Cartilage proteoglycan binding region and link protein

Radioimmunoassays and the detection of masked determinants in aggregates

Anthony RATCLIFFE and Tim HARDINGHAM
Kennedy Institute, Hammersmith, London W6 7DW, U.K.

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Antibodies have been raised in rabbits to the hyaluronate-binding region and link-protein components of aggregated proteoglycans from pig laryngeal cartilage. The anti-(binding region) antibodies did not bind ^{125}I -labelled link protein, nor was ^{125}I -labelled binding region bound by the anti-(link protein) antibodies. The antisera were applied in sensitive inhibition radioimmunoassays to determine binding region and link protein in purified proteoglycan preparations. With intact proteoglycan aggregates, the antigenic sites of link protein, and to a lesser extent binding region, were masked. Heat treatment in the presence of sodium dodecyl sulphate (0.025%, w/v) was found to overcome this masking, thereby allowing the determination of link protein and binding region in aggregated proteoglycan preparations in pure and impure samples.

Cartilage proteoglycans are complex macromolecules in which many glycosaminoglycan chains and oligosaccharides are linked to an extended protein core (Hardingham, 1981). Three separate domains have been identified in the protein core: a highly folded globular region (mol.wt. approx. 80000; 70% protein and 30% carbohydrate) that forms the hyaluronate-binding site (Perkins *et al.*, 1981), a short extended keratan sulphate-rich region (protein mol.wt. approx. 20000) and a long extended chondroitin sulphate-bearing region (protein mol.wt. approx. 50000–150000) (Heinegård & Axelsson, 1977). Cartilage proteoglycans form aggregates by binding to hyaluronate via a highly specific site on the binding region, and a separate globular link protein (mol.wt. 48000 or 43000) interacts with both components to strengthen the proteoglycan–hyaluronate bond (Hardingham & Muir, 1972; Hardingham, 1979; Tang *et al.*, 1979).

Antibodies have been raised against different purified proteoglycan components, which have been shown to contain distinct antigenic determinants (Caterson *et al.*, 1979; Wieslander & Heinegård, 1979, 1980; Poole *et al.*, 1980). With such antisera, hyaluronate-binding region, keratan sulphate-rich region, chondroitin sulphate-bearing region and link protein have been detected. In all preparations of proteoglycan there is polydispersity and heterogeneity, molecules have been characterized with a high or a low content of keratan sulphate

(Heinegård & Hascall, 1979) and there is evidence of considerable variation in length of the chondroitin sulphate-bearing region (Hardingham *et al.*, 1976; Heinegård, 1977; Buckwalter & Rosenberg, 1982). Molecules may thus respond rather differently to antisera raised against different domains. In the present study, in order to determine aggregating proteoglycans selectively, antisera were raised against purified hyaluronate-binding region and link protein, and techniques have been developed to measure their presence in intact aggregates.

Materials and methods

Materials

All reagents were of analytical grade, except for carbazole and guanidinium chloride (BDH Chemicals, Poole, Dorset, U.K.). The guanidinium chloride was purified with activated charcoal (Norit GSX; Hopkin and Williams, Chadwell Heath, Essex, U.K.). Chondroitinase ABC was obtained from Worthington [Millipore (U.K.) Ltd., London N.W.10, U.K.]. Trypsin (diphenylcarbamoyl chloride-treated) (EC 3.4.21.4), 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, disodium EDTA, benzamidine hydrochloride, carbazole and bovine serum albumin (fraction V) were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Bovine serum albumin for radioimmunoassays was treated with Norit GSX. Sepharose gels, Sephacryl S-300, CNBr-activated Sepharose 4B and Protein A–Sepharose CL-4B were obtained from

Abbreviation used: SDS, sodium dodecyl sulphate.

Pharmacia (Uppsala, Sweden), and Bio-Gel P-6 was from Bio-Rad Laboratories (Watford, Herts., U.K.). Freund's adjuvant was from Difco Laboratories (Detroit, MI, U.S.A.) and *Staphylococcus aureus* (Cowan 1) was generously given by Mr. Andrew Lew, London School of Hygiene and Tropical Medicine. Na¹²⁵I (IMS30) was obtained from Amersham International (Amersham, Bucks., U.K.).

General analytical procedures

Uronic acid was determined by an automated procedure (Heinegård, 1973) of the modified carbazole reaction (Bitter & Muir, 1962) with glucuronolactone as standard. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Radioactivity was measured in an LKB Wallac 80000 gamma counter.

