Interactions between Different Corneal Proteoglycans

By PIETRO SPEZIALE, MARIACRISTINA SOSSO SPEZIALE,
LEONARDO GALLIGANI and CESARE BALDUINI

Institute of Biological Chemistry, Faculty of Sciences, University of Pavia, 27100 Pavia, Italy

(Received 24 January 1978)

Proteoglycans were extracted from bovine cornea with 4M-guanidinium chloride and purified by CsCl-density-gradient centrifugation. Under associative conditions two fractions were found: one capable of forming assemblies of high molecular weight and another lacking this property. The heavier fraction (density 1.59g/ml) was eluted as a single retarded peak from Sepharose 2B, but on DEAE-Sephadex chromatography, gave two peaks: the first (eluted with 0.75M-NaCl) contained mainly proteochondroitin sulphate and the second (eluted with 1.25M-NaCl) mainly proteokeratan sulphate. Each of these proteoglycans was more retarded on Sepharose 2B than was the original sample from density-gradient centrifugation. Re-aggregation was obtained by recombination of the two fractions. The lighter fraction (density 1.44g/ml), containing predominantly keratan sulphate chains, was eluted from DEAE-Sephadex as a single peak with 1.25M-NaCl and was retarded on Sepharose 2B: this fraction was not able to form aggregates with proteochondroitin sulphate. Chemical analyses of the carbohydrate and protein moieties of the proteoglycans from DEAE-Sephadex confirmed that, in the cornea, different subunits are present with characteristic aggregation properties and hydrodynamic volumes.

Proteoglycans are typical macromolecules of the extracellular spaces and are therefore present in greatest abundance in all connective tissues. Most of the studies on their structure have been carried out on proteoglycans extracted from cartilage tissues. These macromolecules show a large polydispersity and heterogeneity and are able to form assemblies of high molecular weight, which probably have significance in the organization of extracellular matrices (Hascall & Sajdera, 1969; Sajdera & Hascall, 1974; Heinegård & Hascall, 1974; Muir & Hardingham, 1976; Hardingham *et al.*, 1976).

Little is known about the structure of corneal proteoglycans, although descriptions of the polysaccharide part of the molecules have been given. It was reported that at least two different proteoglycans can be separated, the first enriched in keratan sulphate chains and the second containing mainly chondroitin sulphate and dermatan sulphate (Axelsson & Heinegård, 1975). Our knowledge is incomplete with respect to the aggregation properties of the subunits and to the hydrodynamic volume of the aggregates.

In the present investigation proteoglycans were extracted under both associative and dissociative conditions from bovine cornea and the chemical composition of the different subunits and their ability to interact to form aggregates was studied.

Experimental

Bovine corneas were excised directly at the slaughterhouse after the death of the animals and kept in cold 0.05M-Tris/HCl buffer, pH7.5. The cleaned tissue was cut into thin slices and extracted at room temperature (20°C) for 30h with 10vol. of 0.05M-Tris/HCl buffer, pH7.5, containing 4M-guanidinium chloride and 10mM-EDTA. The extract was then filtered through gauze (Hascall & Sajdera, 1969).

Density-gradient centrifugation

For dissociative density-gradient centrifugation solid CsCl was added to the 4M-guanidinium chloride extract to obtain a final density of 1.5g/ml. For associative density-gradient centrifugation the 4M-guanidinium chloride extract was dialysed overnight against 9vol. of 0.05M-Tris/HCl buffer (pH7.5)/10mM-EDTA, then solid CsCl was added to a final density of 1.5g/ml.

Density determinations were done by weighing in a 50 μ l constriction pipette.

Density-gradient centrifugation was performed in 5ml tubes at 20°C for 44h at 40000 rev./min (192000 g) in a Spinco-Beckman L2 50B ultracentrifuge with a SW 50.1 rotor (Hascall & Sajdera, 1969). Five 1ml

fractions were collected from each tube with a Pasteur pipette and numbered 1–5 starting from the bottom. After density determination, each fraction was dialysed overnight against 0.05 M-Tris/HCl buffer, pH 7.5.

Gel chromatography

Proteoglycans were chromatographed on a column (0.9 cm × 50 cm) of Sepharose 2B, and eluted with 0.05 M-sodium acetate buffer, pH 5.8, containing 0.5 M-guanidinium chloride. A constant flow rate of 8 ml/h was obtained with a peristaltic pump. Fractions (1 ml) were collected and the hexuronate and hexose contents measured. Proteoglycan aggregates from nasal septum were used as markers of the void volume of the column.

