A study of the interaction between cartilage proteoglycan and link protein

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The interaction between proteoglycan and link protein extracted from bovine articular cartilage (15–18-month-old animals) was investigated in 0.5 M-guanidinium chloride. The proteoglycans, radiolabelled as the aggregate (A1 fraction), were fractionated by two ‘dissociative’ density-gradient centrifugations (A1D1D1) followed by a rate-zonal centrifugation (S1) to yield an A1D1D1S1 preparation. At least 65% of these proteoglycans were able to bind to hyaluronate, but only 52% were able to bind to link protein as assessed by chromatography on Sepharose CL-2B. Over 80% of the [³H]link-protein preparation, radiolabelled as the aggregate, was able to interact with proteoglycan as assessed by chromatography on Sepharose CL-4B. Equilibrium-boundary-centrifugation studies performed at low link-protein concentrations (2.42×10^{-9} M– 5.93×10^{-8} M) were analysed by Scatchard-type plots and indicated a K_d of 1.5×10^{-8} M and a stoichiometry, $n = 0.56$, i.e. approx. 56% of those proteoglycans capable of binding to link protein had a strong site for link protein if a 1:1 stoichiometry were assumed. However, experiments performed at higher link-protein concentrations (3.5×10^{-7} M and 8×10^{-7} M) yielded stoichiometry values which were link-protein-concentration-dependent. Non-equilibrium binding studies using chromatography on Sepharose CL-2B and rate-zonal centrifugation yielded apparent stoichiometries between 0.6 and 7.5 link-protein molecules per proteoglycan monomer as a function of increasing link-protein concentration. It was concluded that a proportion of the proteoglycan molecules had a strong site for binding a single link protein (K_d 1.5×10^{-8} M) and that at high link-protein concentrations a weaker, open-ended, process of link-protein self-association nucleated upon the strong link-protein–proteoglycan complex occurred. Hyaluronate oligosaccharides appeared to abolish a proportion of this self-association {as observed by Bonnet, Dunham & Hardingham [(1985) *Biochem. J.* 228, 77–85] in a study of link-protein–hyaluronate–oligosaccharide interactions} so as to leave a link protein: proteoglycan stoichiometry of 2. It is not clear whether this second link-protein molecule binds directly to the proteoglycan or to the first link protein.

INTRODUCTION

Proteoglycan aggregates result from the non-covalent interaction between proteoglycan and hyaluronate; this binding is further stabilized by link-protein molecules (Hardingham, 1979). In aggregates formed *in vitro*, the ratio of proteoglycan to link protein has been estimated as approx. 1:1 (Heinegård & Hascall, 1974; Bonnet *et al.*, 1985). Link protein may also bind to hyaluronate (Heinegård & Hascall, 1974) and proteoglycan (Caterson & Baker, 1978; Franzén *et al.*, 1981) individually. Although the interactions between proteoglycan and hyaluronate (Cleland, 1979; Nieduszyński *et al.*, 1980; Nieduszyński & Sheehan, 1981) and hyaluronate and link protein (Tengblad, 1981; Lyon & Nieduszyński, 1983) have been investigated, little binding data are available for the proteoglycan–link-protein interaction.

Link proteins extracted from bovine and human articular cartilage have been identified as three major species with M_r values of 40000, 44000 and 48000 (Treadwell *et al.*, 1980; Roughley *et al.*, 1982), and the largest link protein has been separated from the two smaller species by wheat-germ-agglutinin affinity chromatography (Choi *et al.*, 1985). The three link proteins are thought to arise from a single gene product and differ only in their degree of oligosaccharide

substitution and proteolytic cleavage at the *N*-terminus of the molecules (Le Glédic *et al.*, 1983; Poole *et al.*, 1984; Mort *et al.*, 1985). Recently the largest (48000) and smallest (40000) link proteins from human articular cartilage have been shown to be phosphorylated (Oester *et al.*, 1986). Link protein may self-associate (Tang *et al.*, 1979; Lyon & Nieduszyński, 1983; Bonnet *et al.*, 1985), and the extent of oligomerization is probably concentration- and solvent-dependent.

The amino acid sequences of link protein (Deák *et al.*, 1986; Doege *et al.*, 1986a; Neame *et al.*, 1986) and segments of the proteoglycan core protein (Périn *et al.*, 1981, 1984; Doege *et al.*, 1986b) have been determined, and a common amino acid sequence has been demonstrated between link protein and a portion of the *N*-terminus of the proteoglycan core protein, i.e. the hyaluronate binding-region (Neame *et al.*, 1985).

