THE ISOLATION OF CROSSLINKED PEPTIDES OF COLLAGEN INVOLVING α1-CB6*

Saryu N. Dixit** and Howard B. Bensusan

Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106

Received February 27, 1973

On cooling a solution of the peptides from a CNBr digest of insoluble steer-corium collagen a precipitate was formed which represented about 20% of the total protein. No such precipitate formed when reconstituted soluble collagen was similarly treated. Amino acid analysis of the washed precipitate showed that there was an approximate equivalence between the content of 3-hydroxyproline and homoserine, indicating a mixture of α1-CB6 and other peptides. Three fractions were isolated from the precipitate. One was analyzed to be two α1-CB6 peptides linked to one α1-CB(0,1)', another was one α1-CB6 plus one α1-CB(0,1). The third was a combination of α1-CB6 with a small CNBr peptide containing homoserine.

Collagen is an extended molecule measuring 2900 x 14 A and is composed of three parallel protein chains. In the case of the most common mammalian collagen, two of the chains are the same (α1 chains) and one is distinct (α2 chain). In the fiber the molecules are aligned with the N-terminal end of one molecule overlapping the C-terminus of another by about 9% of its length. The linear aggregates are staggered at quarter intervals to create the 640 A repeat spacing. Considering that the molecules are packed in a three dimensional structure, it can be shown that any area along the molecule comes in contact with three to four different regions on adjacent molecules. Therefore, if intermolecular crosslinks form they are likely to occur between different portions of the collagen molecule. The identification of various regions along the collagen mole-

---

*This research was supported by NIH grants AM-01825 and HD-00669 from the United States Public Health Service.

**Present address: Veterans Administration Hospital, Memphis, Tennessee 38104

cule has been made easier by the separation, identification and ordering of the peptides obtained by the CNBr cleavage of both $\alpha 1$ and $\alpha 2$ chains (1). The isolation and identification of two covalently bound peptides which are not sequential in their order in a chain, constitutes proof of an intermolecular crosslink.

To our knowledge, only two crosslinked peptides have been reported. Miller (2) reported the presence of a crosslink between peptides 4 and 9 of cartilage collagen, a collagen with three identical chains. Analyses indicated that the crosslink is derived from two hydroxylysine residues in either an aldimine or aldol condensation product. Kang (3) found a crosslink between an $\alpha 1$-CB1 and $\alpha 1$-CB6. In both of these cases the crosslink must have been intermolecular since they connected different portions of identical chains.

This paper describes the isolation of three peptides which appear to be involved in intermolecular crosslinks with the $\alpha 1$-CB6 peptide.

MATERIALS AND METHODS

Steer corium collagen, purified according to Veis et al. (4), was cut into fine shavings with a razor blade. The collagen (5 g) was suspended in 100 ml of water and buffered by titrating with 2 M Tris to pH 8.5. The suspension was cooled in an ice bath and 150-200 mg of $NaBH_4$ was added and the mixture was stirred 4 h in the ice bath. The unreacted hydride was destroyed by adjusting the pH to 2.5 with N HCl. The collagen was washed thoroughly with water and dried in vacuo. CNBr cleavage of the collagen was accomplished in a solution of 5 g of CNBr in 500 ml of 70% formic acid at 37° for 8 h (5,6). The mixture was then diluted with an equal volume of water, taken to dryness in vacuo and then twice redissolved in 500 ml of water and dried in vacuo.

The collagen peptides were dissolved in 600 ml of cold water, centrifuged at 90,000 x g for 30 minutes, diluted to 1,000 ml, adjusted to pH 8.1 with 2 M Tris and stored at 4° overnight. The turbid mixture centri-

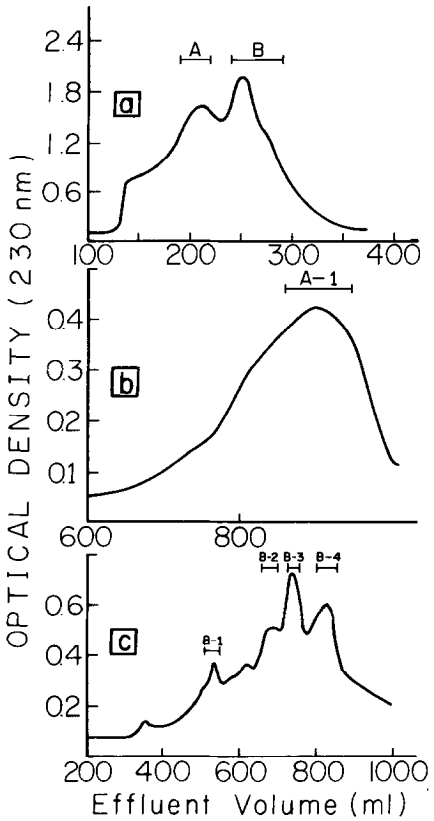

Figure 1. Fractionation of precipitated CNBr peptides. (a) The elution diagram of the dissolved precipitate chromatographed on a 1.9 x 140 cm column of BioGel A 1.5 at room temperature. Molar $CaCl_2$ in 0.05 M Tris buffer, pH 7.5, was used to dissolve the precipitate, equilibrate the column and to elute the fractions. Elution was carried out at a flow rate of 15 ml/hr. (b) Column chromatography of fraction A on a 2.5 x 10 cm column of CM-cellulose, maintained at 42°. The elution was carried out with a linear gradinet 0-0.14 M NaCl in 0.01 M acetate buffer, pH 3.8. (c) The elution diagram of fraction B. The conditions were the same as for (b). In all cases the collections pooled are indicated.

