Isolation and characterization of the precursor of type M collagen

V. C. DUANCE, S. F. WOTTON, C. A. VOYLE and A. J. BAILEY
AFRC Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

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A 225000- M_r peptide has been purified from rat chondrosarcoma which is immunologically and biochemically related to type M collagen. Rotary shadowing shows this molecule to be twice the length of the type M molecule and has a prominent kink close to one end. We believe this molecule represents parent type M, the form of the molecule *in vivo*.

The 'minor' collagens recently identified in cartilage are proving to possess unusual properties and pose some interesting possibilities as to their function. These collagens were first isolated from pig cartilage, 'type M collagen' (Shimokomaki *et al.*, 1980), from bovine cartilage, 'CSP1' (Ayad *et al.*, 1981), and later from avian cartilage, 'HMW' (Reese & Mayne, 1981; von der Mark *et al.*, 1982). Additional collagenous peptides have also been identified in chondrocyte cultures, G, H and J (Gibson *et al.*, 1983).

After our initial identification of type M, we demonstrated that (i) the molecule is one-third the length of the type II collagen molecule, (ii) the triple helix is stabilized by disulphide bonds, and (iii) the collagen is confined to the pericellular region of the chondrocytes in the hypertrophic zone of the articular cartilage (Duance *et al.*, 1982). This latter finding suggests that this collagen may play a role in the phenotypic expression of the chondrocytes. It is therefore of considerable interest to determine the molecular organization of the type M collagen around the chondrocytes. To date all these minor collagens have only been isolated from cartilage after enzymic degradation involving cleavage at sensitive sites; hence the form of type M collagen *in vivo* could be considerably larger. Bruckner *et al.* (1983) have made a tentative identification of what they believe to be the precursor of HMW based on immunoblotting of the products of cartilage cultured *in vitro*.

Here we describe the isolation and identification of the parent molecule of type M collagen extracted without enzymic degradation from a lathyritic-rat cartilaginous sarcoma (Smith *et al.*, 1975).

Abbreviations used: β -APN, β -aminopropionitrile fumarate; SDS, sodium dodecyl sulphate; e.l.i.s.a., enzyme-linked immunosorbent assay; p type M, parent type M collagen.

Materials and methods

The rat chondrosarcoma was grown subcutaneously in Sprague–Dawley rats. The rats were fed on a powdered diet containing 0.5% (w/w) β -APN (Aldrich Chemical Co.) to inhibit collagen cross-link formation. Tumours were excised from the rats after 4–5 weeks growth.

Preparation of parent M

Tumours were homogenized in a buffer containing 0.05M-Tris/1M-NaCl, pH 7.5, and a 'cocktail' of proteinase inhibitors including 1mM-phenylmethanesulphonyl fluoride, 1mM-sodium iodoacetate, 10mM- α -aminohexanoic acid, 5mM-benzamidine, soya-bean trypsin inhibitor (1mg/l) and 10mM-EDTA and left stirring at 4°C for 16h. The insoluble residue was separated from the solubilized material by centrifugation at 2000g for 30min, and the solubilized collagens precipitated with 45%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was redissolved in 0.05M-Tris/0.5M-NaCl, pH 7.5, and fractionally salt-precipitated with 2.0M- and 4.0M-NaCl. These precipitates were redissolved in 0.5M-acetic acid and then salt-fractionated sequentially with 0.7, 1.2, 2.0 and 3.0M-NaCl. These fractions were redissolved in 0.5M-acetic acid, dialysed against 0.05M-acetic acid and freeze-dried. A flow diagram is shown in Fig. 1.

Rat type M collagen was prepared from rat sternal cartilage and non-lathyritic chondrosarcoma by previously described procedures (Shimokomaki *et al.*, 1980).

DEAE-Sepharose chromatography

DEAE-Sepharose chromatography was performed on an 8cm $\times$ 2.5cm column equilibrated in 0.05M-Tris/2M-urea/0.02M-NaCl, pH 8.0. The column was run at 20°C at a flow rate of 120ml/h.

Fig. 1. Flow diagram of the salt-fractionation procedure for the purification of parent M collagen

Samples (30mg) were loaded in the starting buffer and bound protein was eluted with a 500ml linear salt gradient from 0.02 to 0.4M-NaCl.

