Separation and characterization of two populations of aggregating proteoglycans from cartilage

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Intermediary gel immunoelectrophoresis was used to show that purified aggregating cartilage proteoglycans from 2-year-old steers contain two distinct populations of molecules and that only one of these is immunologically related to non-aggregating cartilage proteoglycans. The two types of aggregating proteoglycans were purified by density-gradient centrifugation in 3.5M-CsCl/4M-guanidinium chloride and separated by zonal rate centrifugation in sucrose gradients. The higher-buoyant-density faster-sedimenting proteoglycan represented 43% of the proteoglycans in the extract. It had a weight-average M_r of 3.5×10^6 , did not contain a well-defined keratan sulphate-rich region, had a quantitatively dominant chondroitin sulphate-rich region and contained 5.9% protein and 23% hexosamine. The lower-buoyant-density, more slowly sedimenting, proteoglycan represented 15% of the proteoglycans in the extract. It had a weight-average M_r of 1.3×10^6 , contained both the keratan sulphate-rich and the chondroitin sulphate-rich regions and contained 7.3% protein and 23% hexosamine. Each of the proteoglycan preparations showed only one band on agarose/polyacrylamide-gel electrophoresis. The larger proteoglycan had a lower mobility than the smaller. The distribution of chondroitin sulphate chains along the chondroitin sulphate-rich region was similar for the two types of proteoglycans. The somewhat larger chondroitin sulphate chains of the larger proteoglycan could not alone account for the larger size of the proteoglycan. Peptide patterns after trypsin digestion of the proteoglycans showed great similarities, although the presence of a few peptides not shared by both populations indicates that the core proteins are partially different.

Cartilage proteoglycans are complex macromolecules that contain a central protein core with several different kinds of carbohydrate substituents. Three regions of the core protein, having different amino acid and carbohydrate composition, have been identified (Heinegård & Hascall, 1974a; Heinegård & Axelsson, 1977). The hyaluronic acid-binding region at one end of the core contains *N*-glycosidically linked oligosaccharides of the complex type, but no glycosaminoglycan side chains. It can specifically interact with hyaluronic acid, important for the formation of proteoglycan aggregates (Hardingham & Muir, 1972, 1973; Hascall & Heinegård, 1974; Heinegård & Hascall, 1974a). The chondroitin sulphate

chains and a proportion of the keratan sulphate chains are attached to the chondroitin sulphate-rich region at the other end of the core protein (Heinegård & Axelsson, 1977). The chondroitin sulphate chains are most probably grouped in clusters along the protein (Heinegård & Hascall, 1974b). A keratan sulphate-rich region is located between the other two regions and contains a large proportion of the keratan sulphate chains. The regions containing the glycosaminoglycans also have substituents of *O*-linked oligosaccharides of the mucin type (Lohmander *et al.*, 1980; Nilsson *et al.*, 1982).

The chondroitin sulphate-rich region constitutes more than half of the protein in the average proteoglycan, and appears to have a variable length (Heinegård, 1977; Wieslander & Heinegård, 1980). The proteoglycans are therefore extremely polydisperse and vary with respect to

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size and charge (Heinegård, 1977). The extreme polydispersity has hampered attempts to identify subpopulations. Nevertheless there are indications that more than one population of proteoglycans is present in cartilage, especially as electrophoresis of proteoglycans on agarose/polyacrylamide gels shows two or three bands (McDevitt & Muir, 1971; Pearson & Mason, 1977; Stanescu *et al.*, 1977). Considering the polydispersity of the proteoglycans, these bands are surprisingly sharp. To identify subpopulations of proteoglycans in the polydisperse spectrum, however, it is essential to have a marker for distinct structural features that differ between the populations.

A low- M_r cartilage proteoglycan could thus be isolated because of distinguishing features such as its relatively low degree of polydispersity, low apparent M_r of its core protein (Heinegård *et al.*, 1981) and its high electrophoretic mobility (Stanescu & Sweet, 1981). It has a M_r of about 76000, does not interact with hyaluronic acid and appears to represent a separate gene product (Heinegård *et al.*, 1981). A distinct population of large proteoglycans could be isolated because of the inability of these molecules to interact with hyaluronic acid and form aggregates (Heinegård & Hascall, 1979). They share many structural features with the aggregating proteoglycans, but do not contain the hyaluronic acid-binding region (D. Heinegård, unpublished work) and have a lower substitution with glycosaminoglycans (Heinegård & Hascall, 1979). Preliminary experiments showed that antibodies raised against this proteoglycan population react in immunoelectromigration with only a portion of the aggregating proteoglycans, thus providing a tool for identifying subpopulations of these in fractionations.

