Cartilage proteoglycan aggregate formation

Role of link protein

Ahnders FRANZÉN, Sven BJÖRNSSON and Dick HEINEGÅRD

Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden

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Cartilage proteoglycan aggregate formation was studied by zonal rate centrifugation in sucrose gradients. Proteoglycan aggregates, monomers and proteins could be resolved. It was shown that the optimal proportion of hyaluronic acid for proteoglycan aggregate formation was about 1% of proteoglycan dry weight. The reaggregation of dissociated proteoglycan aggregate A1 fraction was markedly concentration-dependent and even at 9 mg/ml only about 90% of the aggregates were reformed. The lowest proportion of link protein required for maximal formation of link-stabilized proteoglycan aggregates was 1.5% of proteoglycan dry weight. It was separately shown that link protein co-sedimented with the proteoglycan monomer. By competition with isolated hyaluronic acid-binding-region fragments, a proportion of the link proteins was removed from the proteoglycan monomers, indicating that the link protein binds to the hyaluronic acid-binding region of the proteoglycan monomer.

Several cartilage proteoglycan monomers can interact with hyaluronic acid to form aggregates (Hardingham & Muir, 1972). The hyaluronic acid-binding region of the proteoglycan can specifically bind to a decasaccharide or larger sequence of hyaluronate (Heinegård & Hascall, 1974a; Hascall & Heinegård, 1974b; Christner *et al.*, 1978; Hardingham, 1979). Although the K_D of the interaction is in the order of 10^{-8} (Christner *et al.*, 1978; Cleland, 1979; Nieduszynski *et al.*, 1980), an additional protein, the link protein, is present to stabilize the interaction. Present data though indirect, indicate that the link protein interacts both with the proteoglycan monomer and with the hyaluronate (Hascall & Heinegård, 1974b; Hardingham, 1979).

The present study was initiated to determine some of the parameters important for proteoglycan aggregation. Ratios of link protein and hyaluronic acid to proteoglycan, optimal for aggregate formation, were determined. The interaction of link proteins with proteoglycan monomers was studied.

Experimental

Proteoglycan monomers (A1-D1 fraction) and aggregates (A1 fraction) were isolated as described previously (Heinegård, 1972; Heinegård & Hascall,

Abbreviations used: HA-14, hyaluronic acid oligosaccharides containing seven disaccharide units; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

1979). Hyaluronic acid-binding-region fragment was isolated after trypsin digestion of proteoglycan A1 fraction as described previously (Heinegård & Axelsson, 1977; Heinegård & Hascall, 1974a).

Link proteins were prepared from the top fraction (A1-D4) of the dissociative CsCl density gradient of the A1 fraction. The A1-D4 fraction was re-centrifuged in a CsCl/4 M-guanidinium chloride gradient with a starting density of only 1.35 g/ml to remove any remaining proteoglycans. The top fraction was chromatographed on Sephadex G-200, eluted with 4 M-guanidinium chloride, essentially as described previously (Heinegård, 1972; Heinegård & Hascall, 1974a), to yield a pure link-protein fraction. High-molecular-weight hyaluronic acid (Healon) was a gift from Pharmacia AB, Uppsala, Sweden. Hyaluronic acid oligosaccharides were prepared from hyaluronic acid by digestion with testicular hyaluronidase, followed by gel chromatography on Sephadex G-50 as described previously (Hascall & Heinegård, 1974b).

Zonal rate centrifugation was done in linear gradients of 10–30% (or 10–50%)-sucrose/0.5 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, as indicated in the Figures. Samples (100 μl) dissolved in 0.15 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, were layered on the gradient and centrifuged in an MSE 6 $\times$ 4.2 ml swing-out rotor at 20°C and 50000 rev./min (240000 g; r_{av} , 8.802 cm) for the times indicated in the Figures. After centrifugation the tubes were emptied from the bottom and the A_{206} of the effluent was moni-

tored with a Uvicord-S u.v.-monitor (LKB, Bromma, Sweden).

Aggregation of proteoglycans with hyaluronic acid

The sedimentation properties of complexes of hyaluronic acid and proteoglycan monomers were studied. A proteoglycan monomer (6 mg/ml) stock solution in 0.15 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, was prepared. Portions (50 μl) were incubated with 50 μl of the buffer, containing various amounts of hyaluronic acid for 12 h at 4°C. The solutions were layered on top of 10–50%-sucrose/0.5 M-NaCl gradients and centrifuged for 36 min.