In the present study the interaction between cartilage proteoglycan and link protein was addressed by using equilibrium and non-equilibrium methods. The complications due to the presence of link-protein self-association and the problems of accurately determining both the molarity of polydisperse proteoglycan preparations and the biological activities of link proteins and proteoglycans were considered.

Abbreviation used: HABR, hyaluronate-binding region.

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EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) was purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.) and further purified by treatment of 8 M stock solutions with activated charcoal. Benzamidine hydrochloride, dimethyldichlorosilane, high-purity bovine serum albumin and *N*-ethylmaleimide were also from Sigma. Caesium chloride, 6-aminohexanoic acid and disodium EDTA were purchased from BDH Chemicals (Poole, Dorset, U.K.). Beckman Ready-Solv MP (Beckman-RIIC, High Wycombe, Bucks., U.K.), 1,9-dimethyl Methylene Blue (Koch-Light Laboratories, Colnbrook, Slough, Berks., U.K.), [³H]- and [¹⁴C]-acetic anhydride (Amersham International, Amersham, Bucks., U.K.) (500 and 20 mCi/mmol respectively) and Sepharose CL-2B and CL-4B (Pharmacia, Uppsala, Sweden) were also purchased.

Methods

Analytical methods. (a) Chemical. Uronate was assayed by an automated procedure (Heinegard, 1973) based on the modified carbazole assay (Bitter & Muir, 1962) with glucuronolactone as the standard. Hexose was determined by an automated anthrone assay (Heinegard, 1973) with galactose as the standard. Sialic acid was assayed by the procedure of Lohmander *et al.* (1980) with *N*-acetylneuraminic acid as the standard. Amino acids (Lyon, 1986) and hexosamines (Lyon *et al.*, 1983) were determined after hydrolysis under N₂ with an LKB 4101 amino acid analyser. Proteoglycan was assayed by using 1,9-dimethyl Methylene Blue (Farndale *et al.*, 1982). Radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter with Beckman Ready-Solv MP as the scintillant. All glass columns and vessels used with link protein were treated with 1% (v/v) dimethyldichlorosilane in diethyl ether (Schwartz & Zabin, 1966) to decrease losses by adsorption.

(b) Electrophoresis. Discontinuous polyacrylamide-gel electrophoresis was performed on 10%-(w/v)-polyacrylamide slab gels (Laemmli, 1970).

(c) Electron microscopy. Length-distribution data on the [¹⁴C]proteoglycan preparation, spread in benzyl-dimethylalkylammonium chloride, were obtained as described by Thornton *et al.* (1986).

Preparations. (a) [³H]Link protein. [³H]Link protein, from bovine femoral-head cartilage (15–18-month-old animals), was prepared essentially as described by Lyon & Nieduszynski (1983), except that 0.01 M-*N*-ethylmaleimide was present in the inhibitor mixture. This yielded a [³H]link-protein preparation (in 4 M-guanidinium chloride) at a concentration of 0.892 mg/ml, determined by using a specific absorption coefficient ($A_{280}^{1\%}$) in 4 M-guanidinium chloride of 1.39 (Tang *et al.*, 1979), with a specific radioactivity of 9.9×10^6 c.p.m./mg (approx. 0.4 modified acetyl groups per molecule). Three major bands were observed on SDS/polyacrylamide-gel electrophoresis with apparent M_r values of 40000, 44000 and 48000. An inhibition enzyme-linked immuno-adsorbent assay (Bassett *et al.*, 1987) indicated that the link-protein preparation contained 0.025 mol of hyaluronate-binding region per mol of link protein. The proportion of the [³H]link-protein preparation able to

Table 1. Chemical data for the [¹⁴C]proteoglycan fraction (A1D1D1S1)

The ratio of total hexosamine to protein was 3.28.

Component	Proportion (% dry wt.)
Protein	11.0
Hexosamine	36.1
Galactosamine	26.6
Glucosamine	9.8
Uronate	23.4
Hexose	20.6
Sialic acid	1.34

interact with proteoglycan (A1D1) was determined by chromatography on Sepharose CL-4B (Fig. 1, below) as described by Lyon & Nieduszynski (1983). Recoveries of link protein from the column were $\geq 90\%$.

