Interaction of Cartilage Proteoglycans with Collagen-Substituted Agarose Gels

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1. Rat tail-tendon collagen was coupled to activated Sepharose 4B at 2.5 mg of collagen/ml of gel. Chromatographic columns of this gel were calibrated with T2 virus (V_0) and Dnp-alanine (V_i). 2. The chromatographic behaviour of cartilage proteoglycans on the collagen-substituted gel was studied under conditions of varying ionic strength. Proteoglycan subunit obtained from bovine nasal cartilage, the proteoglycan obtained after digestion with chondroitinase ABC and purified chondroitin sulphate were all retarded on the collagen gel by an interaction that was abolished at 1.0 M. Purified keratan sulphate and hyaluronic acid were not retarded. 3. A strong ionic interaction between cartilage proteoglycan and collagen was demonstrated to depend on the structure of the protein core of the proteoglycan.

The major macromolecular constituents of the extracellular matrix of cartilage are collagen and the class of macromolecules known as proteoglycans. The relationships that these molecular types bear to each other and to the water content of the matrix probably account for the physical properties of the tissue. Consequently, a variety of techniques has been used to study collagen–proteoglycan interaction. These include attempts to extract soluble collagen–proteoglycan complexes (Steven *et al.*, 1969*a,b*; Kobayashi & Pedrini, 1973; Brandt & Muir, 1971), electrophoretic studies (Mathews, 1964; Podrazky *et al.*, 1971; Anderson & Jackson, 1972), investigation of collagen-fibril formation in the presence of proteoglycans (Disalvo & Schubert, 1966; Toole & Lowther, 1967, 1968; Mathews & Decker, 1968), chromatography of chondroitin sulphate on granulated collagen artificially cross-linked by treatment with glutaraldehyde (Wasteson & Öbrink, 1968; Öbrink & Wasteson, 1971) and circular-dichroism spectroscopy of collagen–glycosaminoglycan mixtures (Gelman & Blackwell, 1974).

Affinity chromatography has been used widely as a preparative technique for purification of materials that bind to a suitable immobilized ligand. With collagen as the ligand, the method has been used to purify collagenase (Bauer *et al.*, 1971*a,b*). In the present report, we describe analytical studies of the chromatographic behaviour of a well-characterized proteoglycan from bovine nasal cartilage, and some derivatives of this proteoglycan, on a column of agarose coupled covalently with collagen as the attached ligand.

Experimental

Materials

Rat tail-tendon collagen was solubilized in 0.04% acetic acid by the method of Bachra & Fischer (1968). Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Miles Research Laboratories, Kankakee, Ill., U.S.A., supplied T2 virus (42.5 E_{260} units/ml), purified chondroitin sulphate type A, and chondroitinase ABC. Hyaluronic acid was a commercial preparation from Schwarz/Mann, Orangeburg, N.Y., U.S.A. Dr. Fred Kieras, of the Rockefeller University, kindly provided us with purified keratan sulphate. All other reagents were of the best analytical grades available.

Analytical techniques

Hydroxyproline was measured by the technique of Stegemann (1958), uronic acid by the carbazole method (Bitter & Muir, 1962) and hexose by the anthrone reaction (Yemm & Willis, 1954).

Preparation of columns

Sepharose 4B was activated with CNBr and made to react with rat tail-tendon collagen as described by Bauer *et al.* (1971*b*). After 18 h of mixing in the cold, the gel was washed with water until the absorbance of the filtrate at 230 nm was less than 0.02 and it was free of hydroxyproline. Portions of the settled gel were hydrolysed in 6 M-HCl overnight at 110°C for hydroxyproline analysis; the extent of coupling and absolute content of collagen per ml of settled gel were calculated by using a value of 9.42% for the hydroxy-

proline content of rat tail-tendon collagen (Eastoe, 1967). Samples of the gel were examined by light microscopy before and after coupling, and no evidence of gross physical disruption of the beads was noted. To assess the degree of denaturation of the collagen bound to the gel, the comparative ability of collagenase and pepsin to solubilize hydroxyproline from the coupled gel was measured. The Sepharose-bound collagen proved to be susceptible to collagenase digestion but not to pepsin proteolysis.

Sepharose 4B and its collagen-substituted derivative (2.5mg of collagen/ml of gel) were then equilibrated in sodium phosphate buffer, pH 7.2, $I = 0.1$. All experiments were performed in this buffer with the addition of NaCl to adjust the ionic strength as described below. The gels were packed at 15 cm head pressure into identical chromatographic columns (1.5 cm $\times$ 30 cm), and the columns were always operated at this pressure with collection of fractions of identical volume (0.8 ml). The flow rate of the collagen-Sepharose 4B column was generally 20 ml/h, about twice as fast as the column of Sepharose 4B.

