation of the crosslinked labelled peptide from a CNBr digest of corneal collagen*

Collagen from adult bovine corneal stroma was labelled by borohydride reduction, and digested with cyanogen bromide [4]. The digest was fractionated on CM-cellulose [4] to give a chromatogram (fig.2) similar to that found for unreduced bovine corneal collagen [3]. The fractions shown in fig.2 were pooled, dialysed and freeze dried to give 44 mg peptide with 10% of the recovered activity. This peptide fraction was loaded onto a column (103 X 1.3 cm) of agarose (Sepharose 6B, Pharmacia, London W5 5SS). The column was eluted with 1 M $CaCl_2$/50 mM Tris–HCl (pH 7.5) and monitored for A_{234} and radioactivity. This fractionation was repeated; the chromatogram from the second separation (fig.3) shows that most of the label put on the column was in the major fraction eluted after ~70 ml which is close to the elution volume of α2-CB(3–5), the largest expected CNBr fragment. After each gel chromatography step the major fractions had the same composition as α1-CB6 from bovine skin (table 2). Corneal α1-CB6 [3] has an elution position corresponding to ~103 ml on this column (N.A. Panjwani, unpublished result) so the major fraction in fig.3 is larger than α1-CB6 but has the same composition, which is distinct from the composition of other CNBr fragments; it must be a crosslink peptide consisting of two or more chains of α1-CB6 joined together and corresponds to component X from sclera. The yield of component X from cornea represents almost 50% of the theoretical yield of α1-CB6.

3.4. *Isolation of the crosslinked labelled peptide from a CNBr digest of bovine scleral collagen*

CM cellulose chromatography of the scleral CNBr digest gave an elution pattern (fig.4) similar to that for corneal collagen (fig.2), thus further substantiating that scleral collagen is essentially all type I. Fraction C10 (fig.4) was examined by SDS–polyacrylamide gel electrophoresis showing two major peaks, peak X and a peak of the mobility of α1-CB8. Fraction C10

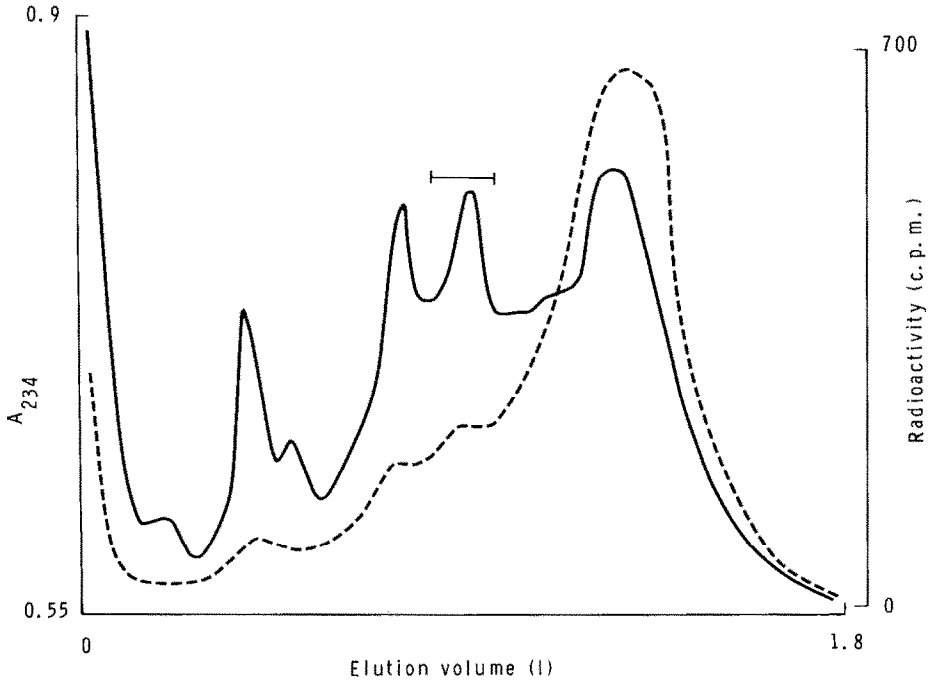

Fig.2. CM cellulose chromatogram of CNBr-cleaved peptides of labelled bovine corneal collagen. Elution was at 43°C with a linear gradient (20–160 mM in 2 l total vol.) of NaCl in 20 mM sodium citrate (pH 3.6). The flow rate was 80 ml/h; Fractions (8 ml) were collected; the bar indicates the fractions pooled. Column load 386 mg. (——) A_{234}; (- - -) radioactivity.

