CHARACTERIZATION OF BASEMENT MEMBRANE COLLAGEN OF BOVINE ANTERIOR LENS CAPSULE VIA SEGMENT-LONG-SPACING CRYSTALLITES AND THE SPECIFIC CLEAVAGE OF THE COLLAGEN BY PEPSIN

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1. Introduction

Basement membranes (BM) contain a class or classes of collagen distinct from the Type I, II and III interstitial collagens. The differences can be grouped under two general categories: first, the peptide chain sequences of the collagen, and their heightened content of 3-hydroxyproline and glycosylated hydroxylysine; and second, the presence of covalently attached non-collagenous protein moieties [1,2]. The molecular weights of individual α-chains of BM collagen are greater, even after pepsin digestion, than the α-chains of Type I collagen [2,3], although the presence of a second species of BM collagen with a mol. wt 55 000 has been proposed [3].

The essential question to us is structural. BM collagens exist in the form of non-filamentous sheets rather than in striated D-periodic fibrils as in the interstitial collagens. Which features of the BM collagen prevent the formation of periodic fibrils, the non-collagenous moieties, or the distribution of interactive groups in the collagen helical region? Electron microscopy of the interstitial collagens has provided many insights into their molecular structure and intermolecular interactions, but little data of this nature has been available [4,5] relative to collagens of BM origin.

We have succeeded in producing segment-long-spacing precipitates (SLS) from bovine anterior lens capsule BM collagen [6]. This technique enables us to examine BM collagen at several stages of enzymic pretreatment and compare the band pattern of the lens capsule collagen SLS directly with the pattern of Type I interstitial collagen. In addition, we find that BM collagen is susceptible to specific cleavage by pepsin, giving rise to the appearance of pepsin resistant half-molecules.

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2. Materials and methods

2.1. *Preparation of BM collagen*

Anterior lens capsules were dissected from 200 fresh bovine eyes and placed in 0.15 M sodium chloride. The dissected capsules were sonicated briefly (<1 min) and then collected by low speed centrifugation. The capsules were suspended in 50 ml 0.075 M sodium citrate, pH 3.7, containing 0.001 M phenylmethylsulfonyl fluoride (PMSF). After stirring for 48 h at 10°C the residual capsular material was again collected by low speed centrifugation.

The supernatant of the centrifugation was made 4.0 M in sodium chloride. The precipitate which formed was collected and redissolved in the pH 3.7 buffer, reprecipitated once again with sodium chloride, and finally dissolved in 0.5 N acetic acid. This solution was desalted by dialysis and lyophilized.

The residual capsular material was suspended in 50 ml 0.5 M acetic acid, pH 2.5 at 4°C and, after dialysis against more 0.5 M acetic acid to remove the PMSF, 0.1 mg pepsin (Worthington) was added. After 24 h the supernatant was decanted and dialyzed against 0.9 M sodium chloride [7]. The precipitate was discarded and the remaining solution taken as the pepsin solubilized BM collagen fraction (P-I).

2.2. *Second pepsin digestion*

P-I was reduced and alkylated following the procedure in [5], but in the absence of urea. The reduced and alkylated collagen was then digested a second time with pepsin (1:50, pepsin:collagen) at pH 2.5 for 16 h at 10°C, following the general scheme in [7]. The collagen produced in this treatment is designated P-II.

2.3. *Collagen digestion*

To determine the collagenous character of certain fractions and components, samples were digested with bacterial collagenase (Worthington) purified further by the method in [8]. Digestion was carried out for 4 h at pH 7.5 in Tris buffer containing *N*-ethyl-maleimide to inhibit non-specific proteolysis.

2.4. *Electrophoresis and amino acid analysis*

Polyacrylamide gel electrophoresis in sodium-dodecylsulfate followed the procedure in [9]. Buffer solutions were used both with and without mercaptoethanol.

Samples for amino acid analysis were hydrolyzed at 105°C for 22 h in 6 N HCl in sealed, nitrogen-flushed tubes. Analyses were carried out in a JEOL 6AH analyzer with a single column program.

