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Matrix Proteins Bound to Associatively Prepared Proteoglycans from Bovine Cartilage

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Proteoglycans were extracted from bovine tracheal cartilage by high-speed homogenization, the use of dissociative solvents being avoided. The homogenate was fractionated by gel chromatography, sucrose-density-gradient centrifugation and ion-exchange chromatography. A previously unrecognized protein, cartilage matrix protein, was identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It co-fractionated with the proteoglycans in all systems, indicating an interaction. The cartilage matrix protein–proteoglycan complex was dissociated by treatment with 4M-guanidinium chloride. The complex again formed when the guanidine was removed. The cartilage matrix protein has a mol.wt. of more than 200000. On reduction it yields subunits with a mol.wt. of approx. 60000.

During the last few years a more detailed picture of the molecular structure of cartilage proteoglycans and collagen has evolved. One of the main cartilage constituents, collagen, forms a fibrillar network in which aggregates of the other major constituent, cartilage proteoglycans, are entangled. The aggregates are formed by the specific interaction between hyaluronic acid and the protein-rich hyaluronic acid-binding region of the proteoglycan monomer (Hascall & Heinegård, 1974*a*,*b*; Heinegård & Hascall, 1974; Hardingham & Muir, 1972, 1973*a*,*b*). The interaction is stabilized by link proteins (Keiser *et al.*, 1972; Hascall & Heinegård, 1974*b*; Baker & Caterson, 1977; Caterson & Baker, 1978). Along one molecule of hyaluronic acid a large number of proteoglycan monomers can be bound.

In most studies of cartilage proteoglycans the dissociative preparation procedure designed by Hascall & Sajdera (1969) has been used. This involves extraction with concentrated solutions of chaotropic salts such as guanidinium chloride or $MgCl_2$, followed by separation of proteoglycans from other cartilage components by equilibrium centrifugation in a CsCl gradient. Such procedures have been shown to give a proteoglycan preparation less degraded than the one obtained when proteoglycans were solubilized by high-speed homogenization in water or weak salt solutions, techniques commonly used earlier (Malawista & Schubert, 1958). The dissociative preparation procedure, however, has some inherent disadvantages, when detailed studies of the interactions forming the proteoglycan aggregate and the stoicheiometry of the participating molecules are attempted. Aggregates are obtained by recombining the dissociated components in the guanidinium chloride extract by lowering the guanidine concentration. It cannot be taken for granted that the recombined aggregates are in all features identical with the native ones. The spacing and the relative distribution of the constituents along the hyaluronic acid might be altered in the recombined aggregate. Proteins participating in the organization of the three-dimensional network of proteoglycans and collagen in the tissue might be irreversibly denatured or otherwise lost in the preparative procedure.

The purpose of the present study was to demonstrate cartilage proteins, other than the link proteins, capable of binding to proteoglycans. To avoid dissociative solvents proteoglycans were prepared by high-speed homogenization. The proteoglycans obtained were purified by mild physicochemical methods and the proteins purified with the proteoglycans were studied.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Materials

Tracheal rings from adult cows were obtained directly from the slaughterhouse. The tracheal cartilage was dissected free from perichondrium and other surrounding tissue, frozen in liquid N_2 and ground in a Wiley mill. This procedure was completed within a few hours after slaughter. The ground cartilage was stored frozen at −20°C until used.

Sepharose gels and protein standards for molecular-weight determination were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (Whatman DE-52) was from W. and

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R. Balston, Maidstone, Kent, U.K.; chondroitinase ABC (Seikagaku) was from Miles Laboratories, Elkhart, IN, U.S.A.; papain (type III) was from Sigma Chemical Co., St. Louis, MO, U.S.A. All chemicals used were of analytical grade.