The void volume (V_0) of the columns was determined with commercial T2 virus by monitoring the absorbance of the fractions at 260 nm. The total column volume (V_t) was measured with Dnp-alanine (Sigma Chemical Co., St. Louis, Mo., U.S.A.) by monitoring absorbance at 340 nm; the mobility of this substance on the collagen column was essentially indistinguishable from that of [^{14}C]glucose. In general the 1.5 cm $\times$ 30 cm columns, packed to bed heights of 26–28 cm, reproducibly yielded values for V_0 and V_t of 12 ml and 29.6 ml respectively.

Preparation of proteoglycans

The major proteoglycan studied in these experiments was prepared from bovine nasal cartilage (Hascall & Sajdera, 1969). The fraction referred to as proteoglycan subunit was dialysed exhaustively against deionized water and adjusted to a concentration of 1.2 mg of hexuronate/ml for routine use. For one series of experiments, the proteoglycan preparation was further fractionated by centrifugation at 26000 rev./min for 66 h ($2.50 \times 10^8 \text{ g} \cdot \text{min}$) at a starting density of 1.62 g/ml as described by Hascall & Sajdera (1970). This procedure yielded three fractions varying widely in their ratio of E_{280} /hexuronate content.

In addition, a sample of proteoglycan subunit was subjected to limit digestion with chondroitinase ABC as described by Hascall *et al.* (1972). This modified proteoglycan, referred to as proteoglycan digest, contained no appreciable hexuronate after exhaustive dialysis. The keratan sulphate content was unaffected by this procedure and its elution from the columns was monitored by the anthrone reaction.

Chromatographic technique

To study the chromatographic behaviour of various substances on either Sepharose 4B or on its collagen-substituted derivative, a sample (200–250 μl) was layered on the top of the column with a small syringe fitted with a blunt needle and a 10 cm length of flexible tubing. The total sample generally consisted of undiluted T2 virus suspension (25 μl), Dnp-alanine solution (100 μl , 1 mg/ml in 4 M-NaCl), and the substance of interest (100 μl). The small amount of 4 M-NaCl had no appreciable effect on the overall ionic strength of the system, but was helpful in ensuring even layering of the sample on the surface of the gel. Absorbance measurements on the collected fractions were made directly at 280 and 340 nm on a Beckman DU-2 spectrophotometer to detect the markers, and the fractions were then assayed by the carbazole or anthrone methods, as appropriate. Results were expressed by the parameter $K_d = (V_e - V_0)/(V_t - V_0)$ where V_e was the peak elution volume of the proteoglycan or glycosaminoglycan. At V_0 , $K_d = 0$, and at V_t , $K_d = 1$. The columns were thoroughly equilibrated with elution buffer before the actual chromatography. All experiments were performed at room temperature. The T2-virus marker gave no reactivity in the carbazole assay at concentrations equivalent to those obtained in the void-volume fractions.

For certain experiments, the proteoglycans were eluted from the collagen column with a linear gradient of ionic strength. Phosphate buffer, pH 7.2, $I = 0.1$ was the starting buffer, and the same buffer with an additional 0.2 M-NaCl was the limit buffer. In these cases, the Cl^- concentration of the fractions was assayed directly with an ion-specific electrode (Orion, Cambridge, Mass., U.S.A.) by using volumetric chloride standards to which was added proteoglycan subunit at a concentration similar to that found in the fractions.

Results

Proteoglycan subunit was eluted from unsubstituted Sepharose 4B in the void volume (Fig. 1a). On the collagen column, however, it did not appear in the first 90 fractions at $I 0.1$, giving K_d a value greater than 3.5, even though V_0 and V_t were unchanged (Fig. 1b). With the addition of NaCl, the proteoglycan was eluted at positions between V_0 and V_t as a function of ionic strength; a typical experiment at $I 0.135$ is shown in Fig. 1(c). Addition of increasing amounts of NaCl gradually lowered the K_d value until at $I 0.17$ the proteoglycan was eluted at V_0 , as it was on Sepharose 4B chromatography (Fig. 2). Recovery of the proteoglycan from the collagen column also appeared to be a function of ionic strength. At values of I greater than 0.16, when K_d was less than 0.04,

Fig. 1. Chromatographic profiles of proteoglycan subunit on columns of Sepharose 4B or collagen-substituted Sepharose 4B

Elution was with phosphate buffer, pH 7.2, I 0.1, and 0.8 ml fractions were collected. (a) Elution of proteoglycan subunit, T2 virus, and Dnp-alanine from Sepharose 4B. (b) Elution of the same mixture from the collagen column. Despite prolonged elution, proteoglycan was not eluted in the first 87 fractions. (c) Elution as in (b) with addition of 0.035M-NaCl. The K_d of the proteoglycan at this value of I was 0.77. Absorbance scales: ●—●, 280nm; ○—○, 360nm; ●—●, 530nm in the carbazole reaction.

recovery was always greater than 65%. At high values of K_d , recovery was generally less than 30%.