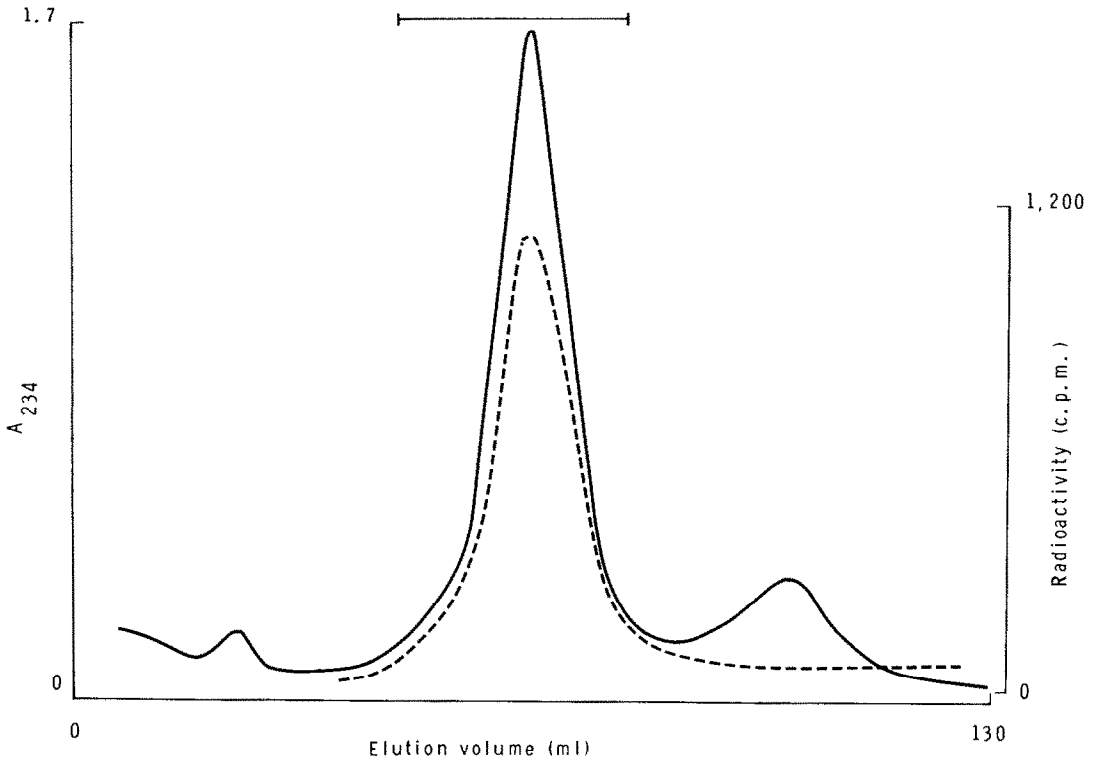

Fig.3. Second gel chromatography, on a column (103 × 1.3 cm) of Sepharose 6B, to give corneal component X. Elution was achieved with 1 M $CaCl_2$/50 mM Tris–HCl (pH 7.5). (——) A_{234}; (- - - - -) radioactivity. The bar indicates the fractions pooled to give component X.

was then subjected to DEAE-cellulose chromatography (fig.5). The staining profile after SDS–gel electrophoresis of the second component showed a single band with the mobility of component X. Its amino acid composition is identical to that of α1-CB6 (table 1) but at 38 ± 1.05 kdaltons is double that of fragment α1-CB6. We propose that this fraction X is a crosslinked dimer of α1-CB6. There was no tyrosine, certainly <0.05 residues/ α1-CB6 chain, obtained in any analysis of X, even if 10 × overloaded on the analyser. The presence of some radioactivity in component X isolated from SDS–gels and from CM- and DEAE-cellulose chromatography indicates that a proportion of the crosslinks in the dimer are reducible. The crosslink(s) may be either inter- or intra-molecular. The yield of this crosslinked peptide from bovine sclera represented 74% of the theoretical yield of fragment α1-CB6.