Preparative procedure

Ground cartilage (10g) was suspended in 200ml of ice-cold 0.15M-NaCl / 5mM-sodium phosphate / 10mM-EDTA / 0.1M-6-aminohexanoic acid / 1mM-phenylmethanesulphonyl fluoride, pH7.4, and homogenized for 30min at top speed in a Sorvall Omnimixer. During homogenization the sample container was submerged in an ice/water mixture. The homogenate was clarified by centrifugation for 30min at 25000$g_{av.}$ at 4°C. The pellet was suspended in 200ml of 4M-guanidinium chloride/5mM-sodium phosphate/10mM-EDTA/0.1M-6-aminohexanoic acid/1mM-phenylmethanesulphonyl fluoride, pH7.4, and stirred at 4°C for 20h. Unextractable material was recovered by centrifugation as above, extensively dialysed against distilled water and freeze-dried. In a control experiment the homogenization was omitted and the cartilage was directly extracted with 4M-guanidinium chloride containing the proteinase inhibitors. In all instances, portions of homogenate and extracts not immediately used were frozen and not thawed until further processed.

Analytical methods

Hexuronic acid and protein. Hexuronic acid content in column effluents was determined by an automated version (Heinegård, 1973) of the carbazole procedure (Bitter & Muir, 1962). Protein was determined as the absorbance of solutions at 280nm.

Chondroitin sulphate. Chondroitin sulphate content was determined after papain digestion of samples in 0.05M-sodium phosphate / 5mM-EDTA / 5mM-cysteine hydrochloride, pH6.5. Approx. 2μl of a crystalline papain suspension (25mg of protein/ml) was used per mg of material. Samples were digested for 72h at 60°C and then applied to an ion-exchange DEAE-cellulose column, which was eluted with 0.02M-HCl and 6M-HCl. The 6M-HCl eluate contained the chondroitin sulphate and was immediately evaporated to dryness. These samples were then hydrolysed in 4M-HCl under argon at 100°C for 10h. The content of galactosamine in the hydrolysates was determined by using an automatic amino acid analyser and taken as a measure of chondroitin sulphate content.

Gel chromatography. Sepharose CL-2B columns (0.8cm×150cm) were eluted at 4°C with 0.15M-NaCl/5mM-sodium phosphate, pH7.4 (associative buffer), and 4M-guanidinium chloride/5mM-sodium phosphate, pH7.4 (dissociative buffer), respectively. Fractions of volume about 1.2ml were collected.

Sucrose-density-gradient centrifugation. A 1ml portion of homogenate was layered on a continuous gradient (10ml) of 10–50% (w/v) sucrose in the associative buffer. The sample was centrifuged for 6h at 38000 rev./min (180000$g_{av.}$) at 18°C in an MSE SS65 ultracentrifuge with the 6×14ml titanium swing-out rotor. Fractions of volume 1ml were collected from the bottom of the tubes by using an LKB Varioperpex peristaltic pump and a drop counter. Before analysis the sucrose was removed by extensive dialysis against water.

Ion-exchange chromatography. A column (10ml packed volume) of DEAE-cellulose was equilibrated with the starting buffer (0.15M-NaCl/5mM-sodium phosphate, pH7.4). The sample (1ml of cartilage homogenate) was applied and the column was eluted with 20ml of starting buffer at 4°C. Elution was continued with a linear gradient of 0.15–1.0M-NaCl/5mM-sodium phosphate, pH7.4, the total volume being 100ml. Fractions of volume approx. 2.25ml were collected.

Gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed on 8% acrylamide gels essentially as described by Neville (1971). The samples had to be pretreated with chondroitinase ABC as follows. Freeze-dried samples were dissolved in 0.1M-Tris/0.1M-sodium acetate, pH7.3. Chondroitinase ABC (4munits/mg of sample) was added and the digestion mixtures were incubated at 37°C for 6h. The digests were then dialysed against distilled water, freeze-dried, dissolved in twice-concentrated upper-reservoir buffer supplemented with 10% (w/v) sucrose and 0.01% EDTA (when reducing conditions were desired also containing 10% 2-mercaptoethanol) and incubated at 37°C for 2h before electrophoresis. The gels were stained with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Phosphorylase *b* (mol.wt. 94000), serum albumin (mol.wt. 67000), catalase subunit (mol.wt. 60000), ovalbumin (mol.wt. 43000), lactate dehydrogenase subunit (mol.wt. 36000), carbonic anhydrase (mol.wt. 30000) and soya-bean trypsin inhibitor (mol.wt. 20100) were used as standards for molecular-weight determination.