2.5. *Electron microscopy and SLS formation*

Collagen solutions, at 0.1 mg/ml in 0.5% acetic acid, were dialyzed against 0.4% adenosine triphosphoric acid (ATP) (Aldrich) in the cold, from 24–48 h. Precipitates did not develop rapidly, as with Type I collagen, but appeared only after many hours. Droplets of precipitate suspension were placed on carbon/formvar coated grids and negatively stained with 2% phosphotungstic acid containing 100 μg bacitracin, adjusted to pH 7.5 with NaOH. Grids were viewed on an Hitachi HU-11A microscope.

3. Results

Little protein was recovered from the initial citrate buffer extraction of the lens capsules. Although a collagen was isolated from a similar preparation [5], amino acid analyses indicated that very little of the citrate extracted protein was collagenous. However, fraction P-I was collagenous. Its amino acid composition, including 44 residues hydroxylysine, 10.7 residues 3-hydroxyproline, 134 residues 4-hydroxyproline and 296 residues glycine per 1000 amino acids, was virtually identical with that of a standard BM collagen preparation from bovine lens capsule basement membrane, generously supplied by Dr N. A. Kefalides. All further work began with the P-I preparation.

Acrylamide gel electrophoresis, in SDS showed P-I contains a mixture of γ-like high molecular weight

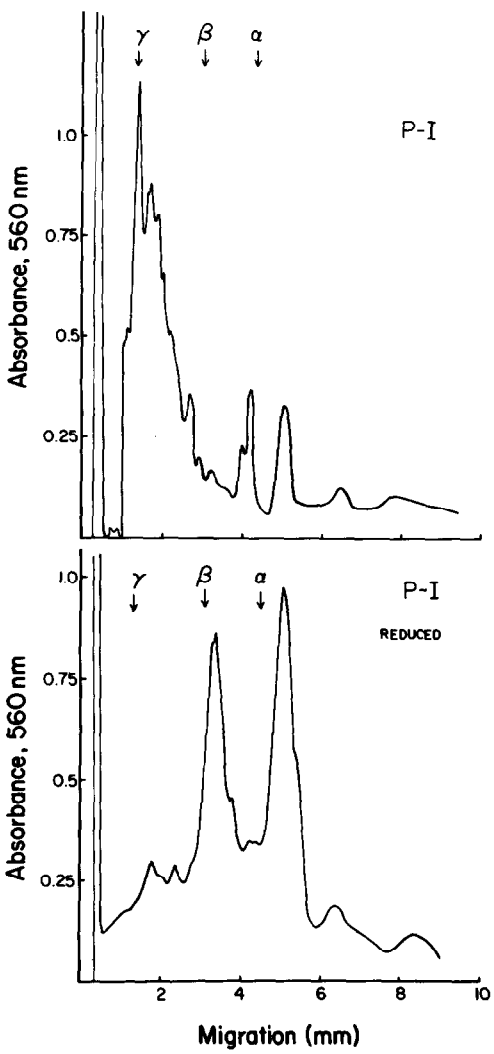

Fig.1a. Polyacrylamide gel electrophoresis in SDS of the collagenous material derived from the first pepsin digest (P-I) of bovine anterior lens capsule. Fig.1b. P-I, with 1% mercaptoethanol in the running buffer.

components, some material with weights between α(I) and β(I) chain weights, and a small amount of lower molecular weight material, fig.1a. Electrophoresis carried out in the presence of 1% mercaptoethanol, fig.1b, showed that most of the γ components were converted to a single chain type migrating between α1(I) and β_{12}(I) positions at app. mol. wt 160 000 and three lower molecular weight fractions with app. mol. wt 115 000, 85 000 and 50 000. All of the major bands are removed upon digestion with bacterial collagenase. Hence these components are BM collagen chains or chain fragments.