(b) [¹⁴C]Proteoglycan. [¹⁴C]Proteoglycans (A1D1D1) were prepared from bovine femoral-head cartilage (15–18 month-old animals) as described by Thornton *et al.* (1986). It proved necessary for the subsequent binding studies to fractionate the A1D1D1 preparation to facilitate separation from link protein in sedimentation experiments and to obtain a more homogeneous and monodisperse population of molecules with a number-average M_r ($\bar{M}_n$) value similar to that of the measured weight-average M_r ($\bar{M}_w$). The A1D1D1 preparation was fractionated by preparative rate-zonal centrifugation in 4 M-guanidinium chloride/CsCl gradients (density 1.15–1.4 g/ml) and the fast-sedimenting, larger, proteoglycans (A1D1D1S1) were pooled (Thornton *et al.*, 1986). This yielded a [¹⁴C]proteoglycan preparation, in 4 M-guanidinium chloride, at a concentration of 1.9 mg/ml and a specific radioactivity of 29 100 c.p.m./mg (i.e. approx. 0.6 modified acetyl groups per molecule). The proteoglycan concentration was determined after exhaustive dialysis and freeze-drying of a portion of the stock solution (Lyon *et al.*, 1983). The chemical data for the proteoglycan are presented in Table 1. An inhibition enzyme-linked immuno-adsorbent assay (Bassett *et al.*, 1987) indicated that the proteoglycan preparation contained 0.0012 mol of link protein per mol of proteoglycan.

The proportion of the [¹⁴C]proteoglycan preparation able to form link-protein-free (Fig. 2a, below) and link-protein-containing aggregates (Fig. 2b, below) was determined by chromatography on Sepharose CL-2B. Recoveries of link protein and proteoglycan from the column were $\geq 90\%$. Analytical ultracentrifugation was also employed to assess the proportion of the [¹⁴C]-proteoglycans able to form link protein-containing aggregates before or after exposure of the proteoglycans to high pH (Plaas & Sandy, 1986). Mixtures of [¹⁴C]-proteoglycan, 1% (w/w) hyaluronate ($\bar{M}_w$ 1×10^6) and 5% (w/w) link protein, pre-equilibrated for 16 h at 20 °C in 0.5 M-guanidinium chloride/5 mM-sodium phosphate, pH 7.4, were centrifuged in an MSE Centrican 75 analytical ultracentrifuge at 30 000 rev./min and 20 °C. The proportions of monomer and aggregate were determined by measurement of the areas under their respective peaks (schlieren monitoring).

(c) Proteoglycan. A1D1 proteoglycan was prepared by Lyon *et al.* (1983).

Sedimentation and diffusion studies. (a) Analytical ultracentrifugation. Sedimentation-velocity experiments were performed in an MSE Centriscan 75 analytical ultracentrifuge at 40000 rev./min and 20 °C. The apparent sedimentation coefficients, for proteoglycan and proteoglycan-link-protein complex, were determined from peak positions (schlieren monitoring). Sedimentation coefficients were obtained from plots of $1/s_{app}$ against proteoglycan concentration (0.108–1.9 mg/ml) with linear least-squares regression and corrected to standard conditions (20 °C, water). The $\bar{M}_w$ values for the [^{14}C]proteoglycan, in 4 M-guanidinium chloride and 0.2 M-NaCl, were calculated by using the equation described by Rowe (1977).

(b) Diffusion studies. The translational diffusion coefficients (D_t) for proteoglycan and proteoglycan-link-protein complex were determined as described by Lyon *et al.* (1983).

Binding studies. (a) Equilibrium (boundary centrifugation experiments). Mixtures of [^{14}C]proteoglycan and [^3H]link protein, in 0.5 M-guanidinium chloride/0.1 % (w/v) bovine serum albumin/5 mM-sodium phosphate, pH 7.4, were equilibrated for 16 h at 20 °C. The solutions were divided into equal portions; one half was made up to 5 % (w/v) in sucrose and the other half to 25 % (w/v) in sucrose. The solution densities (final volume 3.0 ml) were adjusted to 1.24 g/ml by addition of solid CsCl. Linear 5–25 % (w/v) sucrose gradients (5.0 ml) were formed and centrifuged in a swing-out rotor (6 × 5.5 ml, aluminium) at 180000 g_{av} and 20 °C for 3.5 h. Gradients were unloaded from the top via a capillary tube and fractions (550 μl) were assayed for ^{14}C and ^3H radioactivity.

(b) Non-equilibrium (rate-zonal centrifugation experiments). Mixtures of [^{14}C]proteoglycan and [^3H]link protein, in 0.5 M-guanidinium chloride/5 mM-sodium phosphate, pH 7.4, were equilibrated for 16 h at 20 °C before centrifugation on linear 5–25 %-(w/v)-sucrose gradients (4.75 ml) in a swing-out rotor (6 × 5.5 ml, aluminium) for 4 h at 20 °C and 180000 g_{av} . Gradients were unloaded from the bottom via a capillary tube, and fractions (250 μl) were analysed for [^3H]link protein (radioactivity) and proteoglycan (1,9-dimethyl Methylene Blue).