SDS/polyacrylamide-gel electrophoresis

Samples were either run on 8% (w/v)-polyacrylamide gels with a 3% stacking gel or a 7–15% gradient gel with a 3% stacking gel. The discontinuous buffer system described by Laemmli (1970) was used. Samples were reduced with 2% (v/v) mercaptoethanol. M_r values were calculated from the mobilities of collagenous standards.

Pepsin digestion

Samples were dissolved in 0.5M-acetic acid at 1mg/ml and digested with pepsin (enzyme/substrate, 1:10, w/w) at 20°C for 30h. The digests were neutralized with NH_3 , freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis.

Bacterial collagenase assay

This assay was based on the method of Timpl *et al.* (1979). Collagen samples were dissolved in 0.05M-Tris/HCl (pH 7.5)/0.2M-NaCl/2mM- CaCl_2 at 10mg/ml and treated with collagenase (0.1mg/ml; collagenase ABC form III; Advance Biofractures Corp., Lynbrook, NY 11563, U.S.A.) for 20h at 35°C. Samples were dialysed against 0.05M-acetic acid, freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis.

Rotary shadowing

Samples were studied in the electron microscope after rotary shadowing. The method used was essentially that described by Kuhn *et al.* (1981). The collagen samples (1mg/ml in 0.5M-acetic acid) were diluted to 10 $\mu\text{g}/\text{ml}$ in 0.025M-acetic acid/30% (v/v) glycerol. The diluted collagen solutions were

sprayed on to freshly cleaved mica and dried in a vacuum in an Edwards coating unit. The samples were shadowed with platinum at an angle of 9° and then with carbon at 90° . The samples were floated off the mica on a water surface, collected on electron-microscope grids and viewed in a Philips 400 electron microscope.

Antibody preparation

Antibodies to type M collagen were raised in rabbits and assayed by using an e.i.s.a. assay as previously described (Duance *et al.*, 1982). The antibody activity against type M, type II, ($1\alpha, 2\alpha, 3\alpha$) and parent type M was determined by using a doubling dilution of anti-(type M collagen) antibodies from 1:100 to 1:6400.

Results

The use of lathyritic-rat chondrosarcoma enabled the solubilization of considerable amounts of material without the use of dissociative and degradative procedures. The protocol outlined in Fig. 1 allowed the fractionation of the solubilized collagens. A peptide that fitted the criteria required for parent type M is present predominantly in the fraction P2, i.e. the material precipitated at 4.0M-NaCl in neutral salt and 1.2M-NaCl in 0.5M-acetic acid. Some material is also present in the 2.0M-NaCl (neutral salt)/1.2M-NaCl (acetic acid) precipitate. P2 also contains some ($1\alpha, 2\alpha, 3\alpha$), whereas most of the type II is present in the 0.7M-NaCl precipitates.

The major reducible component in P2 is a protein with an apparent M_r of 225 000, which is

Fig. 2. DEAE-Sephacel chromatography of fraction P2. The column was equilibrated in 0.05M-Tris/2M-urea/0.02M-NaCl, pH 8.0, and the protein eluted with a salt gradient (500ml) of 0.02–0.04M-NaCl. The column was washed with the limit buffer at pH 2.0. 'G' indicates the start of the gradient.

sometimes apparent as a doublet. This component has been further purified by anion-exchange chromatography on DEAE-Sephacel. A typical elution profile is shown in Fig. 2. The 225 000- M_r peptide is present in several fractions, but the unbound fraction (peak 1) is essentially pure (Fig. 3, tracks a and b), whereas material eluted with the salt gradient contains additional components.

On reduction the 225 000- M_r peptide gives a major component with an M_r of 60 000 and minor component of M_r about 70 000. After digestion of P2 with pure bacterial collagenase, the 225 000- M_r peptide completely disappears, demonstrating its collagenous nature.

Limited pepsin digestion of the 225 000- M_r protein yields peptides with M_r values of 150 000, 135 000, 120 000 and 35 000 before reduction and values of 90 000, 73 000, 52 000 and 33 000 after reduction (Fig. 3, tracks c and e). Identical peptides are observed for pepsin-extracted rat type M collagen prepared by the procedure of Shimokomaki *et al.* (1980) (Fig. 3, tracks d and f).