The aim of the present investigation was to isolate and characterize the two populations of aggregating proteoglycans that do and do not, respectively, react with the antibodies raised against the population of large non-aggregating proteoglycans.

Materials and methods

Proteoglycans were isolated from bovine nasal-septum cartilage from 2-year-old steers by extraction with 4M-guanidinium chloride containing proteinase inhibitors and *N*-ethylmaleimide, as described by Heinegård *et al.* (1981).

Isolation of proteoglycans

Proteoglycan A1 fraction was prepared by CsCl-density-gradient centrifugation in 0.4M-guanidinium chloride essentially as described by Heinegård & Hascall (1979). The A1 fraction was dialysed against water, sodium acetate, pH 7, and

water, successively. Samples of the dialysis residue were freeze-dried and weighed in the dried state to determine their content of proteoglycan. The remaining A1 fraction was kept frozen at about 10mg/ml until further fractionated.

In an alternative procedure, proteoglycan monomers were directly isolated from extracts by CsCl-gradient centrifugation. The 4M-guanidinium chloride extract was adjusted to a density of 1.51 g/ml by addition of solid CsCl, and the sample was centrifuged as described above. The tube was divided into four fractions representing, from the bottom (d-D1), $\frac{1}{4}$, $\frac{1}{4}$ (d-D2), $\frac{1}{4}$ and $\frac{1}{4}$ of its contents, by using a tube slicer. The material in the bottom two fractions was separately desalted by dialysis against sodium acetate, pH 7.0, and then water. Contents of proteoglycans were determined as described above, and the samples were stored frozen as stock solutions in water at 10mg/ml (d-D1) and 6mg/ml (d-D2).

Preparation of proteoglycan aggregates

Proteoglycan aggregates were separated from proteoglycan monomers by zonal rate centrifugation as described by Heinegård & Hascall (1979) and used for analytical experiments. Larger amounts of proteoglycan aggregates were prepared by zonal rate centrifugation of A1 preparations in sucrose gradients. Samples (2ml) containing 4mg of proteoglycan/ml in 0.2M-CaCl₂/1 mM-Tris/HCl, pH 7.5, were layered on top of linear 10–50% (w/v) sucrose gradients (14ml) in the same CaCl₂/Tris/HCl solution. After centrifugation in an MSE 8 × 25 angle rotor at 50000 rev./min (190000g, r_{av} 6.886cm) and 20°C for 55min the tubes were emptied as described by Franzén *et al.* (1982). Aggregates sedimented to the bottom of the tube, as detected by their A_{206} , and the monomers showed a peak in the middle of the tube (results not shown). The aggregate fraction was extensively dialysed against water and freeze-dried. Typically one centrifugation of 64mg of A1 fraction yielded 48mg of pure aggregates (A1-S1).

Preparation of aggregating proteoglycan monomers

The purified aggregates (A1-S1) were dissolved at 2mg/ml in 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, also containing CsCl to a density of 1.55g/ml. After centrifugation in a Beckman 42.1 rotor at 33000 rev./min (83900g, r_{av} 6.88cm) at 19°C for 66h, the tubes were divided into five fractions, of 5.7ml (A1-S1-D1), 6.4ml (A1-S1-D2), 6.3ml (A1-S1-D3), 10.5ml (A1-S1-D4) and 10.4ml (A1-S1-D5), with a tube slicer. Approx. 80% of the uronic acid was recovered in the bottom fraction (A1-S1-D1), and the A1-S1-D2 fraction contained another 11%. A large proportion of the protein was recovered in the top fraction

(A1-S1-D5). The bottom (A1-S1-D1) fraction was dialysed against water, sodium acetate, pH 7, and water. A sample was freeze-dried, and its weight was used to calculate that 350 mg of pure proteoglycan monomer was recovered from 460 mg of aggregate.

Separation of subpopulations of aggregating proteoglycans

Preparative zonal rate centrifugation was done in 11.4 ml linear gradients of 10–30% sucrose in 0.2 M-CaCl₂/1 mM-Tris/HCl, pH 7.5. Samples (500 μ l; 2 mg/ml) in the same salt solution were centrifuged for 6 h at 40 000 rev./min (195 000 *g*, *r*_{av}, 11.102 cm) at 20°C in an MSE 6 $\times$ 14 titanium swing-out rotor. Alternatively, 38 ml linear gradients of 10–30% sucrose in the same salt solution, with 2 ml of sample at 2 mg/ml, were centrifuged for 16 h at 26 000 rev./min (89 500 *g*, *r*_{av}, 11.82 cm) in a Beckman SW 28.1 swing-out rotor at 20°C. Tubes were emptied as described by Franzén *et al.* (1982). In some experiments 0.1 ml of sample solution (1 mg/ml) was centrifuged in 4 ml linear gradients of 10–30% sucrose in the CaCl₂/Tris/HCl solution at 60 000 rev./min (370 000 *g*, *r*_{av}, 9.17 cm) for 2 h 30 min.