RESULTS

Proportion of [^3H]link protein binding to proteoglycan

The proportion of the [^3H]link-protein preparation capable of interacting with excess proteoglycan, as a percentage of total radiolabel, was determined by chromatography on Sepharose CL-4B and found to be at least 80 % (Fig. 1). It should be emphasized that Lyon (1986) found that, even with a 20-fold higher level of such amino group modification, 73 % of the link-protein molecules retained activity with respect to proteoglycan binding. Indeed, the specific radioactivities of the active and inactive link proteins, measured with respect to A_{280} , were in the ratio of 1.3:1. The link protein that was eluted between K_{av} values of 0.42 and 1.0 was assumed to be inactive with respect to binding proteoglycan, as the experiment was performed under such conditions that virtually all of the link protein capable of interacting with proteoglycan would do so, assuming a dissociation constant, $K_d \leq 10^{-7}$ M.

Fig. 1. Chromatography of [^3H]link protein with an excess of proteoglycan on Sepharose CL-4B

[^3H]link protein (1 μM) was equilibrated with an excess of proteoglycan (A1D1, 3 μM) in 0.5 M-guanidinium chloride/5 mM-sodium phosphate/0.1 % (w/v) bovine serum albumin, pH 7.4, at 6 °C for 16 h. The mixture was chromatographed, at 6 °C, on a column (0.6 cm × 13.5 cm) of CL-4B at a flow rate of 0.6 ml/h. The column was eluted in the same solvent containing proteoglycan (A1D1) at 0.4 mg/ml. Fractions (0.1 ml) were assayed for ^3H radioactivity, proteoglycan/link-protein mixture (●) and link protein alone (○). The V_0 and V_t values were determined with Blue Dextran and glucuronolactone respectively.

Proportions of [^{14}C]proteoglycan able to bind to hyaluronate and to link protein

The proportion of the [^{14}C]proteoglycan preparation capable of interaction with hyaluronate, as a percentage of the total uronate, was determined by chromatography on Sepharose CL-2B and found to be at least 65 % (Fig. 2a). However, a lower proportion, approx. 52 % of the population, was able to form link-protein-containing aggregates (Fig. 2b). The remaining 48 % of the proteoglycans showed insufficient affinity for link protein, as no link protein co-chromatographed with these proteoglycans (Fig. 2b). Thus, for the purposes of calculation in the subsequent binding analyses, it was assumed that 50 % of the proteoglycan preparation was 'active' with respect to link protein.

Analytical ultracentrifugation of mixtures of [^{14}C]proteoglycan (1–2 mg/ml), 1 % (w/w) hyaluronate and 5 % (w/w) link protein, under conditions described in the Experimental section, gave rise to two peaks (results not shown). The faster-sedimenting material, which was assumed to be link-protein-containing aggregate, comprised approx. 40 % of the proteoglycans. This proportion was unaffected by prior exposure of the proteoglycans to high pH (8.5), conditions shown to increase the aggregatability of 'maturing' proteoglycan preparations (Plaas & Sandy, 1986).

Fig. 2. Chromatography of (a) [^{14}C]proteoglycan and [^{14}C]proteoglycan plus 1% (w/w) hyaluronate ($M_w = 1 \times 10^6$) and (b) a mixture of [^{14}C]proteoglycan, 1% (w/w) hyaluronate and 24% (w/w) [^3H]link protein on Sepharose CL-2B

(a) [^{14}C]proteoglycan (700 μg , $\square$) and [^{14}C]proteoglycan (1 mg) plus hyaluronate (10 μg) ($\blacksquare$) and (b) a mixture of [^{14}C]proteoglycan (570 μg), hyaluronate (6 μg) and [^3H]link protein (137 μg) ($\blacksquare$) were equilibrated in 0.5 M-sodium acetate/0.1% (w/v) bovine serum albumin, pH 7.4, for 16 h at 6 $^\circ\text{C}$. The samples (400 μl) were chromatographed, at 6 $^\circ\text{C}$, on a column of CL-2B (1 cm $\times$ 58 cm) at a flow rate of 1.9 ml/h. The column was eluted with the same solvent and fractions (633 μl) were assayed for uronate ($\square$, $\blacksquare$) and [^3H]radioactivity ($\bullet$). The proportion of 'active' to 'inactive' proteoglycans in (b) was 13:12 (uronate) and 16:9 (^{14}C radioactivity, data not shown). V_0 and V_t values were determined with Blue Dextran and glucuronolactone respectively.