Additional evidence that the 225 000- M_r peptide is the precursor of type M was obtained from

Fig. 3. SDS/polyacrylamide-gel electrophoresis of collagen components

Tracks a and b, SDS/8% (w/v)-polyacrylamide gel of peak 1 from the DEAE-Sephacel column (see Fig. 2): track a, unreduced; track b, reduced with 2-mercaptoethanol. Tracks c–f, SDS/7–15%-gradient-polyacrylamide gel of pepsin-digested parent type M and pepsin-extracted rat type M collagen: c, pepsin-digested parent M; d, rat type M collagen; e, reduced pepsin-digested parent M; f, reduced rat type M collagen.

Fig. 4. Anti-(type M collagen) antibody activity determined by e.l.i.s.a. assay on type M- (■), parent M- (▲), (1α,2α,3α)- (●) and type II- (◆) coated wells. The antibody dilution ranged from 1 : 100 to 1 : 6400.

immunochemical studies. As shown in Fig. 4, antibodies to type M collagen react strongly with e.l.i.s.a. plates coated with type M and purified 225000- M_r protein, but only weakly with the other cartilage collagens, type II and (1α,2α,3α). Analysis of the DEAE-Sephacrose-column fractions showed a reactivity towards the anti-(type M) antibodies proportional to the purity of the 225000- M_r protein.

Rotary shadowing of purified 225000- M_r peptide (DEAE-Sephacrose peak 1) revealed a rod-like molecule with an average length of 171 nm. This is equivalent to a peptide with a chain M_r of 60000 when compared with type II collagen, which gives a molecular length of 285 nm for an M_r of 95000. The 225000- M_r peptide frequently shows a prominent kink 40 nm from one end of the molecule (Fig. 5).

Discussion

We have isolated a collagen molecule from lathyrus cartilage with the following properties: (i) it is approximately double the size of pepsin-extracted type M, possessing an M_r of 225000 and a molecular length two-thirds that of the type II molecule; (ii) it gives type M on pepsin treatment; (iii) on reduction it produces a major component of M_r 60000 and peptides similar to type M when reduced after pepsin treatment; (iv) the molecule is immunologically related to type M, giving a positive reaction by e.l.i.s.a.; (v) it possesses a short kinked arm when observed electron-microscopically after rotary shadowing. From this evidence we conclude that the 225000- M_r molecule is the form of type M collagen *in vivo* and have termed it 'parent type M' (p type M).

Fig. 5. Electron micrographs of rotary-shadowed parent type M from DEAE-Sephacrose peak 1 ($\times 50000$). Many of the molecules show prominent kinks ($\rightarrow$) and some dimers are also observed ($\leftrightarrow$).

Pepsin digestion of p type M yields a number of peptides identical with pepsin-extracted rat type M collagen, showing conclusively that the 225000- M_r peptide is the precursor to type M collagen. The peptide pattern of rat type M collagen is more complex than that first described for pig type M (Shimokomaki *et al.*, 1980); similar, but not identical, peptides have been observed in other species (von der Mark *et al.*, 1982; Ricard-Blum *et al.*, 1982). Thus the p type M molecule contains a number of pepsin-sensitive domains that appear to be species-variable (V. C. Duance, M. Shimokomaki, S. F. Wotton & A. J. Bailey, unpublished work).

Before reduction of pepsin-digested p type M, a 35000- M_r peptide is observed (Fig. 3, track c). This peptide is tentatively identified as 'CF2' (Shimokomaki *et al.*, 1981), although further characterization is required for positive identification.

The peptides produced by reduction of p type M are similar to those described by Bruckner *et al.*

(1983) and Gibson *et al.* (1983) from chick chondrocyte cultures. Although differences are apparent, these are probably species variations. Further detailed characterization of these peptides is required before direct comparisons can be made.

Rotary shadowing of p type M frequently revealed the presence of a short kink region near the end of the molecule and occasionally of dimers with kinks at both ends. The kink is not present in pepsin-extracted type M, indicating its sensitivity to pepsin. In contrast, pepsin-extracted avian type M/HMW (von der Mark *et al.*, 1982; Reese *et al.*, 1982) possesses a similar kink, indicating that mammalian type M is more pepsin-sensitive than the corresponding avian molecule. The short arm present in our mammalian p type M is probably analogous to the short arm of avian type M, although this requires further investigation.

We have here described the isolation and purification of a 225000- M_r peptide from a rat chondrosarcoma. Preliminary characterization identifies this molecule as the form of type M collagen *in vivo* and it has been termed 'parent type M'.

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