Column chromatography

A column of Superose 6B (0.65 cm $\times$ 93 cm; Pharmacia Fine Chemicals) was eluted with 0.15 M-Na₂SO₄, and the effluent was continuously monitored for *A*₂₀₆ with a Uvicord S instrument. This column was calibrated with chondroitin sulphate chains of known *M*_r values. The *M*_r values of chains were calculated as described by Wasteson (1971).

Fragmentation of proteoglycans

Proteoglycans were digested with trypsin, or sequentially with trypsin and chymotrypsin, to produce chondroitin sulphate peptide clusters as described by Heinegård & Hascall (1974b). Chondroitin sulphate chains were liberated by treatment with 200 μ l of 0.05 M-NaOH/1 M-NaBH₄ for 48 h at 45°C. Before chromatography on the Superose 6B column, the samples were neutralized with 25 μ l of 10 M-acetic acid.

To test for the presence of a keratan sulphate-rich region, samples were digested in sequence with chondroitinase ABC, trypsin and chymotrypsin as described by Heinegård & Axelsson (1977).

Light-scattering

*M*_r values of samples in 4 M-guanidinium chloride/1 mM-EDTA/1 mM-sodium phosphate, pH 6.5, were determined by light-scattering measurements essentially as described by Sheehan *et al.* (1978). Weighed dilutions were prepared from a stock

solution, which had been previously dialysed against the solvent above. A value of *dn/dc* of 0.146 (Kitchen & Cleland, 1978) was used for calculations, which were performed as described by Tomimatsu *et al.* (1968) and plotted in accordance with Zimm (1948).

Intermediary gel immunoelectrophoresis

Antibodies against proteoglycan preparations were raised in rabbits as described by Wieslander & Heinegård (1979). Intermediary gel immunoelectrophoresis was done as described by Axelsen (1973). Anti-(non-aggregating proteoglycan) antibodies were used in the intermediary gel, and antibodies raised against the total population of proteoglycan monomers (anti-A1-D1) were used in the upper gel. Proteoglycan fractions were digested with chondroitinase ABC at 1 mg/ml before electrophoresis and diluted to contain about 50 μ g of protein/ml.

Agarose/polyacrylamide-gel electrophoresis

Proteoglycans were electrophoresed on agarose/polyacrylamide gels essentially as described by McDevitt & Muir (1971).

Chemical methods

Uronic acid contents of fractions from density-gradient centrifugations were determined by the carbazole method (Bitter & Muir, 1962). Column effluents containing 0.5 M-sodium acetate or 0.25 M-Na₂SO₄ were analysed by an automated version of the carbazole procedure (Heinegård, 1973). Neutral-sugar contents of column effluents were determined by an automated version of the orcinol/H₂SO₄ procedure essentially as described by Kesler (1967). Proteoglycans were dissolved in 6 M-urea and labelled with ¹²⁵I by the chloramine-T method as described by Greenwood *et al.* (1963). A thin-layer electrophoresis and chromatography procedure was used for peptide mapping, as described by Wieslander & Heinegård (1979).

Keratan sulphate and oligosaccharides were quantified after alkaline borohydride treatment of proteoglycans, essentially as described by Inerot & Heinegård (1983). Glycosaminoglycans were separated from oligosaccharides by chromatography on DEAE-cellulose (Whatman DE52), where oligosaccharides were eluted with water and 0.6 M-pyridine, pH 6.5, and glycosaminoglycans were eluted with 2 M-HCl. The number of keratan sulphate chains was calculated from the galactosaminitol content, and their size from the ratio of glucosamine to galactosaminitol of the glycosaminoglycan fraction. The number of *O*-linked oligosaccharides was calculated from the galactosaminitol content of the oligosaccharide fraction, and the number of *N*-linked oligosaccharides

was estimated by subtraction of the estimated glucosamine content of the *O*-linked oligosaccharides from the total glucosamine content of this fraction.

Hexosamine contents were determined after hydrolysis of samples in 4M-HCl at 100°C for 10 h under argon. Amino acid contents were determined after hydrolysis in 6M-HCl at 110°C for 24 h under argon. A Durrum amino acid analyser was used for determinations of hexosamine, hexosaminitol and amino acids.