Determination of [^{14}C]proteoglycan M_r and molarity

It was necessary to determine the molar concentration of proteoglycan for the subsequent binding analyses. Fractionation, as described above, was performed to obtain a more monodisperse population of molecules. The physical data for the preparation provided an estimate for the $\bar{M}_w$ of the population (Table 2 and Fig. 3). Although an $\bar{M}_w$ has been determined, an $\bar{M}_n$ value is required if one is to determine proteoglycan molarities. An estimate for $\bar{M}_n$ has been derived by using the value for $\bar{M}_w$ of 1.74×10^6 obtained in 4 M-guanidinium chloride, conditions which ensure the proteoglycan exists as a monomer, and the assumption of a common mass per unit length for all the proteoglycans. Thus, from hydrodynamic and electron-microscopic studies, the

Table 2. Physical parameters for the [^{14}C]proteoglycan fraction (A1D1D1S1)

Parameter	Value
$\bar{L}_n$	198 nm (A1D1 = 143 nm)
$\bar{L}_w$	233 nm (A1D1 = 218 nm)
$S_{20,w}^0$	16.6 S (0.2 M-NaCl)
	17.0 S (4 M-guanidinium chloride)
$D_{20,w}^0$	$4.51 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ (0.2 M-NaCl)
$\bar{M}_w$	1.9×10^6 (0.2 M-NaCl)*
	2.0×10^6 (0.2 M-NaCl)†
	1.74×10^6 (4 M-guanidinium chloride)*

* Calculated by using the equation of Rowe (1977).

† Calculated using the Svedberg equation; a value for $\bar{v}$ (partial specific volume) of 0.55 ml/g (Hascall & Sajdera, 1970) was used in the calculations of $\bar{M}_w$.

Fig. 3. Sedimentation data for proteoglycan and proteoglycan/link-protein mixtures

Concentration-dependence of the reciprocal apparent sedimentation coefficient (s_{app}) for proteoglycan in 4 M-guanidinium chloride, pH 7.4 ($\blacksquare$), and proteoglycan ($\square$) and proteoglycan/link mixtures ($\bullet$) in 0.2 M-NaCl, pH 7.4. For further details, see the text.

mass per unit length ($\bar{M}_w/\bar{L}_w$) is 7468 nm^{-1} and therefore the $\bar{M}_n$, calculated as $(\bar{M}_w/\bar{L}_w) \times \bar{L}_n$, is 1.48×10^6 . The $\bar{L}_n$ (number-average length) and $\bar{L}_w$ (weight-average length) were obtained from electron-microscopic analysis of the proteoglycan distribution (Table 2) and are described in detail by Thornton *et al.* (1986).

Hydrodynamic studies

Sedimentation and diffusion studies were performed on mixtures of proteoglycan (0.108–0.8 mg/ml) and 3%

Fig. 4. Boundary-centrifugation profiles of (a) [^{14}C]proteoglycan, (b) [^3H]link protein and (c) [^{14}C]proteoglycan-[^3H]link-protein complex

Samples, in 0.5 M-guanidinium chloride/5 mM-sodium phosphate/0.1 % (w/v) bovine serum albumin, pH 7.4, were equilibrated for 16 h at 20 °C. The solutions were divided into equal portions; one half was made up to 5 % (w/v) in sucrose and the other half to 25 % (w/v) in sucrose. The solution densities (final volume 3.0 ml) were adjusted to 1.24 g/ml by addition of solid CsCl. Linear 5–25 % (w/v) sucrose gradients (5.0 ml) were formed and centrifuged as described in the Experimental section. Sedimentation profiles shown are: (a) [^{14}C]proteoglycan before (A1D1D1, ■) and after (A1D1D1S1, □) fractionation; (b) [^3H]link protein (●); (c) [^{14}C]proteoglycan-[^3H]link-protein complex [link-protein (●) profile shown]. The broken line (---) indicates the position of the proteoglycan (a) and link protein (c) before centrifugation.

(w/w) link protein. The reciprocal sedimentation data are shown in Fig. 3 and yield a sedimentation constant ($s_{20,w}^0$) of 17 S. This value is similar to that obtained for the proteoglycan alone (16.6 S) under the same conditions. Measurement of D_t of a mixture of proteoglycan (0.29 mg/ml) and link protein (11 $\mu\text{g/ml}$) yields a value of $4.27 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$, as compared with

$4.51 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$ for the proteoglycan alone. Thus the independent measurements of sedimentation and diffusion coefficients show only slight changes in the presence of link protein, suggesting that link protein is univalent for proteoglycan.