Three fractions of the proteoglycan subunit, with ratios of E_{280} /hexuronate content of 0.61, 0.42 and 0.27, were all eluted from the collagen column at approximately the same ionic strength (Fig. 3), and they all were eluted from Sepharose 4B at V_0 . In addition, values of K_d at each ionic strength from 0.1 to 0.2 in increments of 0.01 were determined individually for each preparation (as in Fig. 2) and all were essentially identical.

The two purified glycosaminoglycans, chondroitin 4-sulphate and keratan sulphate, and the proteoglycan after digestion with chondroitinase ABC, were eluted from both columns at various ionic strengths

Fig. 2. Partition coefficient (K_d) of proteoglycan subunit on the collagen column as a function of ionic strength ($I/2$)

Ionic strength was calculated in each case by direct measurement of the Cl^- concentration in the peak tube containing the proteoglycan by using an ion-selective electrode.

Fig. 3. Elution of three fractions (A, B, and C) of proteoglycan subunit

The ratio of E_{280} /hexuronate content was 0.61, 0.42 and 0.27 for fractions A (●—●), B (○—○), and C (●—●) respectively. Elution from the collagen-Sepharose 4B column was with a linear gradient (—) of ionic strength ($I/2$).

Table 1. *Elution of various substances from Sepharose 4B and its collagen-substituted derivative*

For details see the text. N.D., Not determined.

Substance	$I(\text{mol/l})$	K_d^*	
		Sepharose 4B	Collagen - Sepharose 4B
Proteoglycan	0.1	0	>3.5
subunit	0.17	0	0.04
Chondroitin	0.1	0.63	0.83
sulphate	0.13	N.D.	0.70
	0.18	0.63	0.67
Keratan sulphate	0.1	0.77	0.77
	0.2	0.77	0.74
Proteoglycan digest	0.1	0.25	0.77
	0.2	0.25	0.46
Hyaluronic acid	0.1	0	0.04

* Since $V_t - V_0 = 22$ tubes for both columns, differences in K_d values of 0.03–0.04 represent only one tube difference and are not considered significant.

(Table 1). Chondroitin sulphate was retarded on the collagen column at I 0.1, but addition of extra NaCl brought the value of K_d back to within one tube of its elution profile on Sepharose 4B. Keratan sulphate was eluted from both columns at about the same place regardless of ionic strength. The digest, on the other hand, consisting of the protein core of the proteoglycan plus its associated chains of keratan sulphate, was severely retarded by the collagen column, and this was significantly reversed by addition of excess of NaCl. Hyaluronic acid was eluted at V_0 on both columns.

Discussion

Collagen–proteoglycan interactions have been investigated by various methods, but the only chromatographic studies reported involved the use of umbilical-cord collagen cross-linked with glutaraldehyde and then pulverized for use in columns calibrated with dextran as the void-volume marker (Öbrink & Wasteson, 1971). In the present study, we have avoided the use of glutaraldehyde, which might alter the charge distribution on the collagen by reaction with ϵ -amino groups of collagen lysine residues, and we have used a V_0 marker which does not interact with collagen at ionic strengths less than 0.2 mol/l. Susceptibility of Sepharose-bound collagen to degradation by collagenase, but not by pepsin, indicates that the protein retains the conformation of soluble collagen. This is important because collagen–chondroitin sulphate interaction was shown by electrophoresis to require undenatured collagen (Mathews, 1970).

The present studies indicate that bovine nasal proteoglycan can interact with collagen at two distinct sites. This proteoglycan has been well characterized, and consists of a protein core with a variable number of keratan sulphate and chondroitin sulphate side chains. The intact proteoglycan interacts strongly at neutral pH by an ionic process that is reversible at I 0.17. Proteoglycans separated from a homogeneous but polydisperse preparation into populations of molecules that vary in their degree of substitution with chondroitin sulphate side chains show an identical dependence on ionic strength independent of their chondroitin sulphate content. Further, the interaction of chondroitin sulphate with the collagen can be abolished at I 0.13, whereas retardation of the proteoglycan remains marked until I is raised to 0.17. The technique used here clearly distinguishes between these two phenomena. Öbrink (1970) has also demonstrated that isolated chondroitin sulphate and protein–chondroitin complex behave differently on a cross-linked-collagen column.

The finding that the elution profile of proteoglycan preparations was independent of their content of chondroitin sulphate suggested that interaction with another portion of the molecule was also occurring. Proteoglycan that was digested free of chondroitin sulphate interacted strongly with the collagen despite the loss of its major polyanionic substituent. Hascall (1972) has calculated that the protein–keratan sulphate core contains only 5% of the anionic groups of intact proteoglycan subunit. Since the interaction of the keratan sulphate–protein core was only partially reversible by increasing the ionic strength, and since purified keratan sulphate did not appear to interact electrostatically with the collagen, we conclude that the protein core of the proteoglycan can react directly with collagen. Our finding that the interaction of hyaluronic acid with collagen is negligible at I 0.1 is compatible with that of Mathews (1970).

The technique described here appears to be a sensitive method for study of ionic interactions that may contribute to the stability and organization of connective tissues.

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