SLS precipitates develop from P-I slowly and with difficulty, however, copious amounts of loosely-ordered SLS can eventually be seen, fig.2a. In some isolated SLS spools the band pattern is occasionally very distinct, fig.2b. From the asymmetry of the banding it is evident that each P-I molecule is also asymmetric and that each is aligned and pointing in the same direction within the SLS spool. The BM

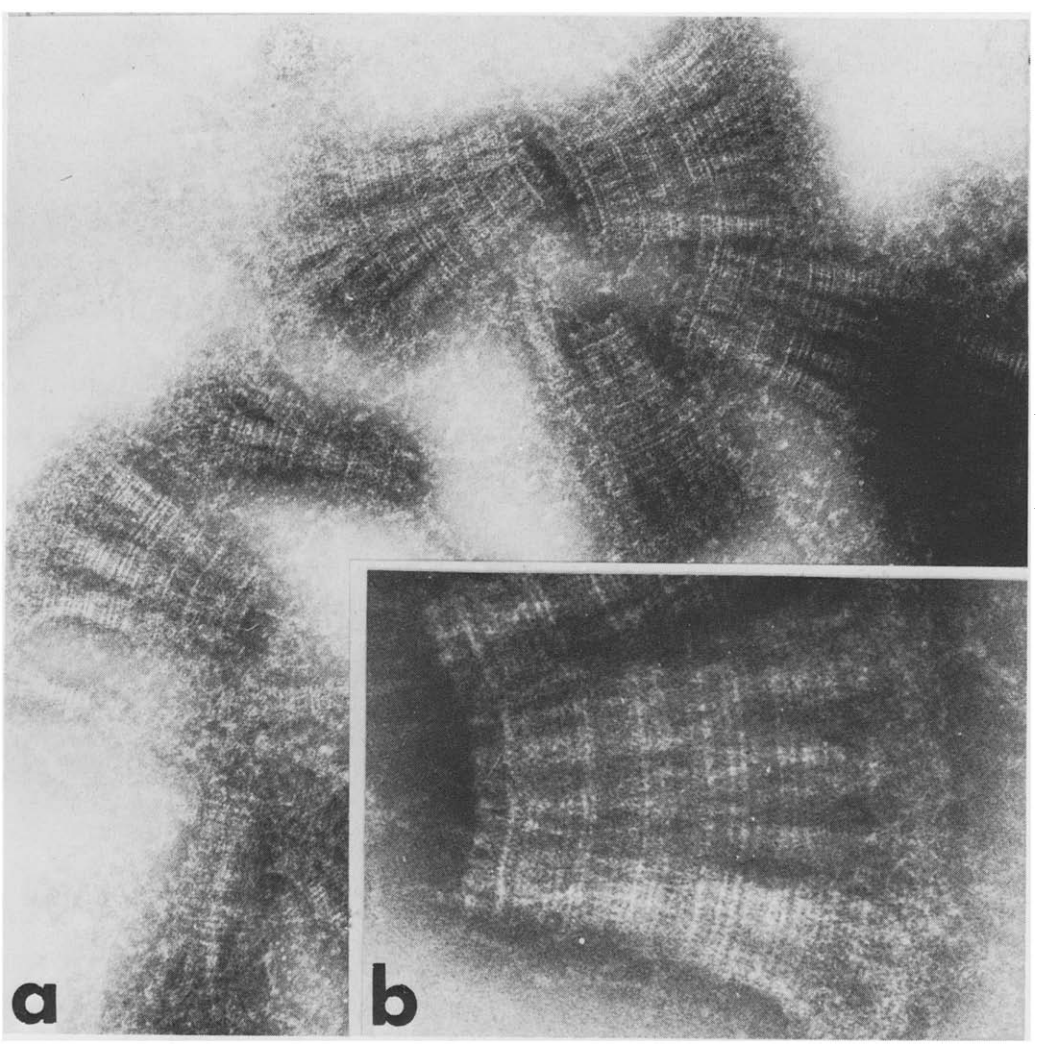

Fig.2a. SLS crystallites of basement membrane collagen after a single pepsin digestion (P-I) × 100 000, 2% PTA, pH 7. Fig.2b. Enlargement of a SLS spool from a similar preparation. Note the different character to each end of the crystallite and the asymmetric banding, × 200 000, 2% PTA, pH 7.

