TRIPLE HELIX FORMATION AND DISULPHIDE BONDING DURING THE BIOSYNTHESIS OF GLOMERULAR BASEMENT MEMBRANE COLLAGEN

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Summary: The ^{14}C-collagen synthesised by isolated rat glomeruli during a 2h labelling period with [^{14}C]proline was found to be digested by chymotrypsin at 25°C suggesting that it was in a random coil form. After a 4h chase period, the ^{14}C-polypeptides were resistant to proteolysis indicating that they were in a triple helical conformation. Gel filtration in sodium dodecylsulphate under reducing and non-reducing conditions suggested that the extent of resistance to digestion by chymotrypsin was closely related to the extent of inter-chain disulphide bond formation. The ^{14}C-collagen was found to be composed of three identical polypeptide chains which were linked by intra-molecular disulphide bonds within the chymotrypsin-resistant triple-helical region of the molecule.

Introduction:

Recent investigations suggest that basement membranes contain a collagenous component (designated type IV collagen) which is distinct from the three types of fibrillar collagen found in vertebrate tissues (1). Studies conducted *in vitro* with embryonic chick lens cells (2-4) and isolated rat glomeruli (5) have demonstrated that the basement membrane collagen is synthesised in a precursor form possessing non-collagenous peptide extensions which may be excised after secretion of the triple-helical molecule. Observations on cell systems synthesising type I and II pro-collagens have similarly established the presence of non-collagenous extensions in these molecules and their role in the folding of the precursor polypeptides into triple-helical procollagen molecules has been demonstrated in several studies (for reviews see refs 6,7). An important feature of the mechanism of assembly of these triple-stranded precursors appears to be the formation of inter-chain disulphide bonds, the timing of

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which is closely related to triple helix formation in embryonic chick tendon, cartilage and lens cells (7).

The results described here provide further data on the relationship between disulphide bonding and triple helix formation during the biosynthesis of glomerular basement membrane (GBM) collagen. Evidence is also presented that this collagen comprises only one type of polypeptide and contains inter-chain disulphide bonds within the chymotrypsin-resistant triple-helical portion of the molecule.

Materials and Methods: Glomeruli isolated from rats (150 ± 10g) by a combination of sieving and Ficoll-gradient techniques (5) were incubated with [^{14}C]proline in Eagle's medium, continually gassed with 5% CO_2/95% air. In general approx. 3 to 4 x 10^5 glomeruli were incubated in 5 ml medium with 50 - 70 μCi [^{14}C]proline for 2h at 37°C and then the hydroxylation of proline and protein synthesis were inhibited by the addition of α,α'-bipyridyl and cycloheximide to final concentrations of 1 μM and 100 μg/ml respectively. In pulse-chase experiments, the glomeruli were collected by centrifugation after labelling for 2h with [^{14}C]proline and then resuspended in medium containing 100 μg/ml [^{12}C]proline and the incubation continued for a further 4h at 37°C.

The molecular size of the collagenous [^{14}C]polypeptides synthesised by the isolated glomeruli and an analysis of the role of inter-chain disulphide bonds in their subsequent aggregation were investigated by gel filtration on sodium dodecylsulphate (SDS)-agarose before and after reduction with mercaptoethanol. The samples for analysis were treated as described previously (5) except that the buffer used was 0.02M-Tris-HCl (pH 7.4) and in experiments where the reducing agent was omitted the samples were made 0.1M in iodoacetamide and applied to the gel filtration column which was eluted with 0.1% SDS in 0.02M Tris-HCl buffer (pH 7.4). Fractions (2.0 ml) were collected and total ^{14}C was assayed by counting an aliquot in a Triton/HCl-containing scintillation fluid (8). Hydroxy-[^{14}C]proline in the fractions was assayed by the method of Juva & Prockop (9).

Whether or not the GBM collagenous [^{14}C]polypeptides were in a triple helical conformation was determined by their susceptibility to limited proteolysis. After incubation with [^{14}C]proline for appropriate time periods the glomeruli were collected by centrifugation, cooled to 4°C,

homogenised in 2 ml 0.4M NaCl in 0.1M Tris-HCl buffer, pH 7.4, and incubated with 300 μg/ml α-chymotrypsin (Sigma Chemical Co. Ltd., London, U.K.) at 25°C for 6h. The reaction was stopped by the addition of SDS to 1% (w/v) and the products of enzymic digestion analysed by SDS-agarose chromatography.

Results and Discussion: In our earlier studies on the molecular size of the collagenous polypeptides synthesised by isolated rat glomeruli incubated with [^{14}C]proline for 2h, it was observed that the hydroxy[^{14}C]-proline-containing polypeptides eluted from an SDS-agarose column ahead of chick tendon pro-α chains when chromatography was carried out under reducing conditions (5). Previous analyses had suggested that the molecular weights of tendon pro-α chains were in the range 120 - 125,000 daltons (10) and it was concluded that the collagenous polypeptides initially synthesised by the glomeruli were approx. 140,000 daltons (5). However, more detailed analyses of the procollagen secreted by matrix-free tendon cells indicate that individual pro-α chains have molecular weights in the range 137 - 145,000 daltons (11) therefore indicating that the basement membrane collagen precursor polypeptides are probably somewhat larger than originally proposed (3,5). In addition, it must now be concluded that the time-dependent conversion of precursor molecules to a size co-eluting with tendon pro-α chains must yield collagenous polypeptides with molecular weights of approx. 140,000 daltons.