Results

Preparation

In the preparative procedure, two soluble fractions, the homogenate and the subsequent guanidinium chloride extract, were obtained. The distribution of chondroitin sulphate between the extracts and the residue was determined as described in the Experimental section (Table 1). For comparison the corresponding results from a direct extraction with 4M-guanidinium chloride with omission of the homogenization step are given. Almost half of the tissue proteoglycans were recovered in the homogenate and another 48% were extracted with 4M-

Table 1. *Distribution of chondroitin sulphate in extracts and residue*

Experimental details are given in the text. Chondroitin sulphate was determined as galactosamine and the distribution expressed as percentage of total recovered tissue galactosamine.

	Distribution of chondroitin sulphate (%)	
	Homogenization followed by guanidine extraction	Guanidine extraction
Homogenate	46.1	
Guanidine extract	47.5	95.0
Residue	6.4	5.0

guanidinium chloride. Only 6.4% of the proteoglycans remained in the residue.

Portions of the homogenate and the subsequent guanidine extract were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 1). In this system most of the proteins of the samples were resolved. The proteoglycans are, however, excluded from the gels and remain at the top of the spacer gel, even after chondroitinase ABC treatment (Fig. 1). In the top portion of the gels one or two bands, probably representing small amounts of α-chains of collagen, can be seen. The most intensely stained band migrates with an apparent molecular weight of 60000. This protein was designated cartilage matrix protein. It was present both in the homogenate and in the subsequent guanidine extract. When less sample was applied it was resolved into two bands, with apparent mol.wts. of 61000 and 59000. The link proteins were also detected in both extracts. Link protein *a* has an apparent mol.wt. of 49500 and link protein *b* one of 45500. Occasionally a faint band was observed at the position corresponding to a mol.wt. of 43500. This component probably represents a subfraction of link protein *b* and may correspond to the protein that Baker & Caterson (1977) suggested to be a third link protein. Three protein bands with mol.wts. of 25000–35000 were observed. At least one of them (mol.wt. 35000) was always present in homogenates and extracts.

The homogenate and the subsequent guanidine extract were also analysed by SDS/polyacrylamide-gel electrophoresis without prior treatment with 2-mercaptoethanol (Fig. 1). A high-molecular-weight protein, not present in the reduced samples, appeared near the top of the gels. Very little protein, on the other hand, migrated to the position of the reduced cartilage matrix protein. Therefore it appears that the intact cartilage matrix protein has a mol.wt. of more than 200000 and may be dissociated to yield subunits when disulphide bridges are reduced. Another component migrated with a somewhat higher

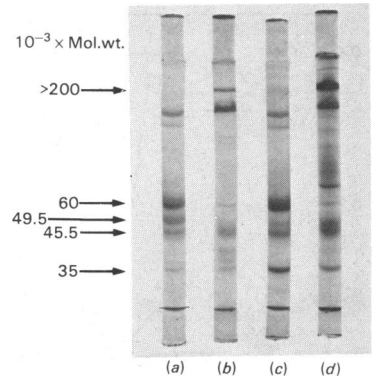


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of extracts of tracheal cartilage*