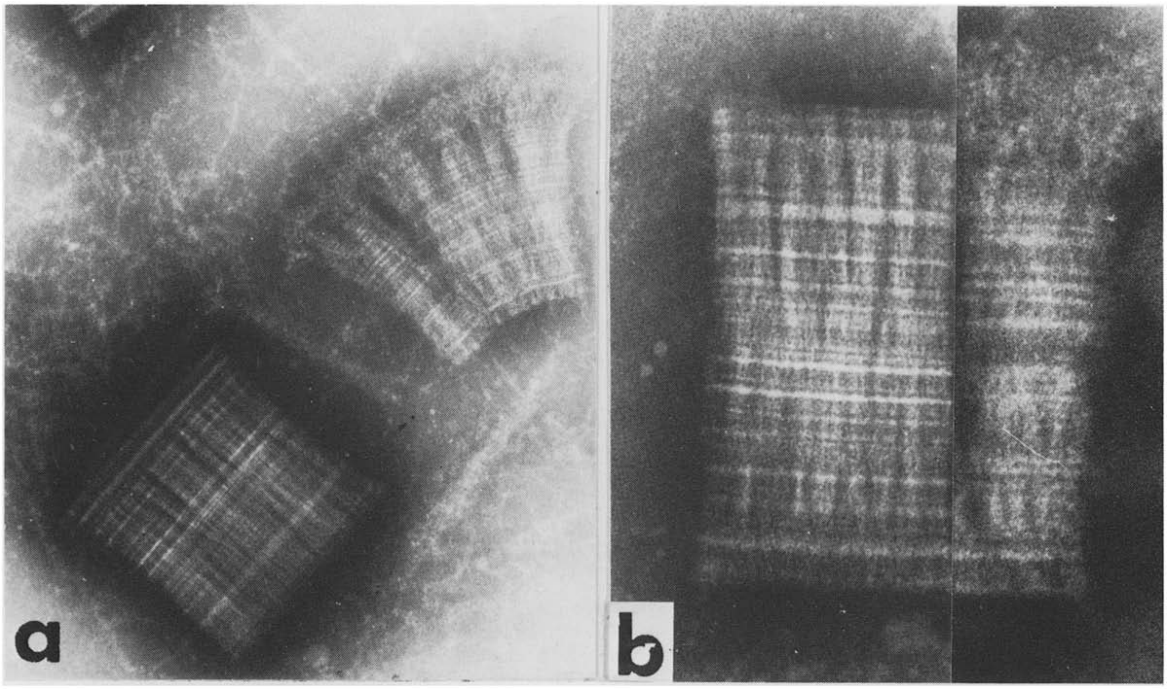

Fig.3a. P-I mixed with salt extracted type I collagen and dialysed against ATP. Upper right is P-I (BM) SLS, lower left is type I SLS, × 100 000, 2% PTA, pH 7. Fig.3b. Comparison of SLS, type I (left) and P-I, basement membrane collagen (right), in the relative orientation of ends which provide the most number of matched bands. The N-terminus of type I is at the bottom of the figure, × 200 000, 2% PTA, pH 7.

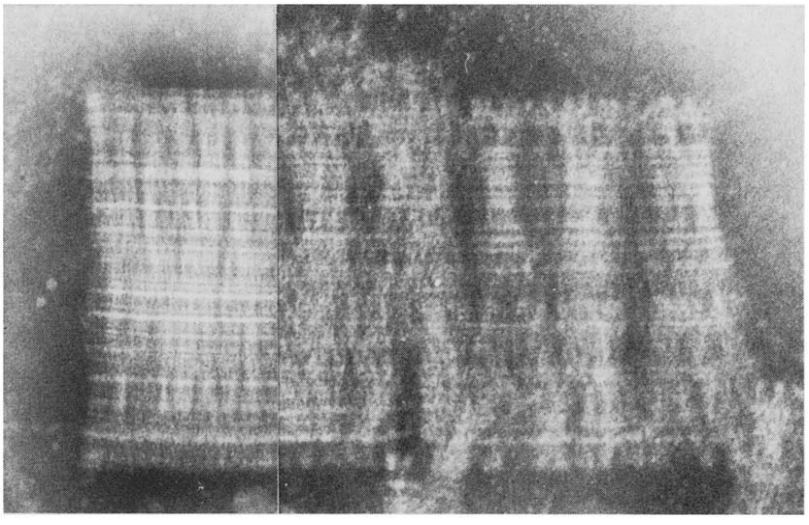

Fig.4. Composite of type I (left), P-I (middle) and P-II, resulting from a second pepsin digestion of P-I after reduction and alkylation (right), × 170 000, 2% PTA, pH 7.