Fractionation of proteoglycans on DEAE-Sephadex A-25

Corneal proteoglycans obtained after associative CsCl-density-gradient centrifugation were dialysed overnight against 0.05 M-Tris/HCl buffer, pH 7.5. The samples were then applied to the column, and a stepwise elution was carried out with water and 0.25 M-, 0.5 M-, 0.75 M-, 1.00 M-, 1.25 M-, 1.5 M-, 1.75 M-, 2.00 M- and 3.00 M-NaCl solutions in 0.05 M-sodium phosphate buffer, pH 6.9 (Bettelheim & Plessy, 1975); samples (15 ml) of each solution were run through the column. All operations were carried out at 4°C.

The fractions were then extensively dialysed against water, dried under vacuum with a rotary evaporator, redissolved in a known volume of water and analysed for hexose, hexuronate, hexosamine, protein and sulphate.

Re-aggregation experiments

Proteoglycans (subunits) recovered after DEAE-Sephadex chromatography were redissolved in a known volume of 0.05 M-sodium acetate buffer, pH 5.8.

Appropriate amounts of the different solutions were mixed together to give a suitable glucosamine/galactosamine ratio (glucosamine/galactosamine, 1.15). The mixtures were incubated at 20°C for 2 h and were then chromatographed on a Sepharose 2B column under the conditions reported above. The K_{av} of the proteoglycans before and after re-aggregation were determined.

Analytical methods

Hexuronate was determined by the carbazole method of Bitter & Muir (1962). Neutral sugars were determined by the method of Trevelyan & Harrison

(1952). Protein was measured by the method of Lowry *et al.* (1951), with serum albumin as standard, and sulphate groups were determined by the method of Terho & Hartiala (1971). Amino acid analyses were performed on a Hitachi-Perkin-Elmer chromatography apparatus by using a Hitachi spherical-resin N.2610 column. Samples were hydrolysed for 24 h at 100°C in 6 M-HCl in sealed glass tubes under N₂. Hexosamines were determined by using the same automatic amino acid analyser. Samples were hydrolysed for 6 h at 100°C in 4 M-HCl in sealed glass tubes under N₂. No corrections were made for the destruction during hydrolysis.

Results

Extraction and purification of proteoglycans by isopycnic centrifugation

In Fig. 1 the distribution of hexosamines and proteins in the CsCl density gradient, under both associative and dissociative conditions, is reported. Under associative conditions hexosamines were mainly recovered in two fractions of the gradient: fraction 2 (density 1.59 g/ml) contained both glucosamine and galactosamine in nearly equal amounts; in fraction 4 (density 1.44 g/ml) mainly glucosamine was present (Fig. 1b). Under dissociative conditions only a broad peak of material was found, corresponding to fraction 4 (density 1.44 g/ml), with coincidence of galactosamine and glucosamine (Fig. 1a).

When fraction 2 obtained from associative density-gradient centrifugation was centrifuged again under dissociative conditions a shift towards lower densities (fraction 4) of the gradient was observed (Fig. 1c).

DEAE-Sephadex chromatography after associative density-gradient centrifugation

In Fig. 2 the elution pattern from DEAE-Sephadex of the proteoglycans obtained under associative conditions from CsCl-density-gradient centrifugation (fractions with densities 1.59 and 1.44 g/ml) is reported.

The heavier fraction (density 1.59 g/ml) gave two peaks: the first, eluted with 0.75 M-NaCl (PG-A), contained mainly galactosamine and the second, eluted with 1.25 M-NaCl (PG-B), was enriched in glucosamine (Fig. 2a).

The lighter proteoglycans (density 1.44 g/ml), chromatographed under the same conditions, were eluted with 1.25 M-NaCl in a single peak (PG-C), containing a predominant amount of glucosamine (Fig. 2b). Chemical analysis of the fractions obtained (Table 1) confirmed that in the 0.75 M-NaCl eluate mainly proteochondroitin sulphate was present, whereas in the two fractions eluted with 1.25 M-NaCl

Fig. 1. CsCl-density-gradient centrifugation of corneal proteoglycans

(a) Dissociative gradient (4M-guanidinium chloride); (b) associative gradient (0.4M-guanidinium chloride); (c) dissociative gradient centrifugation of the fraction with density 1.59 from (b). Centrifugations were performed as described in the text. □, Galactosamine; ■, glucosamine; ▤, protein.

proteokeratan sulphate seems to be the main constituent.