Equilibrium binding studies

Two different series of equilibrium experiments were performed. The first series, at low link-protein concentrations, were to study the dissociation constant (K_d), whereas the second series, at higher link-protein concentrations, were to investigate the stoichiometry.

A novel equilibrium technique involving centrifugation in 5–25 % (w/v) sucrose gradients with a solution density of 1.24 g/ml was employed to investigate the interaction between proteoglycan and link protein. Under the conditions of the experiment (for details, see the Experimental section), the proteoglycan sediments but the link protein remains buoyant, unless it is bound to proteoglycan (Fig. 4). This produces proteoglycan-containing and -depleted zones, which permits calculation of the bound (LP_B) and apparently-free (including inactive) link-protein ($LP_{F, app.}$) concentrations. The sedimentation profile for the [^{14}C]proteoglycan, before (A1D1D1) and after fractionation (A1D1D1S1), is shown in Fig. 4(a) and demonstrates the effectiveness of the proteoglycan fractionation procedure in producing a proteoglycan-depleted zone. The sedimentation profile of [^3H]link protein, in the absence and presence of [^{14}C]proteoglycan, is shown in Figs. 4(b) and 4(c). The difference between the upper plateau (Fig. 4c), which represents total link-protein concentration (LP_T), and

Fig. 5. Scatchard (1949)-type plot of link-protein binding to proteoglycan

Boundary-centrifugation experiments were performed at a fixed concentration of [^{14}C]proteoglycan ($5.9 \times 10^{-8} \text{ M}$) and several concentrations of [^3H]link protein ($2.42 \times 10^{-9} \text{ M}$ – $5.93 \times 10^{-8} \text{ M}$) at 20 °C. For further details, see the Experimental section. B is the concentration of bound link protein; B/F is the ratio of the concentrations of bound and free link protein.

the lower plateau provides a determination of the bound-link-protein concentration (LP_B). The value for the free-link-protein concentration may be determined from the lower plateau after subtraction of the independently determined proportion of inactive link protein.

Several experiments were performed at a constant proteoglycan concentration (5.9×10^{-8} M) and a range of link-protein concentrations (2.42×10^{-9} M– 5.93×10^{-8} M). The values for the concentrations of bound and free link protein obtained at each link-protein concentration studied were treated in the manner of Scatchard (1949). These data yield a value for the dissociation constant at 20 °C, assuming a stoichiometry of one link protein per proteoglycan, of 1.5×10^{-8} M (Fig. 5) and a stoichiometry (n) of 0.56, i.e. approx. 56% of those proteoglycans capable of binding to link protein have a strong site for link protein over the concentration range studied. Experiments performed at higher proteoglycan (2×10^{-7} M) and link protein (3.5×10^{-7} M and 8×10^{-7} M) concentrations indicated that the stoichiometry was dependent on the link-protein concentration. This series of experiments proved too extravagant with the stocks of both [14 C]proteoglycan and [3 H]link protein and was not

Fig. 6. Rate-zonal centrifugation profiles of (a) [3 H]link protein and (b) a mixture of [14 C]proteoglycan and [3 H]link protein

Samples containing (a) [3 H]link protein ($9.7 \mu\text{g}/100 \mu\text{l}$) and (b) [14 C]proteoglycan ($80 \mu\text{g}/100 \mu\text{l}$) and [3 H]link protein ($18 \mu\text{g}/100 \mu\text{l}$), in 0.5 M-guanidinium chloride/5 mM-sodium phosphate, pH 7.4, were equilibrated for 16 h at 20 °C. Samples ($100 \mu\text{l}$) were applied to linear 5–25%-(w/v)-sucrose gradients (4.75 ml) and centrifuged as described in the Experimental section. Fractions ($250 \mu\text{l}$) were assayed for [3 H]link protein (radioactivity, ●) and [14 C]proteoglycan (A_{540} , □).

pursued. Consequently rate-zonal centrifugation, a more economical, but a non-equilibrium, technique, was employed to investigate the stoichiometry further.

Non-equilibrium binding studies

Rate-zonal centrifugation has been employed previously by Franzén *et al.* (1981) for studying the interaction between proteoglycan and link protein extracted from bovine nasal cartilage.

In the present study, centrifugation was performed after equilibration of samples in 0.5 M-guanidinium chloride/5 mM-sodium phosphate, pH 7.4, in 5–25% (w/v) linear sucrose gradients. Experiments were performed by using a fixed concentration of proteoglycan (5.3×10^{-7} M) and a range of concentrations of link protein (10^{-7} M– 5×10^{-6} M). A typical profile is shown for the sedimentation of a link-protein control (Fig. 6a) and a proteoglycan/link-protein mixture (Fig. 6b). The link protein which co-sedimented with the proteoglycan was assumed to be bound. Over the link-protein concentration range studied, the bound-link-protein/'active'-proteoglycan concentration ratio ranged from 0.6 to 6.0 (Fig. 7). Inclusion of 0.1% (w/v) bovine serum albumin in the equilibration mixtures had no effect on this ratio, although it increased the recovery of link protein.