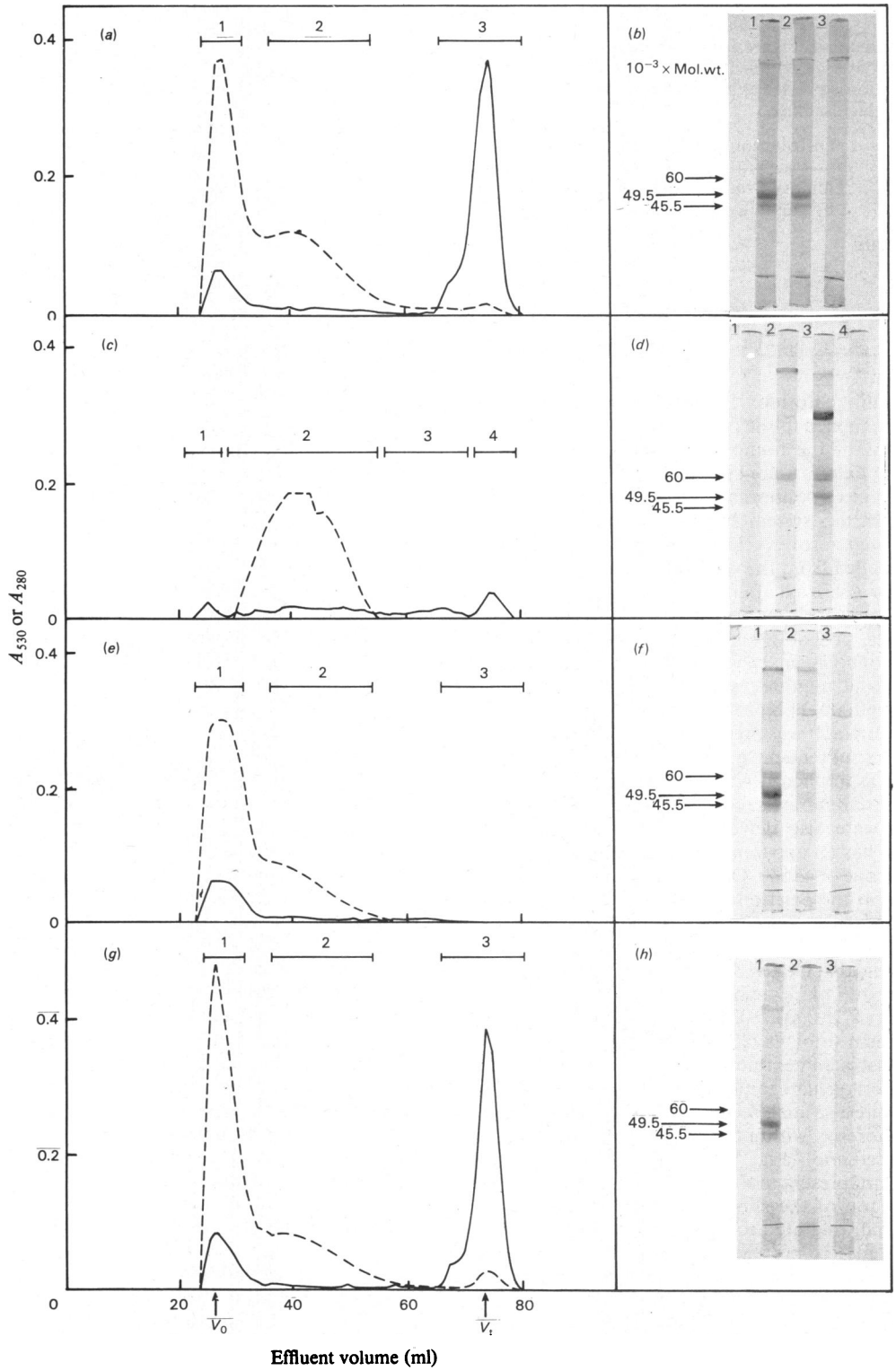
Experimental details are given in the text. (*a*) Homogenate treated with 2-mercaptoethanol; (*b*) identical sample not treated with 2-mercaptoethanol. (*c*) 4M-Guanidinium chloride extract of homogenization residue treated with 2-mercaptoethanol; (*d*) identical sample not treated with 2-mercaptoethanol.

mobility to the position of collagen α-chains with an apparent mol.wt. of about 130000. Some protein did not penetrate the separation gel when the samples were not reduced. This protein may contribute to the bands observed at the position of the matrix protein subunits with the reduced samples.