Preparation of proteoglycan fractions

Proteoglycans were extracted from pig laryngeal cartilage in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, in the presence of proteinase inhibitors as described by Hardingham (1979). The extract was dialysed against 7 vol. of 0.05 M-sodium acetate, pH 5.8, also containing proteinase inhibitors, and the proteoglycan components were separated by equilibrium density-gradient centrifugation (Hardingham & Muir, 1974). Associative equilibrium density-gradient centrifugations were performed in a Sorvall OTD-65 ultracentrifuge with a vertical rotor (8 × 35 ml) at 100 000 *g* for 19 h at 10°C. Each gradient was divided into four fractions: the bottom fraction (A1; 8 ml) and three further fractions each of volume 9 ml (A2, A3 and A4). Re-centrifugation of the A1 fraction under the same conditions gave a more purified A1A1 fraction. To separate link protein from proteoglycan, a solution of aggregate was fractionated by equilibrium density-gradient centrifugation under dissociative conditions in an MSE 65 centrifuge with an 8 × 25 ml angle head at 100 000 *g* for 48 h (Hardingham, 1979). The tubes were frozen and cut into four fractions, the bottom fraction (A1D1, 4 ml) containing proteoglycan monomer, two fractions of volume 5 ml (A1D2 and A1D3) and a top fraction (A1D4; 3 ml) containing the link protein. The link-protein fraction (A1D4) was chromatographed on a column (120 cm × 2.4 cm) of Sephacryl S-300, eluted with 4 M-guanidinium chloride/1 mM-Na₂EDTA, pH 5.8, and fractions containing the link-protein peak were pooled, dialysed and freeze-dried. For routine use, link-protein preparations were stored in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, and the concentration was determined from $A_{275}^{1\%} = 1.40$ (Tang *et al.*, 1979). Residual amounts of binding region in link-protein

preparations were further decreased by adsorption with anti-(binding region) antiserum. A portion (1 ml) of Protein A-Sepharose 4B, suspended in 0.15 M-NaCl buffered with 0.01 M-sodium phosphate, pH 7.4, was added to 2.5 ml of anti-(binding region) antiserum and incubated at room temperature for 2 h with constant mixing. The gel was washed three times with the above-mentioned buffered saline and added to 50 µg of link protein suspended in 1 ml of the incubation buffer (buffer A) used in the radioimmunoassay procedure. This was incubated for 7 h at 4°C with constant mixing. After centrifuging at 2000 *g* for 10 min to pellet the gel, the supernatant containing purified link protein was removed.

Preparation of proteoglycan aggregate

A solution of proteoglycan aggregate depleted of monomer was prepared by chromatography of an A1A1 fraction on a column (175 cm × 1.1 cm) of Sepharose 2B in 0.5 M-sodium acetate, pH 6.8. The *V*₀ (void-volume) fractions were pooled and the retarded peak of free monomer proteoglycan was discarded.

Preparation of proteoglycan binding region and link-protein antigens

Proteoglycan aggregate was digested for 40 min with chondroitinase ABC (2.5 units/g) followed by diphenylcarbamoyl chloride-treated trypsin (2 mg/g) for 5 h, essentially as described by Heinegård & Hascall (1974). The digest was concentrated by partial freeze-drying to one-third the original volume and chromatographed on a column (75 cm × 2.4 cm) of Sepharose CL-6B in 0.5 M-sodium acetate, pH 6.8. Fractions (5 ml) were collected and their uronic acid and protein contents were determined. The protein-rich *V*₀ fractions were pooled, dialysed, freeze-dried, dissolved in 4 M-guanidinium chloride/0.05 M-sodium acetate/1 mM-Na₂EDTA, pH 5.8, and chromatographed on a column (170 cm × 2.4 cm) of Sephacryl S-300 eluted with the same buffer. The column eluate was passed through a flow cell, its *A*₂₃₀ was recorded and 5 ml fractions were collected. The two protein peaks of binding region and link protein, which were partially resolved, were pooled separately, dialysed, freeze-dried and rechromatographed separately on a similar column. Some samples of rechromatographed peaks were further purified by preparative SDS/polyacrylamide-gel electrophoresis with an apparatus similar to that described by Koziarz *et al.* (1978). Binding region, isolated and freeze-dried as the sodium salt, was freely soluble in water; the concentration was determined from $A_{275}^{1\%} = 0.65$ (Perkins *et al.*, 1981). Trypsin-prepared link protein was not freely soluble in water and was kept in 4 M-guanidinium chloride, as described for the gradient preparation.

