Interactions between Bovine Cornea Proteoglycans and Collagen

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Two types of proteoglycan subunits were obtained from bovine cornea, the first mainly composed of proteochondroitin sulphate and the second of proteokeratan sulphate. These two fractions can be obtained from the tissue as an aggregate, and are able to recombine each other after separation, to re-form the original structure. In order to investigate collagen–proteoglycan interactions, type-I collagen was isolated from bovine cornea by pepsin digestion followed by 3.5% (w/v) NaCl precipitation, and was then linked to CNBr-activated Sepharose 4B. Two identical columns were prepared, the first filled with collagen coupled to Sepharose 4B, the second with free Sepharose 4B. The two proteoglycan subunits and the aggregate were chromatographed on the two gels under the same conditions; the elution profiles showed that both the aggregate and the proteochondroitin sulphate subunit are retarded by the collagen coupled to Sepharose. No interaction, however, occurred when proteokeratan sulphate subunit was run through the columns. Chondroitinase digestion of the proteoglycan samples confirmed that chondroitin sulphate chains are mainly responsible for the interaction with collagen; their removal, in fact, completely abolishes any differences between the chromatographic behaviour on the collagen–Sepharose and the control columns.

Collagen and proteoglycans are the main macromolecular components of bovine corneal stroma. Detailed information is available about the structure of these molecules; although essentially type-I collagen is present in adult bovine cornea (Katzman *et al.*, 1974; Panjwani & Harding, 1978), it was reported (Shuttleworth *et al.*, 1978) that other types are also present as minor components. Moreover type-I corneal collagen shows a higher degree of glycosylation than that from other tissues (Spiro, 1969; Grant *et al.*, 1969), and this characteristic is probably responsible for the thickness and uniformity of the fibrils, which are regularly assembled, so as to determine the transparency of the tissue. The proteoglycan components of corneal stroma consist of at least two types of macromolecules, the first mainly containing keratan sulphate chains and the second mainly chondroitin sulphate and dermatan sulphate chains (Axelsson & Heinegård, 1975, 1978; Speziale *et al.*, 1978).

The problem of proteoglycan–collagen interactions is still only partially understood, though different experimental approaches have been used. Soluble proteoglycan–collagen complexes have been isolated and characterized by several groups of workers (Steven *et al.*, 1969*a,b*; Brandt & Muir, 1971; Kobayashi & Pedrini, 1973), and the linkage

between the two macromolecules was investigated by chromatography on agarose–collagen columns (Greenwald *et al.*, 1975; Öbrink *et al.*, 1975). Some experiments, moreover, demonstrate the influence of proteoglycans on collagen-fibre formation (Disalvo & Schubert, 1966; Toole & Lowther, 1967, 1968; Mathews & Decker, 1968). It has also been reported that glycosaminoglycans interact at periodic intervals with collagen fibrils (Trelstad & Coulombre, 1971).

In our laboratory different proteoglycan subunits were isolated and their ability to form aggregates was determined (Speziale *et al.*, 1978). Carrying on these experiments, we have now studied the interactions that can occur between the various corneal proteoglycans and the corneal collagen covalently linked to agarose.

Experimental

Extraction and purification of collagen

Bovine corneas were excised directly at the slaughterhouse after the death of the animals and kept in cold 0.05 M-Tris/HCl buffer, pH 7.5.

The cleaned tissue was swollen in double-distilled water at 4°C for 24 h, and then cut into thin slices,

homogenized and extracted with 10 vol. of 0.5 M-sodium acetate at 4°C for 24 h.

After centrifugation the residue was extracted again with the same procedure with 10 vol. of 0.15 M-sodium citrate buffer, pH 3.4, then the precipitate was suspended in 10 vol. of 0.1% (v/v) acetic acid and dialysed against the same solution for 48 h.

Pepsin (Sigma Chemical Co.) digestion was carried out on this suspension for 60 h at room temperature under stirring (collagen/pepsin ratio 20:1, w/w). The residue was then discarded, and the supernatant containing the solubilized collagen was neutralized with 0.1 M-NaOH to inactivate the enzyme.

Solid NaCl was added to the solution to give a concentration of 3.5% (w/v); after 15 h at 4°C the precipitated collagen was recovered by centrifugation, solubilized in 0.05 M-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl, extensively dialysed against 1% (v/v) acetic acid and finally freeze-dried.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis was carried out by the technique of Furthmayr & Timpl (1971); the sodium dodecyl sulphate concentration was 0.2% (w/v) in the collagen sample and in the gel, 0.1% (w/v) in the eluting buffer. A 5% (w/v) polyacrylamide concentration was used.