Gel chromatography

The SDS/polyacrylamide-gel electrophoresis of the homogenate revealed the existence of several tissue proteins in addition to the link proteins. Therefore it was relevant to determine whether these proteins could interact with the proteoglycans. For this purpose, freshly prepared homogenate (1 ml) was fractionated by sucrose-density-gradient centrifugation, ion-exchange chromatography and gel chromatography. One portion was chromatographed on Sepharose CL-2B in the associative buffer. The proteoglycans were eluted in a typical pattern with an excluded peak of aggregates and an included peak of monomers (Heinegård, 1972) (Fig. 2*a*). Only very small amounts of uronic acid could be detected being eluted late in the chromatogram, indicating that degradation during homogenization was negligible. The peak of u.v.-absorbing material at the total volume of the column was at least partly due to the proteinase inhibitors present in the homogenization buffer. The fractions were pooled as indicated in Fig. 2(*a*), dialysed against distilled water and freeze-dried.

Samples representing an equal proportion of the material in each pool were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 2*b*). Both the link proteins and the cartilage


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
1
2
3
4
0.4
0.2
0
10^{-3} × Mol.wt.
60
49.5
45.5
A_{530} or A_{280}
20
40
60
80
V_0
V_t
Effluent volume (ml)

matrix protein were observed in both the aggregate and in the monomer fractions. Only small amounts of protein were detected in the third pool containing molecules of low molecular weight. The results show that not only the link proteins but also the cartilage matrix protein chromatograph with the larger proteoglycans, indicating an interaction.

A sample of the homogenate was transferred to dissociative conditions by dialysis against 4M-guanidinium chloride/5mM-sodium phosphate, pH7.4, and then chromatographed on a Sepharose CL-2B column eluted with 4M-guanidinium chloride (Fig. 2*c*). The fractions were pooled as indicated. The SDS/polyacrylamide-gel electrophoresis (Fig. 2*d*) of samples representing identical proportions of the pools showed that most of the cartilage matrix protein was eluted in a peak distinct from and more retarded than the proteoglycans. It appears, then, that the interaction of the matrix protein with the proteoglycans is broken by 4M-guanidinium chloride.

To determine whether the dissociation was reversible, another sample of the homogenate was first dissociated as described above and then dialysed back into the associative buffer. When chromatographed on Sepharose CL-2B in the associative buffer it showed a uronic acid profile similar to that of the untreated homogenate (Fig. 2*e*). SDS/polyacrylamide-gel-electrophoretic analysis of identical samples of the pools demonstrated that most of the cartilage matrix protein was eluted with the proteoglycans, indicating that the protein could again interact when the dissociating solvent was removed (Fig. 2*f*).

To determine whether the homogenate contained endogenous proteinases that were not inhibited by the proteinase inhibitors added, a sample was left at room temperature for 48h before chromatography on Sepharose CL-2B in the associative buffer (Fig. 2*g*). No additional uronic acid-containing peak that was eluted late was observed, indicating that the proteoglycans were not degraded during incubation. Indeed the proportion of excluded material increased, indicating that a larger amount of proteoglycan monomers was included in aggregates. SDS/polyacrylamide-gel-electrophoretic analysis revealed that virtually all of the cartilage matrix protein and the link