Results and discussion

Antibodies raised against the non-aggregating proteoglycans (Heinegård & Hascall, 1979) and the whole proteoglycan-monomer population (A1-D1) were used in intermediary gel immunoelectrophoresis (Axelsen, 1973). It was shown that only a portion of purified aggregating proteoglycans cross-reacted with the antibodies to the non-aggregating proteoglycans and formed a precipitate rocket in the intermediary gel (Fig. 1). Another portion of the molecules was not precipitated, but migrated to the upper gel, which contained antibodies precipitating all types of

Fig. 1. Intermediary-gel immunoelectromigration of purified aggregating proteoglycan monomers

The molecules had been digested with chondroitinase ABC before electrophoresis. The intermediary gel contained antibodies raised against the non-aggregating proteoglycans prepared as described by Heinegård & Hascall (1979). The upper gel contained antibodies raised against the whole population of proteoglycan monomers (A1-D1).

aggregating proteoglycans (Fig. 1). Two groups of aggregating proteoglycans with different antigenic properties, and therefore representing distinct subpopulations rather than extremes of a polydisperse spectrum, were thus identified.

A set of experiments was initiated to isolate the two populations of proteoglycans. They were extracted from cartilage by using 4M-guanidinium chloride, containing proteinase inhibitors as well as *N*-ethylmaleimide in a dual function as a proteinase inhibitor and to prevent disulphide exchange (Heinegård *et al.*, 1981). Proteoglycans were then separated from proteins by direct centrifugation in CsCl/4M-guanidinium chloride. The bottom two fractions, which had average densities of 1.67 g/ml (d-D1) and 1.58 g/ml (d-D2), contained 56% and 30% of the total uronic acid in the gradient respectively, and the amounts of proteoglycans were 803 mg and 568 mg. The fractions contained no contaminating proteins, as shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The lower-buoyant-density proteoglycans had higher K_{av} values on Sepharose 2B and higher protein contents (results not shown), corroborating previous data (Heinegård, 1977).

The two fractions (d-D1 and d-D2) were fractionated by zonal rate centrifugation in sucrose gradients. In pilot experiments the sedimentation profiles indicated the presence of more than one component, as shown in Fig. 2, discussed below. The fractionation was optimized by testing sucrose gradients containing various uni- and bi-valent ions of different ionic strength buffered with 1 mM-Tris/HCl, pH 7.0, with proteoglycans dissolved at 1 mg/ml in a solvent of the same ionic composition. The conditions tried were concentrations of 0.05–0.50M-CsCl, -NaCl, -CaCl₂, -BaCl₂, and the buffer alone. Bivalent cations gave somewhat better separation than univalent ions. Optimal fractionation was obtained with 0.20M and higher concentrations of CaCl₂ or BaCl₂ (results not shown). Therefore subsequent centrifugation was done in 10–30% sucrose gradients containing 0.2M-CaCl₂/1 mM-Tris/HCl, pH 7.0. Similar separations were obtained with proteoglycan concentrations of 1 and 2 mg/ml, whereas at 4 mg/ml resolution was impaired. A concentration of 2 mg of proteoglycans/ml was chosen for preparative centrifugations.

The two fractions, isolated by the direct CsCl-gradient centrifugation of guanidinium chloride extracts, were centrifuged preparatively in a 6 × 14 ml swing-out rotor as described in the Materials and methods section. The bottom d-D1-fractions (Fig. 2) showed a major, faster-sedimenting, component plus a minor, slower-sedimenting, component appearing as a trailing shoulder. The less-buoyant (d-D2), fraction, in contrast, showed a slower-sedimenting major

Fig. 2. Zonal rate centrifugation of subfractions [d-D2 (a) and d-D1 (b)] of cartilage proteoglycan monomers prepared by density-gradient centrifugation in CsCl/4M-guanidinium chloride

Samples (0.5 ml; 2 mg/ml) were centrifuged in 10–30% sucrose/0.2M-CaCl₂/1 mM-Tris/HCl, pH 7.5, for 6 h at 40000 rev./min in an MSE 6 × 14 ml titanium swing-out rotor as described in the Materials and methods section.

component plus an additional faster-sedimenting component appearing as a leading shoulder (Fig. 2). An additional, minor, very slowly sedimenting component (component 3) represents the low-*M_r* proteoglycan described by Heinegård *et al.* (1981). Fractions were pooled as indicated in Fig. 2, desalted by dialysis against water and freeze-dried. Intermediary gel immunoelectrophoresis showed that the fast-sedimenting component from the d-D1 fraction (d-D1-S1) essentially contained proteoglycans reacting with the anti-(non-aggregating proteoglycan) antibodies, whereas the slow-sedimenting component in the d-D2 fraction (d-D2-S2) only contained proteoglycans not reacting with these antibodies (Fig. 3). The slowly sedimenting shoulder component from the d-D1-fraction (d-D1-S2) and the fast-sedimenting shoulder component from the d-D2-fraction (d-D2-S1) (Fig. 2) contained a mixture of the two populations of proteoglycans (Fig. 3). It appears, however, that the major proportion of faster-sedimenting material from both fractions d-D1