Small differences exist between the acidic and neutral amino acid contents of the three proteoglycans, and greater variations occur in the basic amino acid content.

Gel filtration on Sepharose 2B

Fraction 2 from the associative CsCl density gradient was retarded on Sepharose 2B and was eluted from the gel in a single peak with a K_{av} of 0.429 (Fig. 3a).

The elution patterns on the same gel of fractions PG-A and PG-B obtained after DEAE-Sephadex

Fig. 2. DEAE-Sephadex chromatography of the fractions obtained from associative CsCl-density-gradient centrifugation

(a) Elution pattern of the heavier peak (density 1.59 g/ml); (b) elution pattern of the lighter peak (density 1.44 g/ml). Chromatography was carried out as reported in the text. □, Galactosamine; ■, glucosamine; ▤, protein.

chromatography of fraction 2 were indicative of K_{av} values of 0.714 and 0.850 respectively (Figs. 3b and 3c). This seems to indicate that fractions PG-A and PG-B were present in fraction 2 as an aggregate, which was resolved into the two subunits by DEAE-Sephadex chromatography.

Recombination experiments (Figs. 3d and 3f) confirmed that aggregates were present in fraction 2. By mixing together fractions PG-A and PG-B, so as to obtain the same glucosamine/galactosamine ratio present in the original aggregate, a complete re-aggregation occurred (Fig. 3d), and a K_{av} value identical with the original one was found. When fractions PG-A and PG-B were mixed together, so as to obtain a glucosamine/galactosamine ratio different from that present in the original aggregate, only a partial re-aggregation of proteoglycans occurred.

Fraction PG-C, obtained after DEAE-Sephadex chromatography of fraction 4 from associative

Table 1. *Chemical composition of corneal proteoglycans fractionated on DEAE-Sephadex after associative density-gradient centrifugation*

Values reported for the polysaccharide moiety are average $\pm$ s.d. of three experiments. Amino acid content was determined in two experiments. Fractions PG-A, PG-B and PG-C were obtained as reported in the text.

Fractions	Content ($\mu\text{mol}/1000\mu\text{mol}$)		
	PG-A	PG-B	PG-C
Hexuronate	149.0 $\pm$ 7.0	39.7 $\pm$ 7.8	17.7 $\pm$ 6.6
Galactosamine	141.7 $\pm$ 9.6	39.7 $\pm$ 8.9	12.1 $\pm$ 2.5
Glucosamine	22.3 $\pm$ 2.4	91.9 $\pm$ 5.4	55.7 $\pm$ 2.3
Hexose	Traces	118.6 $\pm$ 9.6	62.4 $\pm$ 0.6
SO ₄ ²⁻	107.2 $\pm$ 4.8	54.0 $\pm$ 8.6	30.5 $\pm$ 0.8
Aspartic acid	74.8	98.6	96.6
Threonine	24.6	32.9	41.1
Serine	43.1	49.8	54.6
Glutamic acid	59.7	83.5	92.4
Proline	43.1	48.9	46.6
Glycine	44.8	43.2	50.5
Alanine	28.7	37.1	50.2
Valine	31.7	27.5	40.5
Methionine	7.7	12.0	3.0
Isoleucine	31.1	29.8	33.9
Leucine	65.5	83.5	73.5
Tyrosine	13.5	19.7	24.2
Phenylalanine	21.1	27.0	26.1
Lysine	47.5	24.7	80.2
Histidine	19.6	4.6	26.1
Arginine	17.6	4.3	58.6

density-gradient centrifugation, was retarded by Sepharose 2B with a K_{av} of 0.932 (Fig. 3e). Moreover, this fraction showed only a very low degree of interaction with fraction PG-A: the mixture of the two proteoglycans in different ratios, in fact, led to the formation of only a very small percentage of aggregates (Fig. 3f).

Discussion

At least three proteoglycans can be isolated from bovine cornea after extraction with 4 M-guanidinium chloride, CsCl-density-gradient centrifugation under associative conditions and DEAE-Sephadex fractionation of the peak materials from the gradient. The first proteoglycan (PG-A) seems to be composed mainly of chondroitin sulphate chains and the second (PG-B) contains a higher amount of keratan sulphate chains (glucosamine/galactosamine ratio, 2:1).