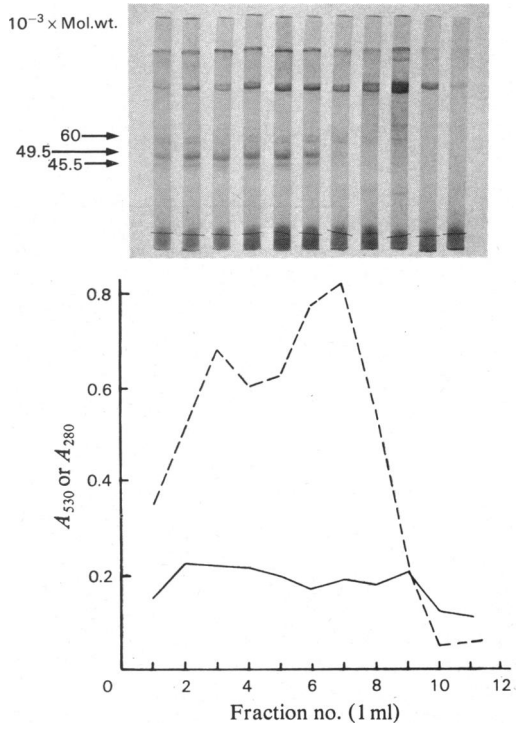


Fig. 3. *Sucrose-density-gradient centrifugation of homogenate of tracheal cartilage*

A 1ml sample of homogenate was subjected to sucrose-density-gradient centrifugation as described in the text. The fractions were analysed by SDS/polyacrylamide-gel electrophoresis after treatment with 2-mercaptoethanol. ----, A_{530} (carbazole reaction); ——, A_{280} (protein).

Fig. 2. *Sepharose CL-2B chromatography of cartilage homogenates*

Experimental details are given in the text. Sepharose CL-2B gel chromatography of homogenate is shown in (*a*). The column was eluted with the associative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (*b*) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate transferred to the dissociative buffer by dialysis is shown in (*c*). The column was eluted with the dissociative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (*d*) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate first dissociated by dialysis against the dissociative buffer and then reassociated by dialysis against the associative buffer is shown in (*e*). The column was eluted with the associative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (*f*) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate incubated at room temperature for 48h before chromatography is shown in (*g*). The column was eluted with the associative buffer. The fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (*h*) after treatment with 2-mercaptoethanol. Gels of the pools are shown with increasing number from left to right. ----, A_{530} (carbazole reaction); ——, A_{280} (protein). V_0, Void volume; V_t, total volume.

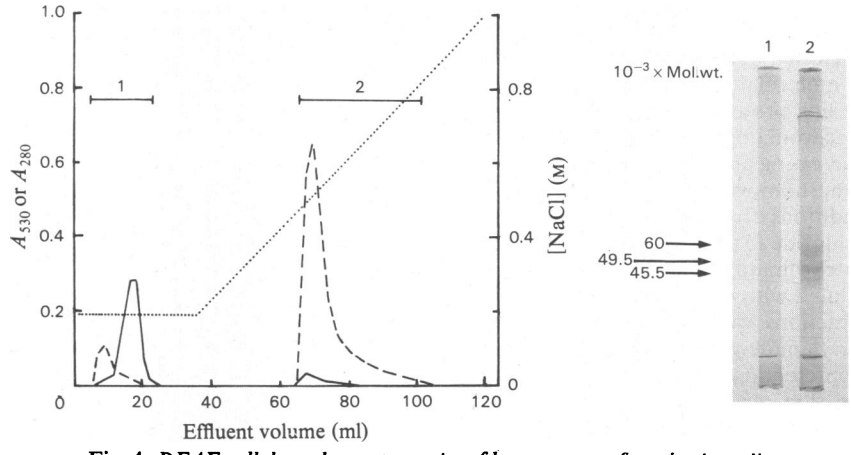


Fig. 4. *DEAE-cellulose chromatography of homogenate of tracheal cartilage*

A 1 ml sample of homogenate was subjected to DEAE-cellulose chromatography as described in the text. The column (packed volume 10 ml) was first eluted with 20 ml of 0.15 M-NaCl/5 mM-sodium phosphate, pH 7.4, and then with a linear gradient of 0.15–1.0 M-NaCl/5 mM-sodium phosphate, pH 7.4. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis after treatment with 2-mercaptoethanol. ----, A_{530} (carbazole reaction); ——, A_{280} (protein); ·····, concn. of NaCl.

proteins were eluted in the aggregate fraction (Fig. 2*h*). The lower proportion of aggregates in the homogenate may be due to a limited disruption of aggregates during homogenization, liberating proteoglycan monomers that were capable of forming aggregates when incubated. Another possibility is that a fraction of the proteoglycans in the tissue are unable to bind to hyaluronic acid for steric reasons. When the collagen network is disrupted during homogenization they are no longer hindered and may eventually bind to hyaluronic acid.