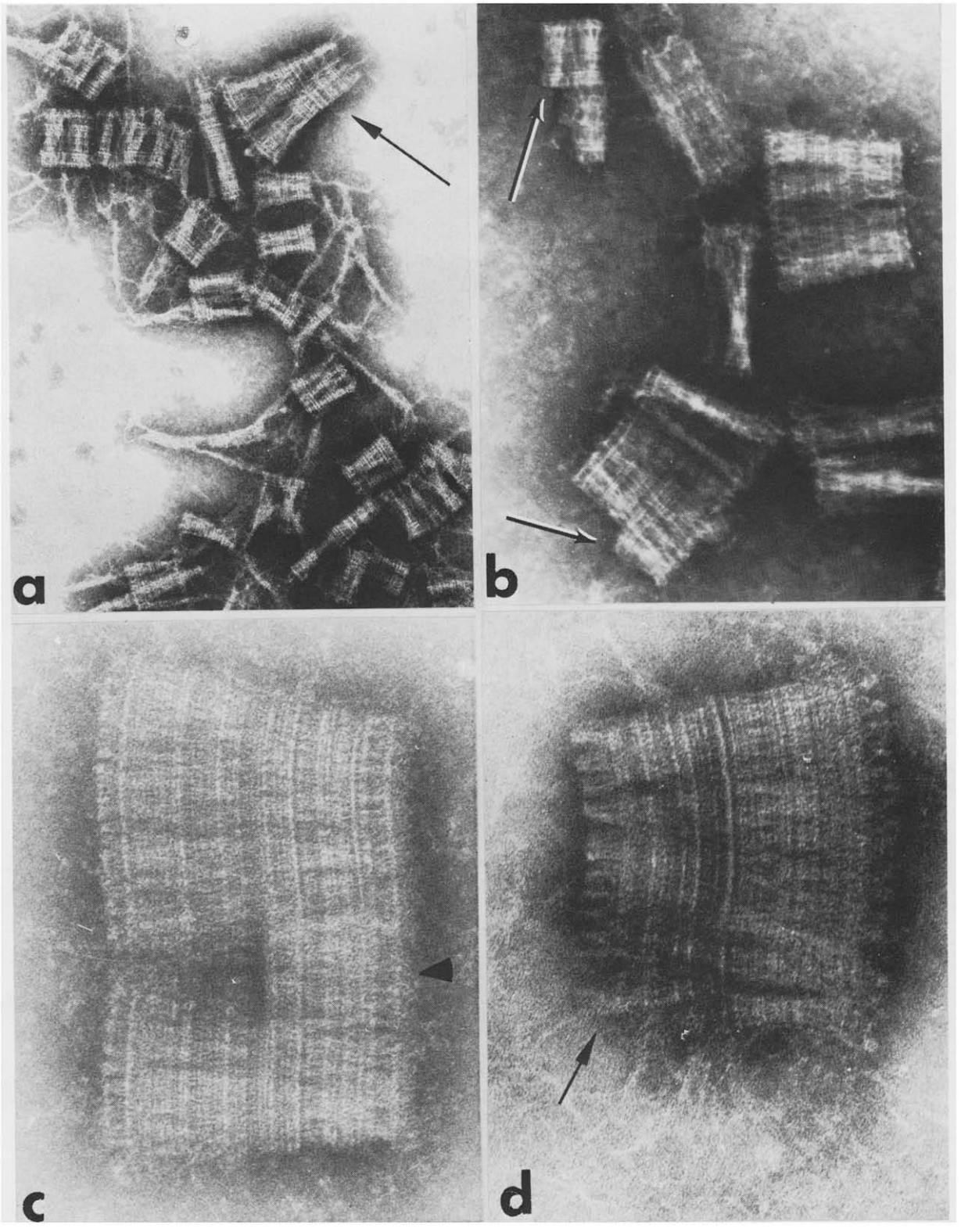

Fig.5

SLS stop abruptly in register at one end but characteristically have a bush-like appendage at the other end. The appendages appear to inhibit close packing of molecules within the spools.