Reaggregation of proteoglycans

The concentration-dependence of proteoglycan reaggregation was determined. Proteoglycan A1 fraction was dissolved at 0.3–9 mg/ml in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8. The samples (200 μl) were dialysed in a constantly rotating microdialysis apparatus. The apparatus had ten chambers, each having a flat dialysis membrane with a surface area of about 1 cm². Dialysis was first against 1500 ml of 0.5 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, for 24 h and then against 1500 ml of 0.15 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, for another 24 h. The samples were, when required, adjusted to constant volume and centrifuged in 10–50%-sucrose/0.5 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, gradients for 36 min. The amounts of aggregate and monomer respectively were determined as the area of the respective peak in the 206 nm scan.

Dependence of aggregate stability on proportion of link proteins

A stock solution of nasal cartilage proteoglycan monomer (A1-D1) at 6 mg/ml in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, was prepared. Portions (100 μl), containing 600 μg , were mixed with equal volumes of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, containing from 0 to 24 μg of link proteins. The mixtures then contained 3 mg of proteoglycan/ml and link protein at 0–4% (w/w) of the proteoglycan. After dialysis against 1500 ml of 0.5 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, for 12 h at 4°C, a constant amount of hyaluronic acid (5.4 μg or 0.9% of proteoglycan dry wt.) was added to each sample. The samples were dialysed against 1500 ml of 0.15 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, for 24 h at 4°C. One set of 100 μl portions representing each concentration of link protein was incubated with hyaluronic acid oligosaccharides containing seven disaccharide units (HA-14) to compete for proteoglycan bound to hyaluronic acid but not stabilized

by link proteins (Hascall & Heinegård, 1974b; Björnsson & Heinegård, 1981). Freeze-dried HA-14 (27 μg) was added to each sample (ten times the amount of hyaluronic acid). They were incubated for 12 h at 4°C before centrifugation in 10–50%-sucrose/0.5 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, gradients for 36 min.

Interaction of link protein with proteoglycan monomer

Stock solutions of proteoglycan monomer (fraction A1-D1; 10 mg/ml), link protein (1 mg/ml), bovine serum albumin (1 mg/ml) and isolated hyaluronic acid-binding region (1 mg/ml) were prepared in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8. A series of mixtures were made, all having a constant concentration of proteoglycan monomer (5 mg/ml) and link protein (0.05 mg/ml; 1% of proteoglycan dry wt.). In addition they contained albumin (0.1 mg/ml; 2% of proteoglycan dry wt.), or isolated hyaluronic acid-binding region (0.1 mg and 0.2 mg/ml respectively; 2 and 4% of proteoglycan dry wt.). The solutions were dialysed in the microdialysis apparatus against 1500 ml of 0.5 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, for 24 h at 4°C and then against 1500 ml of 0.15 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, for 24 h at 4°C. The dialysed solutions were diluted with an equal volume of the buffer used for dialysis, and portions (100 μl), containing 250 μg of proteoglycan, were layered on 10–30%-sucrose/0.5 M-NaCl/5 mM- Na_3PO_4 , pH 7.0, and centrifuged for 4 h at 20°C. The tubes were emptied with continuous monitoring at 206 nm, and 200 μl fractions were collected. Portions (triplicates) were taken for quantification of link proteins by enzyme-linked immunosorbent assay (ELISA; Engvall & Perlman, 1972). The assay included a preincubation of the sample in 0.8% (w/v) SDS and subsequent formation of mixed micelles of the unbound SDS by addition of a tenfold excess (w/w) of Triton X-100 (Chua & Blomberg, 1979). The sample was then preincubated with rabbit anti-(link protein) antibody (Wieslander & Heinegård, 1980) and portions were transferred to microtitre plate wells coated with link protein. After incubation for 1 h, the wells were washed and anti-(rabbit IgG) antibody conjugated with alkaline phosphatase was added. The amount of anti-IgG antibody bound to the well was measured as the enzyme activity and is inversely proportional to the amount of link protein in the sample over a range from about 0.0001 to 10 $\mu\text{g}/\text{ml}$.