These two types of macromolecules are linked together to form an aggregate such as resulted from CsCl-density-gradient centrifugation and Sepharose 2B gel filtration; they can be separated under dissociative conditions or simply by ion-exchange chromatography on DEAE-Sephadex.

Both the subunits and the aggregate are completely retarded on Sepharose 2B, indicating that their hydrodynamic volume is significantly smaller than that of the proteoglycans extracted from several

cartilage tissues. Fractions PG-A and PG-B, when mixed together so as to obtain the same glucosamine/galactosamine ratio as the original aggregate, can completely recombine, as proved by Sepharose 2B gel filtration. However, if the optimum fraction PG-A/PG-B ratio is not reproduced in the mixture, the re-aggregation is not complete and results in a smaller aggregate.

Fraction PG-B is smaller than fraction PG-A; the amino acid compositions of the two subunits differ significantly, especially with respect to basic residues, indicating that two different subunits can interact to form the aggregate.

Fraction PG-C is the third proteoglycan that can be separated from bovine cornea; it bands at lower density under associative conditions. DEAE-Sephadex chromatography of this fraction results in a single peak, which is enriched in keratan sulphate chains, but shows a higher glucosamine/galactosamine ratio (3:1) than fraction PG-B and a different amino acid composition. Moreover, it is smaller than fractions PG-A and PG-B, and shows only a very small interaction with fraction PG-A, as proved by the re-aggregation experiments.

The question arising from our results concerns the reasons why fractions PG-A and PG-B, but not fraction PG-C, can interact together: it can be supposed that the mechanisms of aggregation of corneal proteoglycans are different from those of

cartilage proteoglycans, but at present nothing is known about the type of interactions occurring. It must be mentioned, however, that no proteinase inhibitor, except EDTA, was used during isolation procedures, so that it cannot be excluded that a partial degradation occurred; in particular, it could be hypothesized that the inability of fraction PG-C to form aggregates depends on the action of some degradative enzyme (Pearson & Mason, 1977).

The elucidation of these problems should improve our understanding of the biological role of the different proteoglycans in the structural organization of corneal tissue.

This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Italy.

References

- Axelsson, I. & Heinegård, D. (1975) *Biochem. J.* **145**, 491-500
- Bettelheim, F. A. & Plessy, B. (1975) *Biochim. Biophys. Acta* **381**, 203-214
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330-334
- Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) *Biochem. J.* **157**, 127-143
- Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384-2396
- Heinegård, D. & Hascall, V. C. (1974) *J. Biol. Chem.* **249**, 4250-4256
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Muir, H. & Hardingham, T. E. (1976) in *Biochemistry of Carbohydrates* (Whelan, W. J., ed.), vol. 5, pp. 153-222, Butterworths, University Park Press, London
- Pearson, J. P. & Mason, R. M. (1977) *Biochim. Biophys. Acta* **498**, 176-188
- Sajdera, S. W. & Hascall, V. C. (1974) *J. Biol. Chem.* **244**, 77-87
- Terho, T. T. & Hartiala, K. (1971) *Anal. Biochem.* **41**, 471-476
- Trevelyan, W. E. & Harrison, J. S. (1952) *Biochem. J.* **50**, 298-303

Fig. 3. Gel filtration on Sepharose 2B of corneal proteoglycans

(a) Heavier peak (density 1.59 g/ml) from associative density gradient; (b) proteoglycan A (eluted from DEAE-Sephadex with 0.75 M-NaCl); (c) proteoglycan B (eluted from DEAE-Sephadex with 1.25 M-NaCl); (d) proteoglycan A plus proteoglycan B; (e) proteoglycan C (eluted from DEAE-Sephadex with 1.25 M-NaCl); (f) proteoglycan A plus proteoglycan C. The hexuronate (—) distribution was followed in (a), (b) and (d). The hexose (---) distribution was followed in (c) and (e). Both the assays are reported in (f). V_0 marks the void volume of the column. Gel filtration on Sepharose 2B was carried out in 0.05 M-sodium acetate buffer, pH 5.8, containing 0.5 M-guanidinium chloride as described in the text. K_{av} values for peaks are indicated.