In 0.2 M-NaCl, higher values for the bound-link-protein/'active'-proteoglycan ratio were observed, and, for example, at a link-protein concentration of 3.7×10^{-6} M, a ratio of 12.0 was calculated.

In the presence of a molar excess of hyaluronate oligosaccharides (2×10^{-5} M), the bound-link-protein/'active'-proteoglycan concentration ratio, in 0.5 M-guanidinium chloride, was decreased from > 6.0 to

Fig. 7. Proteoglycan saturation curve

The ratio of the concentrations of bound link protein to 'active' proteoglycan, determined by rate-zonal experiments performed in 0.5 M-guanidinium chloride at a constant proteoglycan concentration (5.3×10^{-7} M) and several link protein concentrations (10^{-7} M– 5×10^{-6} M), is plotted against the total link-protein concentration. The effect of a molar excess, over link protein, of hyaluronate oligosaccharides ($\bar{M}_w$ 20000) is also indicated (★).

approx. 2.0 at a link-protein concentration of 5×10^{-6} M and a proteoglycan concentration of 5.3×10^{-7} M (Fig. 7).

DISCUSSION

In previous studies of the interaction between cartilage proteoglycan and link protein, two different approaches have been taken. One has been to determine the ratio of the two components in the ternary complex, i.e. aggregates or trypsin-treated aggregates (Heinegård & Hascall, 1974; Faltz *et al.*, 1979; Kimura *et al.*, 1980; Ratcliffe & Hardingham, 1983; Bonnet *et al.*, 1985); in the other, investigations have been aimed primarily at the interaction, in the absence of hyaluronate, between proteoglycan and link protein (Caterson & Baker, 1978; Franzén *et al.*, 1981). These latter studies have given no indication of the stoichiometry for the interaction, whereas from the former studies the ratio of 1:1 has clearly emerged.

In our investigation of the interaction the proteoglycan, rather than the proteolytically derived hyaluronate-binding region (HABR), was used. This allowed efficient separation of the link-protein molecules bound to proteoglycan from those free (unbound) in centrifugal and chromatographic experiments, and if there had been site(s) on the proteoglycan for binding link protein, other than the HABR, these might have been detected.

It is important to emphasize, in the context of the present study, that the proportion of 'aggregating' proteoglycans is much lower in articular cartilage (50–60%) than in nasal cartilage (90%) preparations (Heinegård *et al.*, 1986).

The biological activities of the proteoglycan and link-protein preparations were assessed. At least 80% of the link-protein preparation was capable of binding to proteoglycan. The ability of the proteoglycan to bind to link protein was assessed indirectly employing the conventional assumption that the HABR is the site at which link protein is bound. Thus chromatography on Sepharose CL-2B (Fig. 2a) of the proteoglycans plus 1% (w/w) hyaluronate ($\bar{M}_w \times 10^6$) displaced 65% of the molecules into the void fraction and indicates that at least 65% of the proteoglycans have a functional HABR. When an excess of link protein was included in this experiment (Fig. 2b), it was observed to be co-eluted exclusively with the hyaluronate-binding proteoglycans, though surprisingly the proportion of these 'aggregating' proteoglycans was reduced from 65 to 52%. These data confirm that the link protein is bound at, or close to, the HABR, but suggest that not all HABR retain the ability to bind strongly to link protein, and at least 48% of the proteoglycans showed no affinity for link protein at all. Also it would appear (as was noted by Tang *et al.*, 1979) that the presence of excess link protein can displace some proteoglycans from the hyaluronate rather than stabilize them, and this suggests that there may be two types of HABR, one of which does not bind effectively with link protein and indeed can be competitively inhibited from binding to hyaluronate in an excess of link protein. Chromatography experiments on Sepharose CL-2B where high ratios of link protein to proteoglycan were used indicated a stoichiometry considerably greater than 1, though the presence of hyaluronate in such experiments makes the sites of binding for the link protein (i.e. HABR

or hyaluronate) uncertain. A value of 80% for the activity of the link protein towards proteoglycan and 50% for the activity of the proteoglycan towards link protein was adopted for use in the binding analyses.