Fig. 3. Analysis of proteoglycan subfractions. Intermediary gel immunoelectromigration (a) and agarose/polyacrylamide-gel electrophoresis (b) of fractions of proteoglycan monomers prepared by zonal rate centrifugation as shown in Fig. 2.

and d-D2 represents proteoglycans reacting with the antibodies to non-aggregating proteoglycans, whereas a major proportion of the slower sedimenting material represented proteoglycans that did not react with these antibodies. Further support for the purity of the subfractions (d-D1-S1 and d-D2-S2) was obtained by agarose/polyacrylamide-gel electrophoresis (Fig. 3). The high-buoyant-density fast-sedimenting fraction, d-D1-S1, contained only the slowly migrating component, whereas the low-buoyant-density slowly sedimenting fraction, d-D2-S2, contained only the rapidly migrating component described previously (McDevitt & Muir, 1971; Stanescu *et al.*, 1977; Pearson & Mason, 1977) (Fig. 3). Fraction d-D1-S1 contained only a very small amount of the other type of proteoglycan, as detected in the immunological assay, and fraction d-D2-S2 appeared pure. The two fractions represent approx. 43% (d-D1-S1) and 15% (d-D2-S2) of the proteoglycans in the extract.

It is likely that the non-aggregating proteoglycans described above are contained in the d-D1-S1 and to a larger extent the d-D1-S2 fractions. Therefore an alternative procedure was used to purify the two subpopulations further, starting with proteoglycan aggregates (A1-S1) which had been purified by zonal rate centrifugation in sucrose gradients. The aggregating-proteoglycan

monomers were purified by CsCl-density-gradient centrifugation of the purified aggregates (A1-S1) in 4M-guanidinium chloride. The top fraction of this gradient, which contained the link proteins, was not analysed further. Samples of the other fractions were centrifuged in sucrose gradients in 0.2M-CaCl₂ as described above. Only the bottom (D1) fraction contained both proteoglycan populations (Fig. 4). The D2 fraction contained almost only the slow-sedimenting proteoglycans, whereas fraction D3 contained two components. The fast-sedi-

menting component in fraction D3 probably represents proteoglycan aggregates formed with the hyaluronate present, whereas the slow-sedimenting component represents monomers of one of the two subpopulations of aggregating proteoglycans. Almost all of the material in fraction D4 similarly appears to be aggregated, probably reflecting the distribution of hyaluronic acid in the gradient. The profiles show that the proteoglycans with higher buoyant density in CsCl gradients have a higher sedimentation rate in sucrose gradients.

The bottom A1-S1-D1 fraction was isolated on a larger scale by zonal rate centrifugation in sucrose gradients (sedimentation pattern similar to those shown in Fig. 4). The two fractions A1-S1-D1-S1 and A1-S1-D1-S2 were pooled as indicated in Fig. 4, dialysed against sodium acetate, pH7, and water, concentrated by ultrafiltration (Amicon PM-10 membrane) and kept frozen at about 3mg/ml until analysis. Samples were re-centrifuged on sucrose gradients (Fig. 5), showing that

Fig. 4. Sedimentation profiles in sucrose gradients of sub-fractions of purified aggregating proteoglycan monomers. Fractions isolated by density-gradient centrifugation in CsCl/4M-guanidinium chloride of purified proteoglycan aggregates were dialysed against 0.2M-CaCl₂/1 mM-Tris/HCl, pH7.5, and centrifuged in 10–30% sucrose/0.2M-CaCl₂ in an MSE 6×14ml swing-out rotor as described in the Materials and methods section. (a) A1-S1-D4; (b) A1-S1-D3; (c) A1-S1-D2; (d) A1-S1-D1.

Fig. 5. Zonal rate centrifugation of subpopulations of aggregating proteoglycans.