Results and discussion

Hyaluronic acid–proteoglycan monomer interaction

Zonal rate sedimentation centrifugation is very well suited for studies on the formation of proteo-

glycan aggregates, in particular since the relative proportion and size of aggregates formed can be determined simultaneously. In a set of experiments the minimal proportion of hyaluronic acid required to form a maximal amount of hyaluronic acid-proteoglycan complex was determined. Proteoglycan monomers were mixed with various amounts of high-molecular-weight hyaluronic acid, incubated and centrifuged in 10–50% sucrose (Fig. 1). Hyaluronic acid complexes form the broad, faster-sedimenting peak, whereas the proteoglycan monomers remain as a relatively sharp peak near the top of the gradient (Fig. 1). The size of the aggregates remained rather constant up to a proportion of 1% hyaluronic acid (of proteoglycan dry wt.), whereas at larger proportions of hyaluronic acid the size of the aggregates progressively decreased (Fig. 1). The largest proportion of complexes was obtained with 0.9–1.05% (w/w) hyaluronic acid. The data indicate that, at these concentrations, the hyaluronic acid is maximally saturated with proteoglycan monomers and all monomers capable of binding are bound to the hyaluronic acid. When the hyaluronic acid concentration is further increased, the number of proteoglycan monomers bound to each hyaluronic acid molecule progressively decreases and the smaller aggregates formed sediment more slowly (Fig. 1). The optimal concentration of hyaluronic acid for complex-formation with cartilage proteoglycan monomers was previously found to be about

1% (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a), in accordance with the present data.

Concentration-dependence of reassociation of link-stabilized proteoglycan aggregates

The concentration-dependence of the reaggregation of proteoglycan aggregates (A1 fraction) was determined (Fig. 2). Nasal-cartilage A1 fraction was dissociated in 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, and diluted to concentrations ranging from 0.3 mg to 9 mg/ml. The solutions were adjusted to associative conditions by dialysis as described in the Experimental section.

As a control, nasal-cartilage proteoglycan monomers, dissolved in 4M-guanidinium chloride, were mixed with high-molecular-weight hyaluronic acid (0.9% of proteoglycan dry wt.). The solutions were diluted to give a set of proteoglycan concentrations ranging from 0.125 to 3 mg/ml. The samples were dialysed as described for the A1 fractions. All samples were centrifuged in 10–50% sucrose gradients for 36 min and the proportions of aggregate and monomer were determined as the area under the respective peak (Fig. 2). Assuming a

Fig. 1. Zonal rate sedimentation for 36 min in 10–50% sucrose gradients of a constant amount (300 μ g) of proteoglycan monomer with various concentrations of high-molecular-weight hyaluronic acid (HA) (up to 4% of proteoglycan weight)

Fig. 2. Zonal rate sedimentation for 36 min in 10–50% sucrose gradients of nasal-cartilage proteoglycan aggregates (A1 fraction) reassociated from 4M-guanidinium chloride at concentrations of 0.125–9 mg/ml. The proteoglycans were reassociated by dialysis as described in Experimental section. The proportions of proteoglycan aggregates (●) were calculated from the area of the peaks for aggregate and monomer respectively. As a control the proportion of aggregate in the original A1 fraction (83%) is shown (▲). For comparison, the proportion of aggregate formed when proteoglycan monomer at 0.125–3 mg/ml was mixed with a constant proportion of high-molecular-weight hyaluronic acid (0.9% of proteoglycan weight) is shown (▲).

proteoglycan molecular weight of 2.5×10^6 and a dissociation constant of about 10^{-8} for the proteoglycan-hyaluronic acid interaction, maximal complex-formation should be obtained even at proteoglycan concentrations of 0.125 mg/ml. Expectedly then, mixtures of proteoglycan monomer (0.125–3 mg/ml) and hyaluronic acid contained a constant amount (80–85%) of complex (Fig. 2). Proteoglycan A1 fraction, which in addition contained the link proteins, unexpectedly reaggregated much less efficiently at low concentrations (Fig. 2). At the lowest concentration, 0.3 mg/ml, only about 30% of the proteoglycans were aggregated, compared with 83% for the original A1 fraction. At concentrations from 1 mg to about 9 mg/ml, increasing amounts of aggregates were obtained. Surprisingly, even at 9 mg/ml only about 75% of the proteoglycans were aggregated. The proteoglycan concentration in the cartilage extract used to prepare proteoglycan A1 fraction is usually no more than 5 mg/ml, and yields of aggregate are, nevertheless, about 85%. Experiments to improve reaggregation of the A1 fraction by changing the dialysis conditions and the geometry of the dialysis bag were unsuccessful. It is possible that the more efficient reaggregation in the original extract is due to the presence of tissue factors promoting aggregation, or, more likely, the link proteins renature incompletely on dialysis to associative conditions.