Purification of corneal proteoglycans

Proteoglycan aggregate and the subunits were isolated by CsCl-density-gradient centrifugation and DEAE-Sephadex chromatography as previously described (Speziale *et al.*, 1978).

Chondroitinase treatment of proteoglycans

Chondroitinase ABC from *Proteus vulgaris* and chondroitinase AC from *Arthrobacter aureus* (Sigma Chemical Co.) digestions were carried out by incubating proteoglycan solution in 0.1 M-Tris/acetate buffer, pH 7.3, at 37°C for 24 h; 0.04 unit of each enzyme was used per mg of proteoglycan. [One unit of the enzymes is defined as the amount of chondroitinase that catalyses the release of 1.0 μ mol of unsaturated disaccharide/min at pH 7.3 at 37°C (Yamagata *et al.*, 1968).] Our chondroitinase preparations were assumed to be free from any detectable proteinase activity.

After digestion the sample was dialysed overnight at 4°C against 11.7 mM-NaH₂PO₄/29.37 mM-Na₂HPO₄, pH 7.2 and *I* 0.1, to remove the products of chondroitin sulphate breakdown.

Preparation of corneal glycosaminoglycans

Papain (Sigma Chemical Co.) digestion of corneal proteoglycans, after 4 M-guanidinium chloride ex-

traction and separation of glycosaminoglycans on Dowex 1 (X2) was carried out as described by Mathews & Cifonelli (1965).

Preparation of collagen-Sephadex 4B columns

After activation with CNBr, Sephadex 4B was linked to corneal collagen by the procedure of Bauer *et al.* (1971). Collagen solution and gel suspension were stirred for 20 h at 4°C; the gel was washed with distilled water until the eluate did not show any absorbance at 230 nm, and it was then packed in the column. The column was equilibrated with 41.07 mM-sodium phosphate buffer, pH 7.2 and *I* 0.1. The amount of collagen linked to the gel was determined by assaying hydroxyproline after hydrolysis of a suitable amount of the gel with 6 M-HCl for 24 h at 100°C. The amount of collagen linked to the gel was about 1 mg/g of settled wet gel.

Gel chromatography on collagen-Sephadex 4B

Two columns of identical size (0.9 cm $\times$ 50 cm) were prepared with collagen-Sephadex 4B and free Sephadex 4B. After equilibration with 41.07 mM-sodium phosphate buffer, pH 7.2 and *I* 0.1, proteoglycan and control samples were applied to the gels and eluted with the same solution; a constant elution rate of 6 ml/h was obtained by using a peristaltic pump. Fractions of volume 0.8 ml were collected, and their hexuronate, hexose and protein contents measured. All the experiments were carried out at room temperature (20°C). Thyroglobulin (Sigma Chemical Co.), aldolase, catalase (Boehringer Mannheim) and dextran grade B (mol.wt. 1.5×10^5 – 2×10^5 ; BDH Chemicals) were used as control samples.

Analytical methods

Hexuronate was determined by the carbazole method of Bitter & Muir (1962), and hexose by the anthrone method of Trevelyan & Harrison (1952).

Protein was assayed by the method of Lowry *et al.* (1951), with serum albumin as standard. Hydroxyproline was determined by the technique of Leach (1960).

Results

Gel filtration on Sephadex 4B and collagen-Sephadex 4B

As previously reported, in our experimental conditions at least three different proteoglycans samples can be obtained from bovine cornea: (1) a subunit mainly composed of proteochondroitin sulphate (PG-A); (2) a subunit mainly composed of proteokeratan sulphate (PG-B); (3) an aggregate containing both types of subunits (Speziale *et al.*, 1978).

Fig. 1. Gel filtration of corneal proteoglycans on Sepharose 4B and collagen-Sepharose 4B (a) and (b) Aggregate composed of PG-A and PG-B subunits; (c) and (d) PG-A subunits; (e) and (f) PG-B subunits. In the upper part of the Figure the chromatographic behaviour on collagen-Sepharose 4B is reported and in the lower part of the control elution profiles are shown. The hexuronate (—) distribution was followed in (a), (b), (c) and (d). The hexose (---) distribution was followed in (a), (b), (e) and (f). Two columns of identical size (0.9 cm × 50 cm) (collagen-Sepharose 4B and Sepharose 4B) were used for the experiments; the elution was carried out with 11.7 mM-NaH₂PO₄/29.37 mM-Na₂PO₄, pH 7.2 and 10.1, as reported in the text. Fractions of volume 0.8 ml were collected. V_e/V_t values for peaks are indicated; V_e is the elution volume of the sample, and V_t is the total volume of the column.