Proteoglycans (100 µg in 100 µl) were centrifuged in 10–30% sucrose gradients in 0.2M-CaCl₂/1 mM-Tris/HCl, pH7.5, in a Beckman SW 60 rotor at 60000 rev./min for 2 h 24 min as described in the Materials and methods section. (a) A1-S1-D1-S1; (b) A1-S1-D1-S2.

the components sedimented as expected. Other samples were taken for agarose/polyacrylamide-gel electrophoresis, with and without prior trypsin

Fig. 6. Agarose/polyacrylamide-gel electrophoresis of intact and fragmented proteoglycans

Electrophoresis was performed on agarose/polyacrylamide slab gels essentially as described by McDevitt & Muir (1971). Gels stained with Toluidine Blue were dried on Gelbond films (FMC Corp., Marine Colloids Division, Rockland, ME, U.S.A.) and scanned for A_{645} with the tungsten lamp of a Zeineh soft laser scanner. The samples represent, from the top: (a) fast-sedimenting aggregating proteoglycans (S1); (b) slower sedimenting aggregating proteoglycans (S2); (c) trypsin digest of S1 (S1-T); (d) trypsin digest of S2 (S2-T); (e) a mixture of chondroitin sulphate and keratan sulphate peptides (A1-T-A1) prepared from trypsin-digested proteoglycan aggregates as described by Heinegård & Axelsson (1977) [for reference the tracing of free chondroitin sulphate chains (CS) is included (----)]; (f) keratan sulphate-rich region isolated as described by Heinegård & Axelsson (1977) by chondroitinase digestion of chondroitin sulphate plus keratan sulphate peptides (A1-T-A1) as described above.

digestion (Fig. 6). The two fractions prepared by this procedure also contain either the slow-migrating component (A1-S1-D1-S1) or the fast-migrating component (A1-S1-D1-S2). Sepharose-2B chromatography showed that each proteoglycan preparation was eluted as a broad peak, indicating polydispersity (results not shown). Their K_{av} values were 0.36 for A1-S1-D1-S1 and 0.60 for A1-S1-D1-S2. Absolute values for the molecular dimensions were obtained by light-scattering measurements in 4M-guanidinium chloride (Fig. 7). The faster-sedimenting, larger, A1-S1-D1-S1 has a weight-average M_r of 3.5×10^6 and a radius of gyration (R_G) of 83.4 nm, and the slower-sedimenting, smaller, A1-S1-D1-S2 has a weight-average M_r of 1.3×10^6 and a radius of gyration of 57 nm.

Structural features of the two proteoglycans were further studied by enzymic degradations. Sepharose-6B chromatography of sequential trypsin/chymotrypsin digests, i.e. corresponding to the

Fig. 7. Zimm plots of light-scattering data from solutions of subpopulations of aggregating proteoglycans (a) A1-S1-D1-S1 and (b) A1-S1-D1-S2

All measurements were performed in 4M-guanidinium chloride/1 mM-EDTA/1 mM-sodium phosphate buffer, pH 6.5. The following concentrations were used: (a) 0.79, 0.60, 0.40, 0.20, 0.10 mg/ml; (b) 0.78, 0.59, 0.39, 0.20, 0.10 mg/ml. The optical constant was $3.78 \times 10^{-7} \text{ cm}^2 \cdot \text{g}^{-2}$ in both cases.

chondroitin sulphate cluster peptides described elsewhere (Heinegård & Hascall, 1974*b*; Thyberg *et al.*, 1975), gave rather similar patterns (Fig. 8). Interestingly, the fragments produced by trypsin digestion alone differed somewhat. Those from the larger proteoglycan were eluted less retarded on Sepharose 4B (Fig. 8). The size difference between the two proteoglycans and their fragments, respectively, may in part result from the somewhat larger average M_r of the chondroitin sulphate chains of the larger proteoglycan (Table 3). Agarose/polyacrylamide-gel electrophoresis of trypsin digests of the two proteoglycan preparations (Fig. 6) gave different patterns. Both contained faster-moving components, probably chondroitin sulphate peptides, as indicated by a mobility similar to that of the faster-migrating component of a high-buoyant-density fraction (A1-T-A1) of a trypsin digest of a proteoglycan-aggregate fraction [for conditions see Heinegård & Axelsson (1977) and Heinegård & Paulsson (1984)]. The slower-migrating component in this material represents keratan sulphate peptides, as indicated by its similar mobility compared with the keratan sulphate peptides isolated from the A1-T-A1 preparation after chondroitinase ABC digestion (KS-T). A clearly

visible component at this position was only present in the digest of the slower-sedimenting proteoglycan, i.e. A1-S1-D1-S2 (Fig. 6). It appears therefore that the two proteoglycan preparations differ with respect to relative contents of keratan sulphate-rich region and chondroitin sulphate-rich region. Further support for this notion was obtained by chromatography on Sepharose 6B of chondroitinase/trypsin digests of the two proteoglycans (Fig. 9). The smaller A1-S1-D1-S2 contained a more prominent, early eluted, peak corresponding to the keratan sulphate peptides (Heinegård & Axelsson, 1977). The second peak, containing the linkage regions of the chondroitin sulphate chains to protein (Heinegård & Axelsson, 1977), was more prominent in the larger A1-S1-D1-S1.