3.5. *Attempted cleavage of the crosslink from bovine cornea and sclera*

Component X isolated from the cornea (22 mg) was treated with periodate, a reagent that splits some collagen crosslinks [4,6] using a relatively short (5 min) procedure [6] followed by reduction with NaB^3H_4 [7]. The product was chromatographed on the same agarose column and the major fraction was eluted at the same elution volume as before (68 ml), indicating that the crosslink had remained intact. A second attempt was made to split the crosslink, this time heating to 70°C for 1 h. The product was loaded on the same agarose column and appeared after 69 ml. This product and that after periodate treatment, both had the amino acid composition of α1-CB6 (table 2), but are larger than α1-CB6. The crosslinked peptide (15 mg) isolated from the sclera was treated with HIO_4 for 15 min by the method in [6]. The

Table 2
Amino acid compositions of the large crosslinked peptides from cornea

Amino acid	Major fraction from fig.3. i.e., X	Periodate-treated X	Heat-treated X	Skin[a] α1-CB6
Hydroxyproline	118	86	104	106
Aspartic acid	40	44	46	45
Homoserine	0	0[b]	0	0
Threonine	19	22	20	19.5
Serine	39	39	33	38
Glutamic acid	65	70	75	67
Proline	142	125	117	133
Glycine	304	335	341	328
Alanine	114	128	135	113
Valine	15	21	18	16
Isoleucine	9	11	9	11
Leucine	21	19	19	20.5
Tyrosine	0	0	0	0
Phenylalanine	11	10	12	10
Hydroxylysine	10	5	4.8	5.6
Lysine	32	29	24	28
Histidine	3.3	trace	1	3.6
Arginine	60	56	43	56

[a] Taken from [8]
[b] Absence of homoserine confirmed by gross overloading

Values are expressed as residues/1000 residues

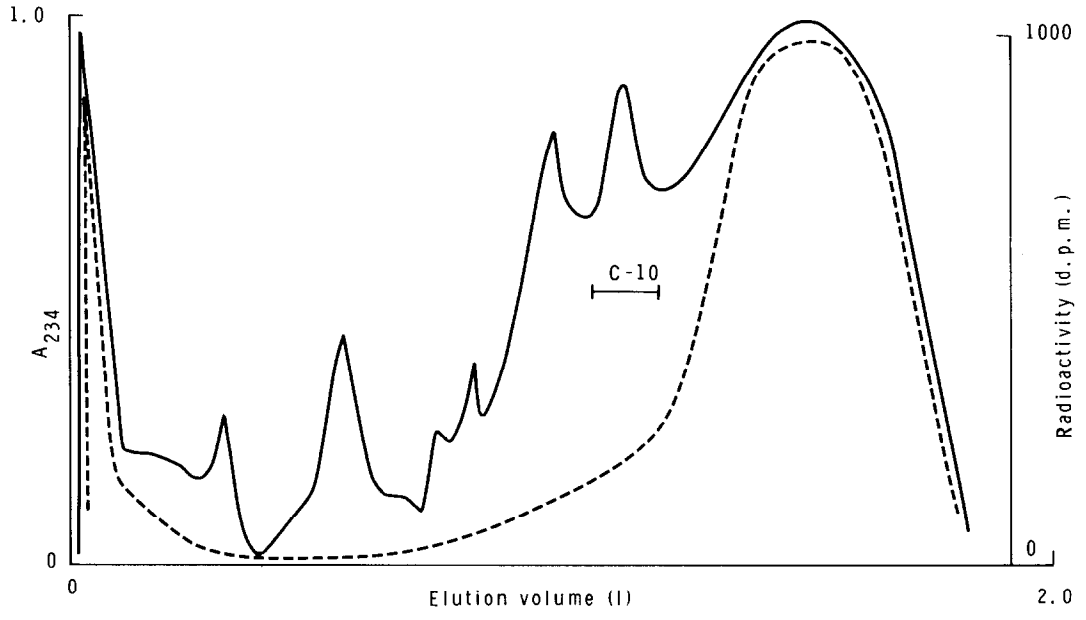

Fig.4. CM-cellulose chromatography of CNBr-cleaved peptides of labelled bovine scleral collagen (400 mg). Elution was as described in the text. (——) A_{234}; (- - -) radioactivity in dpm. The horizontal bar indicates the fractions pooled to give fraction C10.