---

fuged at 4° for 60 minutes at 90,000 x g. The pellet was dissolved in 600 ml of water at room temperature, the pH was adjusted to pH 8.1 with 2 M Tris and the solution was stored overnight at 4°. The mixture was

centrifuged and the precipitate was dissolved and reprecipitated as before.

The initial fractionation of the precipitated material dissolved in M $CaCl_2$ in 0.05 M Tris buffer, pH 7.5, was performed as indicated in Fig. 1a. Collections were made for 10-minute intervals. The elution pattern is given in Fig. 1a. Fractions A and B were collected, dried in vacuo, dissolved in 2-4 ml of water and dialyzed against water.

Fractions A and B (Fig. 1a) were subjected to CM-cellulose chromatography using the technique of Piez et al. (7). A single fraction was collected from the eluate of fraction A (Fig. 1b) whereas four fractions were collected from the eluate of fraction B (Fig. 1c). The volumes indicated were collected, dried, dissolved in a small amount of water and dialyzed against water.

Gel electrophoresis was performed on the fractions A-1, B-1, B-2, B-3 and B-4 in the presence of sodium dodecylsulfate (SDS) as described by Furthmayr and Timpl (8). CNBr peptides from isolated α1 chains, and α1 chains isolated according to Bornstein and Piez (5) were used as standards. Since each fraction was found to contain one major component by SDS electrophoresis, this technique was used on a larger scale for the final purification of the major components. The bands were located by short-term staining, the area containing the major component was removed by cutting the gel and was hydrolyzed in 6 N HCl at 110° for 18 h under nitrogen. Amino acid analyses were performed on a BioCal 200 instrument. Control studies on α1 chains subjected to SDS gel electrophoresis showed that there was no abnormal destruction of any amino acid, except methionine, by this treatment.

RESULTS AND DISCUSSION

It is very important that we establish the fact that the precipitate obtained by cooling the CNBr digest of insoluble collagen is a phenomenon associated with the insolubility of the corium collagen. A similar precipitate was not obtained from reconstituted fibers of salt-soluble or acid-

soluble collagen; the solutions remained perfectly clear on cooling. On the other hand, the yield of precipitate from insoluble collagen represented 19-23% of the original material. Further, the amino acid composition of the total precipitate contained 29-32% glycine and about 9.5% hydroxyproline, thereby indicating that we are dealing with portions of collagen rather than impurities.

As an example of our calculations we can use the data obtained for peptide A-1. SDS-gel electrophoresis indicated that this fraction contained an average of 465 residues (Fig. 2). The amino acid analysis data was calculated to give the number of moles of each amino acid/465 moles total. It was found that there were about two moles of 3-hydroxyproline and one mole of homoserine. Since there is only one residue of 3-hydroxyproline per α1 chain, located in the C-terminal peptide α1-CB6, it is apparent

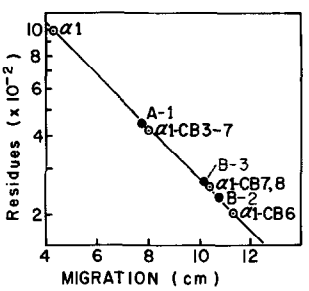

Figure 2. The SDS-gel electrophoretic migrations of fractions A-1, B-2 and B-3 compared with those of α1 chains and the CNBr peptides from α1 chains.

---

that two α1-CB6 peptides are in this fraction. In addition, α1-CB6 has 200 residues, making a 400-residue contribution to fraction A-1. As a C-terminal peptide, α1-CB6 contains no homoserine, indicating that a small peptide must be contributing the single residue of homoserine. Since Volpin and Veis report no tyrosine in α1-CB6 (6), the extra peptide could be the N-terminal 14-residue peptide α1-CB(0,1)', which contains tyrosine. A total number of residues was assumed to be approximately 420 for A-1 in keeping with our deduction that it is composed of two chains of α1-CB6

and one chain of α1CB(0,1). The amino acid composition was then adjusted accordingly. The results are presented in Table I together with the values calculated from the data of Volpin and Veis. The fit appears reasonable.

Rauterberg and Kuhn have also published data for the CNBr peptides of the α1 chain of bovine skin collagen (9). We have used the data of Volpin and Veis since the collagen used in these studies was a generous gift from their laboratory and since Rauterburg and Kuhn did not include data for the peptides of the α2 chain.