The chemical composition of the two proteoglycans gave further information on the differences between them. The larger proteoglycan had higher contents of serine and glycine (Table 1), amino acids that are enriched in the chondroitin sulphate attachment region (Heinegård & Axelsson, 1977), whereas the smaller proteoglycan had a higher content of glutamic acid/glutamine, and less markedly of proline, amino acids that are enriched in the keratan sulphate-rich region (Heinegård &

Fig. 8. *Sepharose 4B* chromatograms of trypsin and *Sepharose 6B* chromatograms of sequential trypsin/chymotrypsin digests, respectively, of subpopulations of aggregating proteoglycans

Samples were sequentially digested with trypsin/chymotrypsin (T-C, left panels) and only with trypsin (T, right panels) and chromatographed on *Sepharose 4B* or *6B* columns ($0.6 \text{ cm} \times 147 \text{ cm}$ and $0.6 \text{ cm} \times 144 \text{ cm}$) eluted with 0.5 M -sodium acetate, pH 7. (a) and (c) are A1-S1-D1-S1; (b) and (d) are A1-S1-D1-S2. Effluents were analysed by using the carbazole procedure. Absorbance values are in arbitrary units.

Axelsson, 1977). The contents of glucosamine and protein were higher in the smaller proteoglycan, whereas the larger proteoglycan had a higher content of galactosamine (Table 1).

Samples of the proteoglycans were treated with alkaline borohydride. Each keratan sulphate chain and each *O*-linked oligosaccharide chain contain one galactosaminitol residue formed by reduction with borohydride. Oligosaccharides were separated from glycosaminoglycans by ion-exchange chromatography on DEAE-cellulose. The two fractions obtained from each proteoglycan were analysed for contents of hexosamines and hexosaminitols (Table 2). The compositional data of the two proteoglycans were used to calculate several molecular parameters (Table 3). Protein contents and M_r values of the proteoglycans were used to

calculate the M_r of the protein core. The larger proteoglycan has a core of M_r about 200000, in accordance with previously published data (Hascall & Riolo, 1972), whereas the core of the smaller proteoglycan is of approximately half that size. The number of chondroitin sulphate chains, calculated from their number-average M_r and glycosaminoglycan-hexosamine contents, was considerably higher in the larger proteoglycan. The smaller proteoglycan contains almost the same

Table 1. Amino acid and hexosamine compositions of two populations of aggregating cartilage proteoglycans

	Content (residues/1000 residues)	
	A1-S1-D1-S1	A1-S1-D1-S2
Aspartic acid	72	67
Threonine	62 (41%)*	58 (34%)*
Serine	139 (52%)*	119 (43%)*
Glutamic acid	138	151
Proline	96	104
Glycine	136	123
Alanine	68	72
Cysteine	—	—
Valine	57	62
Methionine	—	—
Isoleucine	41	41
Leucine	77	74
Tyrosine	21	24
Phenylalanine	33	38
Histidine	13	12
Lysine	20	21
Arginine	28	33
Protein†	59	73
Glucosamine†	12.5	22.4
Galactosamine†	217	206

* Values in parentheses represent percentages of residues destroyed by alkaline β -elimination.
† Values represent $\mu\text{g}/\text{mg}$ of proteoglycan.

Fig. 9. Sepharose 6B chromatograms of sequential chondroitinase/trypsin/chymotrypsin digests of subpopulations of aggregating proteoglycans. Samples were digested as described in the Materials and methods section and chromatographed on Sepharose 6B eluted with 0.5M-sodium acetate, pH7. (a) A1-S1-D1-S1; (b) A1-S1-D1-S2. ----, orcinol reaction; —, carbazole reaction. Absorbance values are in arbitrary units.

Table 2. Hexosamine and hexosaminitol contents of oligosaccharide and glycosaminoglycan fractions of two populations of aggregating proteoglycans

Values are expressed as nmol/mg of proteoglycan.

	A1-S1-D1-S1		A1-S1-D1-S2	
	Oligosaccharide	Glycosaminoglycan	Oligosaccharide	Glycosaminoglycan
Galactosaminitol	12.1	8.5	9.7	12.1
Glucosamine	20.0	49.7	23.9	100.9
Galactosamine	4.6	1184	3.3	1124

Table 3. Calculated M_r values of protein core and numbers of different side chains per molecule of two populations of aggregating proteoglycans

	Al-S1-D1-S1	Al-S1-D1-S2
Core protein (M_w)	207800	94400
Chondroitin sulphate		
No. of chains	97	41
M_w	19700	16400
M_n	15400	12300
Keratan sulphate		
No. of chains	30	16
Size*	5.9	8.3
O-Linked oligosaccharides		
No. of chains	42	13
N-Linked oligosaccharides		
No. of chains†	7 (6)	5 (4)

* Calculated from glucosamine/galactosaminitol ratio.