The band pattern of the BM collagen was compared with Type I SLS banding in a preparation in which P-I and Type I SLS were mixed and deposited together on the same grid. All aspects of subsequent preparation for viewing, staining, drying, magnification and so on are thus identical. It is evident, fig.3a, that the two types of collagen have distinctly different staining patterns. A direct comparison, fig.3b, with the sharply defined BM SLS segment, end matched with the amino terminal end of the Type I SLS, shows that while some striations appear to match, the central regions of the two collagens, in particular, are very different. The alternate orientation of Type I provides a still greater mismatch of band pattern. The bush-like appendage makes the P-I SLS about 40 nm longer than Type I SLS.

Reduction and alkylation of P-I, followed by a second pepsin digestion to produce P-II, brings about two modifications in the BM collagen. First, the appendage is removed and both ends of the SLS become sharply defined. As shown in the composite set of micrographs of fig.4, the clearly banded P-II SLS is almost the same length as the Type I SLS.

A second, and most striking observation, fig.5a, is that in addition to the ∿300 nm full length BM SLS, there are large numbers of partial segments, with lengths close to 135 nm or 45% the length of the intact P-II SLS. Almost all of the partial segments have the same band pattern. Moreover, where the partial segments coprecipitate alongside full segments, figures 5b and 5c, it is evident that the partial segments correspond to the end of the BM molecule which originally had the bush-like extensions. Although one may occasionally find what looks like an isolated half-segment corresponding to the 'NH_2-terminal' end of the BM collagen, fig.5d, more than 95% of the partial segments are from the bush-like end region and are of uniform length. In the original P-I preparation both the extra-helical depleted and the 45% SLS could be found but to a much lesser extent than after the second pepsin digest. Occasionally SLS ∿85% of the P-II were also seen in P-I preparations, indicating cleavage in the helical region and possibly corresponding to the 85 000 mol. wt peak seen in the SDS gels.

4. Discussion

Bovine anterior lens capsule basement membrane collagen released in acid-soluble form by limited pepsin digestion contains collagenous and non-collagenous parts. The non-collagenous region can be removed from the collagenous part by a combination of disulfide bond rearrangement, accomplished by reduction and alkylation, followed by a second pepsin digestion. SLS precipitates of this collagen, P-II, fig.4, show that the triple helical region has a length virtually identical to that of intact Type I collagen although the pattern of cross-striation of the SLS, and hence the sequence in the helical region, is distinctly different from that of Type I.

The non-collagenous region, seen in SLS precipitates of P-I collagen, that is before cleavage of the disulfide bonds and after only a single pepsin treatment, is located at one end of the SLS spools, fig.2 and fig.3. The presence of the pepsin-sensitive extension region interferes with the close packing of molecules and obscures the band pattern at its end of the SLS spool. This is similar to the effect of the non-helical extensions in Type I procollagen SLS [10,11]. At this point we cannot say whether the end-extension region is a continuation of the main peptide chains, as in Type I procollagen, or a separate disulfide-linked protein as sometimes suggested [1]. Removal of the appendage appears to be facilitated by reduction of disulfide bonds but extensive pepsin treatment alone can accomplish the same result. Since pepsin was used in the initial stage of extraction we

Fig.5a. P-II, demonstrating full-length SLS (arrow) and numerous 45% length, fragment SLS, × 69 300. Fig.5b. Fragment SLS (arrows) along side full size SLS, × 69 300. Fig.5c. A partial SLS (arrowhead) placed between 2 full-length SLS. Along with a and b demonstrating that most fragment SLS are derived from one end of the molecule, tentatively identified as the C-terminal half. × 180 000. Fig.5d. A rare N-terminal half SLS (arrow) that was likely derived from a single pepsin cleavage at the 45–55% point along the collagen molecule, × 180 000. All preparations stained with 2% PTA, pH 7.

cannot rule out either the possibilities that, in vivo, the appendage is larger than demonstrated here, or that there may be an even more labile pepsin-sensitive region at the other end of the BM-collagen molecule prior to extraction.