Dependence of aggregate stability on proportion of link protein

Taking into account the concentration-dependent reaggregation and by using an optimal concentration of hyaluronic acid, the amount of link protein required to stabilize aggregates was investigated. The concentration of proteoglycan used was 3 mg/ml. Further increases of the proteoglycan concentration would have only a limited effect on the proportion of aggregates formed. Proteoglycan monomer (A1-D1 fraction) and link protein were dissolved in 4 M-guanidinium chloride and mixed to give link-protein proportions from 0 to 4% of proteoglycan dry weight. The solutions were dialysed to associative conditions as described in the Experimental section. A constant amount of high-molecular-weight hyaluronic acid (0.9% of proteoglycan dry wt.) was then added to each solution. Consequently only the concentration of link protein was varied. Samples representing each concentration of link protein were incubated with hyaluronic acid oligosaccharides (HA-14; ten times the amount of hyaluronic acid in the sample). The oligosaccharides will displace proteoglycan monomers from high-molecular-weight hyaluronic acid unless the interaction is stabilized by link proteins (Heinegård & Hascall, 1974b; Björnsson & Heinegård, 1981). Therefore all aggregates observed on

Fig. 3. Zonal rate sedimentation in 10–50%-sucrose gradients for 36 min of aggregates formed by incubating a constant proportion of proteoglycan monomer and hyaluronic acid with a variable amount of link protein (Link) (from 0 to 4% of proteoglycan dry weight).

Before centrifugation, samples were incubated with an excess of hyaluronic acid oligosaccharide (HA-14) to dissociate any hyaluronic acid–proteoglycan monomer aggregates not stabilized by link proteins. Experimental details are given in the Experimental section.

centrifugation of the samples in the sucrose gradients are link-stabilized. A progressive increase of the size and the amount of stable aggregates was obtained when the link-protein concentration was increased to 1.5% of proteoglycan-monomer dry weight, whereas further increase did not significantly change the pattern (Fig. 3). The optimal concentration of link protein (1.5%) is close to the proportion estimated by using fragmentation of proteoglycan aggregates (Heinegård & Hascall, 1974a).

Binding of link protein to proteoglycan monomer

The interaction between proteoglycan monomer and hyaluronic acid is well characterized (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a,b; Christner *et al.*, 1978; Cleland, 1979; Nieduszyński

et al., 1980). Evidence indicates that the link proteins can interact with proteoglycan monomers (Baker & Caterson, 1979; Heinegård & Hascall, 1979; Hardingham, 1979; Poole *et al.*, 1980). It is possible that the hyaluronic acid-binding region of the proteoglycan monomer is involved in this interaction.