Sucrose-density-gradient centrifugation

Another method used to fractionate the components of the homogenate was sucrose-density-gradient centrifugation. The proteoglycans were distributed in a large number of fractions (Fig. 3). The faster-sedimenting aggregates can, however, be distinguished from more slowly sedimenting proteoglycan monomers. The absorbance at 280 nm shows a minor peak at the position of the aggregates. The content of various proteins in the fractions was studied by SDS/polyacrylamide-gel electrophoresis (Fig. 3). The cartilage matrix protein co-distributed with both the proteoglycan aggregates and the proteoglycan monomers, whereas the link proteins were predominantly present in the fractions containing aggregates. Some collagen was found even in the rapidly sedimenting fractions. Whether this was due to collagen–proteoglycan interactions was not further studied.

Ion-exchange chromatography

Both gel chromatography and sucrose-density-gradient centrifugation separate molecules according to parameters related to their weight and shape. Therefore it was possible that the co-fractionation of the cartilage matrix protein with the proteoglycans was due to the formation of high-molecular-weight homoaggregates of this protein. On ion-exchange chromatography, however, proteins are eluted at a lower ionic strength than are the highly charged proteoglycans, unless the components are associated by salt-resistant bonds. DEAE-cellulose chromatography resolved the homogenate into two fractions (Fig. 4). The cartilage matrix protein was eluted with the link proteins and the proteoglycans, late in the gradient (Fig. 4), indicating that these components form complexes. This further supports the existence of an interaction between the cartilage matrix protein and the proteoglycans. Furthermore, the interaction seems to be stable also at higher concentrations of NaCl.

Discussion

The choice of gel chromatography and sucrose-density-gradient centrifugation as means of fractionating the cartilage homogenate was determined by a desire to isolate proteoglycans in an ionic environment as close to the physiological one as possible, thereby preserving even weak interactions with other structural components. By this approach a previously

unrecognized protein, present both in tracheal and nasal cartilage (M. Paulsson & D. Heinegård, unpublished work), could be shown to co-fractionate with the proteoglycans in both fractionation procedures. This protein, the cartilage matrix protein, has a high molecular weight and contains what seem to be two different kinds of subunits (with apparent mol.-wts. of 61000 and 59000) probably joined by disulphide bonds. The stoicheiometry of the complex is not yet known. Currently it cannot be precluded that the matrix protein represents more than one protein component not resolved by the techniques used. Only one or two different protein subunits were, however, observed on reduction. The presently available data indicate that the cartilage matrix protein interacts with the proteoglycan monomers as well as with the aggregates. The fact that the protein remains bound to the proteoglycans during ion-exchange chromatography when the ionic strength is substantially increased implies that the interaction is relatively stable. The high concentration of salt used in the conventional CsCl-density-gradient centrifugation, however, breaks the interaction, even though small amounts of cartilage matrix protein can occasionally be detected in the A1 fraction. Most previous investigations of cartilage proteins have been focused on proteins that can be dissociated from the A1 fraction. Therefore the cartilage matrix protein has escaped our attention until recently.

Homogenates and extracts of cartilage also contain other proteins, which, even if they interact with proteoglycans to a smaller extent, still might contribute to stabilizing the proteoglycan aggregates and the proteoglycan–collagen interactions. It is likely that the characteristic properties of cartilage, such as elasticity, are to a large extent the result of multiple interactions between the molecular constituents of the tissue.

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