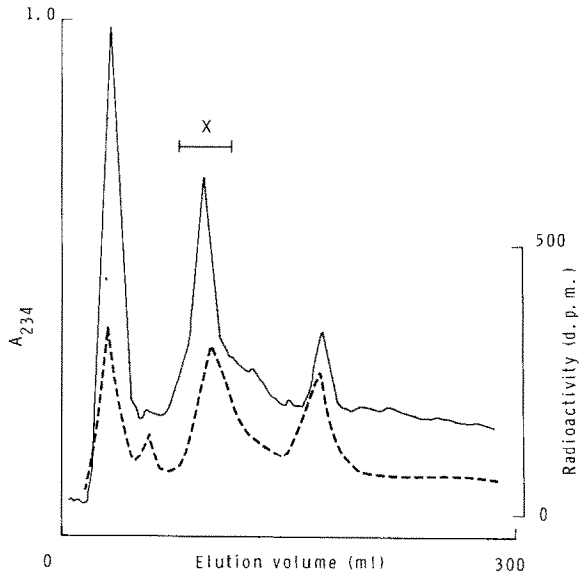

Fig.5. DEAE-cellulose chromatography of fraction C10 from bovine scleral collagen. Fraction C10 (40 mg) dissolved in 2 ml 10 mM Tris–HCl (pH 8.6) was applied to a column (15 × 2 cm) of DEAE-cellulose, at a jacket temperature of 50°C. The column was eluted with a linear gradient from 0–0.2 M NaCl in 2 l 10 mM Tris–HCl (pH 8.6). (——) A_{234}; (- - -) radioactivity in dpm. The short horizontal bar indicates the fractions pooled to give peptide X.

product was chromatographed on Sephadex G-10 and the resulting fractions analysed by SDS–gel electrophoresis. The major peak, which was radioactively labelled, eluted from the column at the same volume as the untreated peptide, and had a mobility on SDS–gels identical to that of fraction X.

4. Discussion

These results indicate that CNBr digests of both corneal and scleral collagen contain a component (X) that is a crosslinked dimer of α1-CB6, the C-terminal peptide of the α1 chain, and represents ⩾50% of the total α1-CB6 in cornea and sclera. The reduced crosslink from both cornea and sclera was stable to a mild periodate treatment and component X from cornea was also stable to heating at 70°C.

Components with mobility similar to X are seen as major bands on many published SDS–gels of CNBr peptides of collagen and although in some digests these bands may be uncleaved peptides it is possible that the crosslink from one α1-CB6 to another may also be present in skin, bone and dentin. Component X was also seen on SDS–gels of digests of unreduced corneal collagen indicating that the unreduced crosslink may be stable.

The absence of tyrosine from the crosslink peptides (tables 1, 2) and from corneal and skin α1-CB6 isolated by conventional procedures [3,8] indicates that the non-helical region has been lost as has been found with other collagens [9–11]. This indicates that the crosslink holding the dimer together goes from the helical region of one chain to the helical region of another chain. The presence of the dimer in cyanogen bromide digests of pepsin-solubilized collagen (J.J.H., unpublished result) seems to confirm this view but we will investigate this point by further breakdown of component X and the precise pinpointing of the crosslink site.

We have now located 2 crosslink sites of corneal collagen, and 3 crosslink sites of scleral collagen to the level of CNBr peptides ([4,5] and present work).

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References

[1] Furthmayr, H. and Timpl, R. (1971) Analyt. Biochem. 41, 510–516.
[2] Scott, P. G. and Veis, A. (1976) Connect. Tiss. Res. 4, 107–116.
[3] Panjwani, N. A. and Harding, J. J. (1978) Biochem. J. 171, 687–695.
[4] Harding, J. J. (1978) FEBS Lett. 87, 251–253.
[5] Crabbe, M. J. C. and Harding, J. J. (1979) FEBS Lett. 97, 189–192.
[6] Robins, S. P. and Bailey, A. J. (1975) Biochem. J. 149, 381–385.
[7] Bailey, A. J., Peach, C. M. and Fowler, L. J. (1970) Biochem. J. 117, 819–831.
[8] Volpin, D. and Veis, A. (1973) Biochemistry 12, 1452–1464.
[9] Kang, A. H. and Gross, J. (1970) Biochemistry 9, 796–804.
[10] Rauterberg, J., Fietzek, P., Rexrodt, F., Becker, U., Stark, M. and Kühn, K. (1972) FEBS Lett. 21, 75–79.
[11] Stoltz, M., Timpl, R. and Kühn, K. (1972) FEBS Lett. 26, 61–65.