Proteoglycans carrying bound proteins can be separated from other proteins by zonal rate centrifugation (A. Franzén, S. Björnsson & D. Heinegård, unpublished work). This technique can therefore be used to discern whether or not link proteins co-sediment with the proteoglycans (indicating binding) and whether or not any bound link proteins can be removed by competition with proteoglycan substructures, primarily the hyaluronic acid-binding region. A number of samples in 4M-guanidinium chloride containing proteoglycan monomers and link proteins were prepared. The proportion of link proteins (1% of proteoglycan dry wt.), is below saturation and was chosen to ensure binding of all link proteins. Hyaluronic acid-binding-region fragment was added at two concentrations (2 and 4% of proteoglycan dry wt.) to compete for binding of link proteins. As a control, bovine serum albumin (2% of proteoglycan dry wt.) was added to another sample. All solutions were dialysed to associative conditions and centrifuged in sucrose gradients as described in the Experimental section. The distribution of protein and proteoglycan in the tube was monitored by using the A_{206} , and the distribution of link protein was determined by using ELISA. The profile of the proteoglycan monomer was very similar in all cases, regardless of whether or not link protein had been added (results not shown). The sample containing only proteoglycan monomer and link proteins showed the same type of profile as the proteoglycan alone with no slow-sedimenting protein component, indicating binding of the link proteins (results not shown). The sample containing proteoglycan monomer, link protein and albumin in addition showed a slow-sedimenting component corresponding to the albumin (Fig. 4a). ELISA for link protein demonstrated that the link proteins sedimented with the proteoglycan monomer (Fig. 4a). The total amount of link protein in the gradient was $2.89\mu\text{g}$ as determined by ELISA, in reasonable agreement with the $2.5\mu\text{g}$ that was originally added. No link protein was detected in the slow-sedimenting protein component. Therefore all the link protein had bound to the proteoglycan monomer. The samples containing proteoglycan monomer, link protein and 2 or 4% of hyaluronic acid-binding-region fragment, showed similar profiles of proteoglycan monomer. In addition, a slow-sedimenting component, representing the added hyaluronic acid-binding region, was observed (Figs. 4b and 4c). ELISA showed that all link proteins present sedimented with the proteo-

Fig. 4. Zonal rate centrifugation in 10–30% sucrose gradients of mixtures of (a) proteoglycan monomer ($250\mu\text{g}$), link protein ($2.5\mu\text{g}$) and bovine serum albumin ($5\mu\text{g}$), (b) proteoglycan monomer ($250\mu\text{g}$), link protein ($2.5\mu\text{g}$) and isolated hyaluronic acid (HA)-binding region ($5\mu\text{g}$), and (c) proteoglycan monomer ($250\mu\text{g}$), link protein ($2.5\mu\text{g}$) and isolated hyaluronic acid-binding region ($10\mu\text{g}$).

The gradient was monitored for A_{206} (---) and for content of link proteins (by using ELISA) (—). The area of the indicated peak (Ø) is a measure of the amount of protein not bound to proteoglycan.

glycan monomers (Figs. 4b and 4c). The amounts of link protein, however, were much lower: $1.52\mu\text{g}$ (2% added hyaluronic acid-binding-region fragment) and $1.45\mu\text{g}$ (4% added hyaluronic acid-binding-region fragment) compared with the expected $2.5\mu\text{g}$ of link protein. Consequently the hyaluronic acid-binding region, in contrast with albumin, was capable of competing for binding of link protein. The complex of link protein–hyaluronic acid-binding region was not recovered, since no link protein sedimented at the expected position of the hyaluronic acid-binding region. Corroborating evidence was the unexpected low recovery of hyaluronic acid-binding-region fragment, particularly when added at lower concentration (Figs. 4b and 4c). Isolated link protein has

a low solubility in low-salt solutions (Tang *et al.*, 1979) and is often lost on surfaces when not bound to proteoglycans. The observed bimodality of the link-protein distribution (Fig. 4) may represent different binding to the two aggregating subpopulations of cartilage proteoglycans, which can be partially separated by using zonal rate centrifugation in sucrose gradients (Heinegård *et al.*, 1981).

The data provide strong evidence that the link proteins bind to the hyaluronic acid-binding region of the proteoglycan monomer. It should be borne in mind, however, that the hyaluronic acid-binding region used had been prepared by fragmentation of the proteoglycan and may not contain all interacting structures.

General discussion

It has been shown that zonal rate centrifugation in sucrose gradients is a useful tool in studies of interactions in proteoglycan aggregation. It appears that proteoglycan monomers interact with hyaluronate at an optimal ratio of about 100:1 respectively, corroborating previous data (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a). The interaction is stabilized by link proteins at an optimal concentration of 1.5% of proteoglycan dry weight, which corresponds to one link-protein molecule per proteoglycan monomer, assuming molecular weights of 45 000 and 2.5×10^6 respectively. The link protein was shown to interact with the isolated hyaluronic acid-binding region of the proteoglycan monomer. Since it also interacts with isolated hyaluronic acid (A. Franzén, S. Björnsson & D. Heinegård, unpublished work), the binding of proteoglycans to hyaluronate in the presence of link proteins is trifunctional.

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