The equilibrium binding of the proteoglycan and link protein was investigated by using a novel centrifugal method which separated bound from free (unbound) link protein by exploiting the difference in buoyant density of proteoglycan and link protein and the difference in the sedimentation rate of link protein and proteoglycan-link-protein complex. The technique is in principle similar to a frontal-gel-chromatography analysis (Winzor & Scheraga, 1964), but is faster and more economical with samples. Experiments performed at low link-protein concentrations (2.42×10^{-9} – 5.93×10^{-8} M), yielded a value for $n = 0.56$ (i.e. 56% of those proteoglycans capable of binding to link protein have a strong site for link protein) and a K_d of 1.5×10^{-8} M at 20 °C. The value for K_d is comparable with those observed for the other two interactions in the ternary complex (Cleland, 1979; Nieduszynski *et al.*, 1980; Tengblad, 1981; Lyon & Nieduszynski, 1983). Why only 56% of the proteoglycans capable of interacting with link protein should show strong binding is not clear. There may be two classes of HABR or, more likely, a proportion of the HABR has a binding site for link protein that is weaker because of variable renaturation (after preparative procedures) or proteolytic damage during extraction and/or within the tissue. Measurements of stoichiometry by equilibrium boundary centrifugation suggested that it varied with link-protein concentration, but the quantities of link protein and proteoglycan required to pursue the problem necessitated different experimental methods.

Rate-zonal experiments were used to examine the concentration-dependence of link-protein binding to proteoglycan over a wide range of link-protein concentrations. Though not rigorous, this method demonstrated that suprastochiometric quantities of link protein co-migrated with proteoglycan. This is in good agreement with the Sepharose CL-2B chromatography data discussed above. The saturation curve (Fig. 7) shows a point of inflection in the region of two to three link-protein molecules per proteoglycan and thereafter reaches a plateau at approx. six link-protein molecules per proteoglycan. On addition of an excess of hyaluronate oligosaccharides a significant decrease in the amount of co-migrating link protein was observed, yielding a stoichiometry of approx. two link-protein molecules per proteoglycan. The explanation of these supra-stoichiometric effects is not clear, but the best hypothesis probably involves link-protein self-association. Indeed, in rate-zonal experiments performed in NaCl, a solvent shown to allow higher oligomers of link protein (Tang *et al.*, 1979; Bonnet *et al.*, 1985), stoichiometries of up to 12 link-protein molecules per proteoglycan were observed. Lyon & Nieduszynski (1983) have shown that, in 0.5 M-guanidinium chloride, and over the link-protein concentration range employed in the rate-zonal experiments, link protein occurs predominantly as dimers. We envisage, therefore, that the link protein strongly bound to proteoglycan acts as a nucleating site for further weaker binding of a link protein to the proteoglycan in the region of the HABR, thus stabilizing a site to which more link proteins (possibly as dimers) can bind.

Unfortunately it has not been possible to test this

Scheme 1. Proposed model for the interaction between proteoglycan (PG) and link protein (LP) in solution

hypothesis directly, since we know of no way of chemically inhibiting or removing self-association without abolishing the link-protein-proteoglycan interaction. However, Bonnet *et al.* (1985) have shown that, for link protein in 1 M-NaCl, the addition of univalent hyaluronate oligosaccharides (10–16 residues long) reduces link-protein multimers to dimers. In the present study, hyaluronate oligosaccharides decreased the link-protein-proteoglycan stoichiometry from approx. 6:1 to approx. 2:1. Thus the stable unit appears to be [proteoglycan+two link-protein molecules+hyaluronate]. It is not possible to establish from these data whether the second link protein binds directly to the proteoglycan or to the link protein, or to both. The interpretation of our data is summarized in Scheme 1.

Conclusions

(1) In the bovine articular-cartilage preparation examined, 65% of the proteoglycans bound to hyaluronate, but only 50% bound to link protein.

(2) At low link-protein concentrations (2.42×10^{-9} M– 5.93×10^{-8} M) in 0.5 M-guanidinium chloride (20 °C) a dissociation constant of 1.5×10^{-8} M was observed. However, the stoichiometry indicated that only 56% of those molecules capable of binding link protein have this strong site.

(3) At higher link-protein concentrations (10^{-7} M– 5×10^{-6} M) in 0.5 M-guanidinium chloride, link-protein/proteoglycan ratios of approx. 6:1 were observed. These high values for the stoichiometry were interpreted as self-association of link-protein molecules nucleated upon the strong link-protein-proteoglycan complex.

(4) The presence of hyaluronate oligosaccharides reduced the stoichiometry to 2:1, and this suggests that there may be a second weaker site for a link protein, either on the HABR of the proteoglycan or on the strongly bound link protein.

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