In a similar way, we have identified fraction B-3 as one α1-CB6 and one α1-CB(0,1) as shown in Table I. This is the same combination of peptides that Kang (3) demonstrated to be crosslinked by dehydrolysinonorleucine. The assignment of a combination of CNBr peptides for fraction B-2 was not as well defined. As indicated in Table I, this fraction could be made up of α1-CB6 linked to α1-CB4 but not to α1-CB(0,1) as indicated by the small amount of tyrosine. As indicated in the footnote of Table I, the smaller peptide could also be α1-CB2, α1-CB5 or α2-CB2. Fractions B-1 and B-4 were mixtures and could not be sufficiently resolved for analysis.

Of particular interest is the fact that the total precipitate contained a somewhat greater content of 3-hydroxyproline than homoserine. Evidently the precipitate is composed of several fractions, most of which are peptides of α1-CB6 crosslinked with peptides from other areas of the molecule. In addition, these crosslinks must be intermolecular since the homoserine-containing peptides must come from regions other than the C-terminal region occupied by α1-CB6. These results are consistent with the findings of Volpin and Veis that there was a marked decrease in the amount of α1-CB6 in the chromatograms of the CNBr peptides from insoluble bovine collagen (10).

Reduction of the precipitated fraction with NaB^3H_4, followed by hydrolysis and chromatography failed to demonstrate any reducible crosslinks.

Table I. The Comparison of the Amino Acid Composition of Peptides of A-1, B-2 and B-3 with Combinations of Known Peptides

Residue	A-1	2 x α1-CB6 + 1 x α1-CB(0,1)	B-3	α1-CB6 + α1-CB(0,1)'	B-2	α1-CB4[a] + α1-CB6
3-Hydroxyproline	2.2	1.4	0.7	0.7	1.2	0.7
4-Hydroxyproline	38	34	19	17	26	22
Aspartic Acid	20	19	9.7	9.6	10	11
Threonine	8.1	8.2	4.8	4.5	4.8	4.5
Serine	15	17	7.5	9.0	12	7.2
Homoserine	0.8	1.0	0.6	1.0	1.0	1.0
Proline	56	62	32	32	36	36
Glutamic Acid	30	28	14	14	19	16
Glycine	133	139	76	71	68	84
Alanine	45	44	26	22	30	25
Valine	11	7.6	3.7	4.2	4.0	3.2
Isoleucine	6.5	5.1	2.4	3.0	2.2	2.1
Leucine	12	9.3	4.1	4.1	3.7	6.0
Tyrosine	1.4	1.4	0.8	0.9	0.6	(0.2)
Phenylalanine	5.8	4.0	2.4	2.0	3.1	2.0
Hydroxylysine	3.0	2.6	0.7	1.3	1.2	1.3
Lysine	9.2	12	5.4	6.3	7.7	7.4
Histidine	2.2	1.6	0.6	0.8	0.4	0.8
Arginine	21	22	9.1	11	11	14
Total calc'd	420	419	220	215	240	246
Total found	465	---	230	---	260	---

[a]Although α1-CB4 is used in this calculation, α1-CB2, α1-CB5 or α2-CB2 would give total values which agree with those of B-3 about as well.

We suggest that the crosslinks are already reduced *in vivo*. It is interesting to note that there are 3 less lysyl residues in fraction A-1 than is found in the sum of the proposed three constituent peptides, while the hydroxylysine content is about the same. These data would be consistent with a crosslink made up of three lysyl residues such as merodesmosine. Periodate treatment of A-1 did not result in an alteration in its migration in SDS-gel electrophoresis. This would be expected if a crosslink such as merodesmosine were present. Although there is not direct evidence as to which crosslinks are involved, the data for the total residue weight and the amino acid content of the fractions combine to show that fractions A-1, B-2 and B-3 must contain crosslinked peptides rather than being the result of the fortuitous summation of free CNBr peptides.

REFERENCES

1. Traub, W., and Piez, K.A., Adv. Protein Chem. 25, 243 (1971).
2. Miller, E.J., Biochem. Biophys. Res. Commun. 45, 444 (1971).
3. Kang, A.J., Biochemistry 11, 1828 (1972).
4. Veis, A., Anesey, J., and Cohen, J., J. Am. Leather Chemists' Assoc. 55, 548 (1960).
5. Bornstein, P., and Piez, K.A., Biochemistry 5, 340 (1966).
6. Volpin, D., and Veis, A., Biochemistry 10, 1751 (1971).
7. Piez, K.A., Eigner, E.A., and Lewis, M.A., Biochemistry 2, 58 (1963).
8. Furthmayr, J., and Timpl, R., Anal. Biochem. 41, 510 (1971).
9. Rauterberg, J., and Kuhn, K., Eur. J. Biochem. 19, 398 (1971).
10. Volpin, D., and Veis, A., Biochem. Biophys. Res. Commun. 44, 804 (1971).