The appearance of a BM collagen component with mol. wt 55 000 in pepsin digested collagen from human aortic intima has indicated the presence of BM collagen in relatively short sequences joined by non-collagenous protein [3]. Our data in the lens capsule system offer at least one alternative explanation.

The collagen solubilized from the lens capsule with one pepsin treatment (P-I) is essentially all in intact α-chain length form. The SLS are predominantly in ∿300 nm lengths with an asymmetric band pattern throughout, fig.2 and fig.3. Upon further pepsin treatment, and after reduction and alkylation, P-I is converted to clean, full length, ∿300 nm asymetrically banded collagen molecules (P-II) plus many ∿135 nm segments which in almost all cases exactly match only one end of the intact molecules in band pattern, fig.4 and fig.5. This sequence of events is consistent with the presence of a pepsin-sensitive region or regions within the triple-helical sequence of the lens capsule BM collagen, similar to the trypsin-sensitive region found in Type III collagen [12] and in dentin collagen [13].

The number of pepsin-sensitive sites in BM collagen is difficult to assess at this time. The remaining 135 nm length segments, corresponding to that end which binds the non-collagenous protein, contains no additional pepsin-sensitive sites. The region which is cleaved and reduced to dialyzable peptides may be a molecular region of low stability after opening of a single pepsin-sensitive triple-helical region or may contain several such regions. Half-segments corresponding to the pepsin-labile helical region have been observed, although very rarely. This might indicate that one site of pepsin susceptibility is at the 55–45% point along the molecule. The presence of a few especially pepsin-sensitive regions in the collagen helical region is supported by the SDS electrophoretic pattern of reduced P-I in which collagenase susceptible components of 115 000, 85 000 and 50 000 are seen. At the same time, the lack of other collagenase sensitive fragments is consistent with the instability of the partly-cleaved molecules.

Acknowledgements

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References

[1] Kefalides, N. A. (1973) Int. Rev. Conn. Tiss. Res. 6, 63–104.
[2] Howard, B. V., Macarak, E. J., Ganoon, D. and Kefalides, N. A. (1976) Proc. Natl. Acad. Sci. USA 73, 2361–2364.
[3] Chung, E., Rhodes, R. K. and Miller, E. J. (1976) Biochem. Biophys. Res. Commun. 71, 1167–1174.
[4] Kefalides, N. A. (1968) Biochemistry 7, 3103–3112.
[5] Olsen, B. R., Alper, R. and Kefalides, N. A. (1973) Eur. J. Biochem. 38, 220–228.
[6] Veis, A. and Schwartz, D. (1977) 1st. Int. Symp. Biol. Chem. Basement Membranes, Philadelphia, 1976, in press.
[7] Dehm, P. and Kefalides, N. A. (1977) 1st. Int. Symp. Biol. Chem. Basement Membranes, Philadelphia, 1976, in press.
[8] Peterkofsky, B. and Diegelmann, R. (1971) Biochemistry 10, 988–994.
[9] Furthmayr, H. and Timpl, R. (1971) Anal. Biochem. 41, 510–516.
[10] Goldberg, B. (1974) Cell 1, 185–192.
[11] Hoffman, H., Olsen, B., Chen, H. and Prockop, D. (1976) Proc. Natl. Acad. Sci. USA 73, 4304.
[12] Miller, E. J., Finch, J. E., jr, Chung, E., Butler, W. T. and Robertson, P. B. (1976) Arch. Biochem. Biophys. 173, 631–637.
[13] Scott, P. G. and Leaver, A. G. (1974) Conn. Tiss. Res. 2, 299–307.
[14] Kefalides, N. A., Comeron, J. D., Tomichek, E. A. and Yanoff, M. (1976) J. Biol. Chem. 251, 730–733.
[15] Grant, M. E., Kefalides, N. A. and Prockop, D. J. (1972) J. Biol. Chem. 247, 3539–3544.