When the ^{14}C-proteins synthesised by glomeruli labelled for 2h with [^{14}C]proline were subjected to limited proteolysis at 25°C, all the hydroxy[^{14}C]proline-containing peptides eluted as small molecular weight species (Fig. 1a) indicating that little, if any, of the newly synthesised collagen had attained a triple-helical conformation. However, following a chase period of 4h, it was found that virtually all of the hydroxy[^{14}C]proline-containing polypeptides were resistant to chymotrypsin digestion and eluted with a molecular weight of approx. 140,000 daltons under reducing conditions (Fig. 1b). These results are consistent with

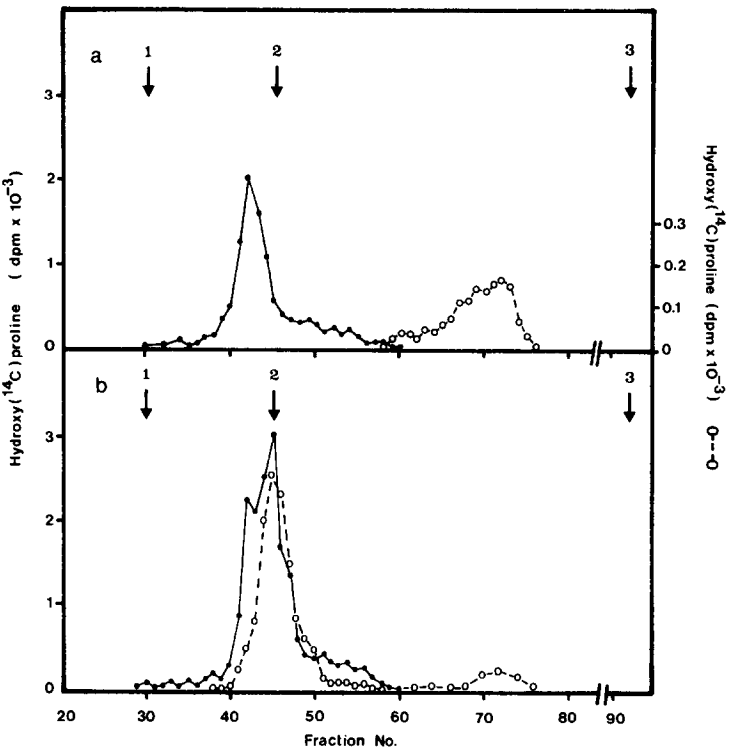

Fig. 1. Gel filtration on SDS-agarose of collagenous ^{14}C-polypeptides subjected to limited proteolysis.
a) Isolated rat glomeruli were incubated for 2h with [^{14}C]proline and then either treated directly with SDS and mercaptoethanol or incubated with chymotrypsin prior to denaturation and reduction and subsequent gel filtration.
b) Glomeruli were labelled for 2h with [^{14}C]proline and then incubated for a further 4h in the presence of 'cold'-proline. Control and chymotrypsin-treated samples were subjected to gel filtration analysis following denaturation and reduction.
●——●, untreated sample; o - - - o, chymotrypsin-treated sample.
The elution positions of dextran blue, tendon pro-α chains (M.Wt. approx. 140000 daltons) and $^{3}H_2O$ are labelled 1, 2 and 3 respectively.

previous observations on chick lens epithelial cells which demonstrated that the majority of intracellular basement membrane collagen is non-helical and that even after a labelling period of 2h very little collagen is to be found extracellularly (3). In the pulse-chase experiment it can be assumed that most of the ^{14}C-collagen is extracellular and the results are therefore consistent with the findings that collagen must be in a triple-helical conformation for normal secretion to occur (7).