† Values for biantennary and triantennary (in parentheses) oligosaccharides respectively.

Fig. 10. Peptide patterns of trypsin-digested populations of aggregating proteoglycans

Samples were [125 I]iodinated, digested with trypsin and electrophoresed on thin-layer plates of silica gel in pyridine/acetate, pH6.4, followed by chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.). Labelled peptides were detected by autoradiography. 'HABR' represents isolated hyaluronic acid-binding region. The arrows indicate the peptides specific for each preparation.

number of keratan sulphate chains per weight of the protein core, although the size of the chains on the smaller proteoglycan is considerably larger, as calculated from ratio of glucosamine to galactosaminitol (Inerot & Heinegård, 1983). The larger proteoglycan, however, has a somewhat higher content of O-linked oligosaccharides, which are structurally related to the linkage region of keratan sulphate to protein.

Values for contents of N-linked oligosaccharides are approximate and based on the assumption that each O-linked oligosaccharide contains one glucosamine residue, whereas it is assumed that all N-linked oligosaccharides are biantennary and triantennary (results in parentheses in Table 3),

respectively. Supportive data on the numbers of O-linked oligosaccharides and glycosaminoglycans were obtained by determination of the number of serine and threonine residues destroyed by β -elimination during the alkaline treatment. The calculated numbers of O-linked side chains were 169 and 70 for the larger and smaller proteoglycan respectively (Table 3), in reasonable accordance with the sums of destroyed hydroxyamino acids of 200 and 65 respectively.

The structures of the protein cores of the two proteoglycans were compared by peptide mapping of trypsin-digested 125 I-labelled proteoglycans (Fig. 10). A great number of labelled peptides were observed, in accordance with results of Oike *et al.*

(1982). The two preparations yielded a large number of identical peptides, but there are a few peptides unique to either preparation, indicated by arrows in Fig. 10. Therefore, it appears that the protein core contains large identical regions and small non-identical regions. The radiolabelled peptides observed are derived both from the hyaluronic acid-binding region and from the glycosaminoglycan-attachment region of the proteoglycan, as shown by the only partial identity with a peptide map of the hyaluronic acid-binding region (Fig. 10). The patterns are distinctly dissimilar to those of the low- M_r cartilage proteoglycan (D. Heinegård, unpublished work; Heinegård *et al.*, 1981).

General discussion

In a number of studies cartilage proteoglycans have been fractionated to yield subfractions differing with respect to composition and size (Hoffman *et al.*, 1975; Heinegård, 1977; Swann *et al.*, 1979; Hoffman, 1979). The data presented in these investigations do not preclude that the samples analysed represent subfractions of a poly-disperse population rather than distinct molecular entities. Strong evidence for the presence of more than one population of proteoglycans in cartilage was provided by electrophoresis on agarose/polyacrylamide gels (McDevitt & Muir, 1971; Stanescu *et al.*, 1977; Pearson & Mason, 1977). Furthermore, Triphaus *et al.* (1980) found two metabolically distinct populations of proteoglycans in human xiphoid cartilage. Immunochemical studies indicated that cartilage extracts contain more than one population of proteoglycans (Wieslander & Heinegård, 1981), and in the present study we have purified two antigenically distinct populations of aggregating proteoglycans.

Various differences in both chemical and physical properties, together with small differences in the peptide patterns of their trypsin-digested protein cores, suggest that the two populations of aggregating proteoglycans represent different gene products, although very closely related. At this time, however, the possibility that the occurrence of the two populations is due to post-translational modifications cannot be entirely excluded. In studies of bovine tracheal cartilage proteoglycans, it has been shown that the proportions of the two populations change with age, i.e. that during fetal life only the larger, chondroitin sulphate-rich, proteoglycan can be electrophoretically detected, whereas during maturation the proportion of the smaller, keratan sulphate-rich, proteoglycan increases (Inerot & Heinegård, 1983). After an age of about 20 months the two proteoglycans remain constant at proportions much like those found in the nasal cartilage studied in the present work. It is

therefore quite possible that the two proteoglycans differ in their function in the cartilage. Interestingly, they have somewhat different extractability in associative solvents (D. Heinegård, unpublished work).

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