Table 1. Gel filtration on Sepharose 4B and collagen-Sepharose 4B

Proteoglycans and glycosaminoglycans extracted from cornea, control proteins and polysaccharides were chromatographed on the two columns as reported in the text. V_e/V_t values are reported.

	V_e/V_t on Sepharose 4B		<i>a/b</i> ratio
	+ collagen (a)	- collagen (b)	
Aggregate	0.853	0.609	1.40
PG-A subunits	0.975	0.706	1.38
PG-B subunits	0.878	0.902	0.97
Aggregate after chondroitinase ABC or AC digestion	0.902	0.926	0.97
PG-A subunits after chondroitinase ABC digestion	0.902	0.902	1.00
Chondroitin sulphate	0.880	0.800	1.10
Keratan sulphate	0.900	0.875	1.02
Thyroglobulin	0.768	0.768	1.00
Catalase	1.000	0.850	1.17
Aldolase	1.000	1.000	1.00
Dextran B	0.825	0.850	0.97

The chromatographic behaviour on collagen-Sepharose 4B of these proteoglycan fractions is reported in Fig. 1 and Table 1. It was observed (Figs. 1a and 1b) that the aggregate is retarded, with respect to the control, by the collagen-Sepharose gel. A similar interaction occurs when proteochondroitin sulphate (PG-A) is used for the experiments (Figs. 1c and 1d). Proteokeratan sulphate (PG-B), on the other hand, is not retarded at all by the collagen-Sepharose gel (Figs. 1e and 1f). Thyroglobulin, aldolase and dextran B behave in the same way on the two columns, though catalase is slightly retarded by the collagen-Sepharose (Table 1).

Chondroitinase digestion

After removal of chondroitin sulphate chains by chondroitinase ABC and AC digestion, the proteochondroitin sulphate completely loses the ability to be retarded by the collagen gel and is eluted in a single peak from the collagen-Sepharose and from the control column (Table 1). The same results were obtained after chondroitinase digestion of the aggregate.

Papain digestion

After removal of the protein core of the proteoglycans by enzyme treatment corneal chondroitin sulphate and keratan sulphate (Laterza *et al.*, 1967) were run through the collagen and control columns. Chondroitin sulphate was only slightly retarded by collagen gel, whereas keratan sulphate showed the same behaviour on the two columns (Table 1).

Discussion

The problem of collagen-proteoglycan interactions in bovine cornea has been only partially investigated, and the results available from the literature are therefore not conclusive; the main reason for this lack of information is reflected in the fact that only comparatively recently (Axelsson & Heinegård, 1975, 1978; Speziale *et al.*, 1978) were 'native' proteoglycans isolated from bovine cornea and characterized in their chemical and physico-chemical properties.

In our laboratory different proteoglycans were extracted by guanidinium chloride and purified by CsCl-density-gradient centrifugation and DEAE-Sephadex chromatography; two types of subunits were obtained: the first (PG-A) was mainly composed of proteochondroitin sulphate and the second (PG-B) of proteokeratan sulphate. These two fractions behave as an aggregate in 0.4 M-guanidinium chloride and are able to recombine with each other, after separation, to form the original aggregate. The results reported in the present paper seem to indicate that the proteochondroitin sulphate subunit is able

to interact with collagen coupled to Sepharose 4B. The degree of interaction is apparently similar both when PG-A fraction is chromatographed alone and when the aggregate is used for the experiments. Proteokeratan sulphate does not show any appreciable binding, at least in our experimental conditions, thus confirming the results of Conochie *et al.* (1975), who proved that keratan sulphate does not interact with collagen.

Chondroitinase digestion of the aggregate supports the evidence that polysaccharide chains of proteochondroitin sulphate are responsible for the interaction with collagen; their removal, in fact, completely abolishes any difference between the chromatographic behaviour on the collagen-Sepharose and the control column.

This interaction seems to be quite specific and not attributable to a modification of the exclusion characteristics of Sepharose when coupled to collagen, since different proteins (thyroglobulin, aldolase and catalase) and dextran B show the same behaviour on Sepharose 4B and collagen-Sepharose gels.

The protein core of the proteoglycans seems to be involved in the interaction with collagen; in fact chondroitin sulphate obtained by papain digestion of corneal proteoglycans shows only a low degree of binding with respect to proteochondroitin sulphate. The role of the protein moiety is confirmed by some preliminary results indicating that, when the protein core of the aggregates is only partially destroyed by trypsin treatment, the degree of interaction with collagen is not modified, although a partial resolution of PG-A subunits from PG-B subunits occurs.

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