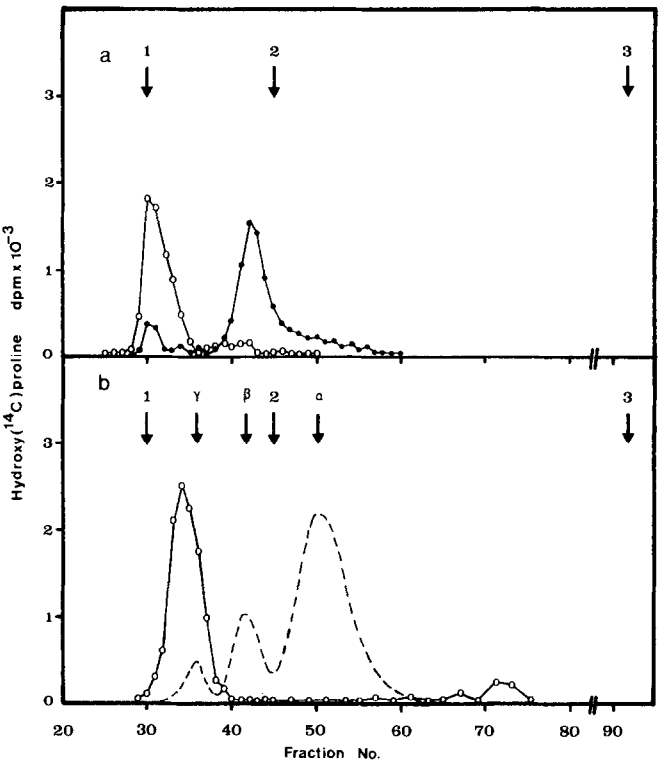

Fig. 2. Gel filtration on SDS-agarose of unreduced collagenous ^{14}C-polypeptides.
a) Elution profiles of labelled polypeptides denatured in SDS and alkylated with iodoacetamide after incubation with [^{14}C]proline for 2h (●——●), and for 2h followed by 4h chase (o——o). Elution positions of standards are as in Fig. 1.
b) Elution profile of unreduced collagen ^{14}C-polypeptides following chymotrypsin treatment. Glomeruli were incubated with [^{14}C]proline for 2h followed by a 4h chase and then digested with chymotrypsin as described in text. Sample was denatured and alkylated prior to chromatography with carrier rat tail tendon collagen. Elution positions of rat tail tendon γ, β and α chains are indicated.

Because earlier studies had demonstrated a relationship between the association of pro-α chains through disulphide bonds and the formation of the collagen triple helix (6,7), it was of interest to examine the state of aggregation of the labelled GBM collagen polypeptides. Gel filtration on SDS-agarose under non-reducing conditions revealed that after a 2h incubation the collagen chains were not disulphide bonded whereas in the pulse-chase experiment the hydroxy[^{14}C]proline-containing polypeptides eluted as large molecular weight aggregates in excess of 450,000 daltons (Fig. 2a). These aggregates could represent complexes of the hydroxy[^{14}C]-

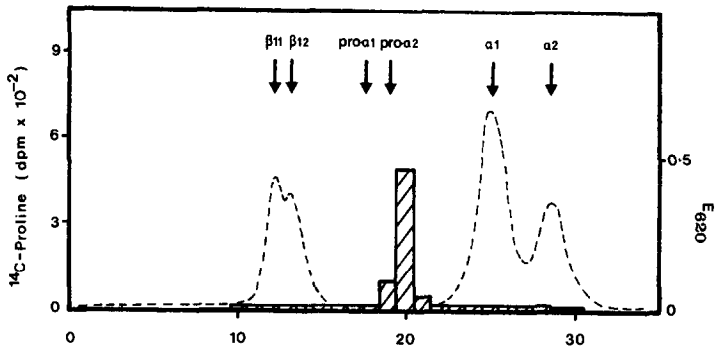

Fig. 3. SDS polyacrylamide gel electrophoresis of chymotrypsin-resistant ^{14}C-polypeptides from pulse-chase experiment. The peak fractions (33-35) from the experiment shown in Fig. 2b were pooled, concentrated, reduced with mercaptoethanol and subjected to electrophoresis on 5% polyacrylamide gels (13). Gels were sliced into 1 mm sections and assayed for radioactivity (hatched histogram). Standards of rat tail tendon collagen and chick tendon procollagen were run on gels in parallel, detected by Coomassie Blue staining and the gels scanned at 620 nm (- - -).

proline-containing polypeptides disulphide-linked to non-collagenous glycoproteins which are known to be present in covalent association with the collagenous components of basement membranes (1). However, when the pulse-chase experiment was repeated and the sample digested with chymotrypsin prior to alkylation and gel filtration, the hydroxy[^{14}C]proline-containing polypeptides now eluted in a position just prior to rat tail tendon collagen γ-chains (Fig. 2b). This observation contrasts with the results obtained when chymotrypsin-resistant polypeptides were chromatographed under reducing conditions (Fig. 1b) and provides the first direct evidence for inter-chain disulphide bonds within the chymotrypsin-resistant helical portion of GBM collagen; and in this respect the type IV collagen appears to resemble type III collagen in that the constituent α-chains are linked by cystine residues located close to or within the triple helix (12).

When the peak of chymotrypsin-resistant collagen obtained from the SDS-agarose column (Fig. 2b) was reduced and analysed by SDS-polyacrylamide gel electrophoresis (13) only one peak of radioactivity migrating in the region of pro-α chains was observed (Fig. 3). This

finding is consistent with the suggestion (1) that the collagenous component of GBM comprises a single species of α-chain which in the extracellular matrix interacts with other non-collagenous glycoprotein subunits. The heterogeneous population of collagenous polypeptides extracted from GBM preparations by other workers (14-16) may be a consequence of the complexity of these covalent and non-covalent interactions.

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