Preparation of antisera

Antisera were raised in female New Zealand White rabbits (1.5 kg body wt.) to trypsin-prepared binding region and link protein, and to SDS/polyacrylamide-gel-electrophoresis-purified preparations of these antigens. All injections were subcutaneous in the back of the neck. Primary injections were given with 0.75 mg of antigen dissolved in 0.5 ml of 0.15 M-NaCl buffered with 0.01 M-sodium phosphate, pH 7.4, and emulsified in 0.5 ml of Freund's complete adjuvant. Booster injections of 0.25 mg of antigen in Freund's incomplete adjuvant were given on day 20 and day 35 and at various intervals subsequently to maintain antibody titres. Blood samples (30–40 ml) were taken 10–12 days after each boost, allowed to clot for 1 h at room temperature and overnight at 4°C, and the serum was separated from the blood clot by centrifugation.

For these studies the antiserum to binding region was raised primarily against native binding region, but SDS/polyacrylamide-gel-electrophoresis-purified antigen was injected in later boosts. The antiserum against link protein was raised against SDS/polyacrylamide-gel-electrophoresis-purified link protein throughout. Of the limited number of animals immunized (eight), half received native protein in primary injections and half received SDS/polyacrylamide-gel-electrophoresis-prepared antigen in all injections. There was considerable variation in antibody titres obtained from the different animals, but this was unrelated to whether native or SDS-treated antigen was used.

Antiserum raised to binding region showed a low titre against link protein. The contaminating antibodies were removed by adsorbing the antiserum with link protein. A portion (1 mg) of link protein (A1A1D3) in 1 M-NaCl was covalently bound to 1 ml of CNBr-activated Sepharose 4B, and washed with 0.15 M-NaCl buffered with 0.01 M-sodium phosphate, pH 7.4. The link-protein-Sepharose 4B (0.1 ml) was added to 1 ml of anti-(binding region) antiserum and mixed continuously for 1 h at room temperature. The mixture was then centrifuged and the adsorbed antiserum removed. The amount of link protein bound to the Sepharose 4B was not determined, but it appeared sufficient to remove anti-(link protein) antibodies from the antiserum, and they were eluted when the gel was washed in 0.1 M-glycine/HCl, pH 2.8.

Radioimmunoassay procedures

The radioimmunoassay used is essentially that described by Caterson *et al.* (1979) in which formaldehyde-treated heat-killed *S. aureus* (Cowan strain 1) with Protein A in its cell wall is used to precipitate all the antigen-antibody complexes (Kessler, 1975). Assays were performed in poly-

styrene tubes (50 mm × 10 mm, capacity 2.5 ml) initially in an incubation buffer A containing 0.5% (w/v) deoxycholate, 0.1% (w/v) Nonidet P40, 0.1% (w/v) bovine serum albumin and 0.03% (w/v) Na₂SO₄ in 0.15 M-NaCl buffered with 0.01 M-sodium phosphate, pH 7.3; later assays were performed in buffer B, which contained SDS (0.025%, w/v) in addition to the components of buffer A.

For titration of the antiserum, ¹²⁵I-labelled antigen (3 ng in 0.1 ml containing 10 000–20 000 c.p.m.) was mixed with 0.1 ml of an antiserum dilution and incubated at room temperature for 90 min. To each solution, 0.1 ml of a 10% (w/v) suspension of *S. aureus* was added with mixing and incubated at room temperature for 15 min. Incubation buffer A (1 ml) was then added and the *S. aureus*-bound antibody-¹²⁵I-labelled-antigen complexes were pelleted by centrifugation at 12 000 *g* for 5 min. The supernatant was removed, the pellet washed twice with incubation buffer A and both the free and bound ¹²⁵I-labelled antigen were counted for radioactivity. The results were plotted as percentage of ¹²⁵I-labelled antigen bound versus dilution of antiserum.

Inhibition radioimmunoassays were performed as follows: 0.05 ml of ¹²⁵I-labelled antigen (3 ng) was mixed with 0.1 ml of unlabelled antigen and 0.05 ml of an antiserum dilution which was able to bind 50% of the maximum amount of ¹²⁵I-labelled antigen bound by the antiserum. After incubation at room temperature for 15 min, 0.03 ml of the 10% suspension of *S. aureus* was added to precipitate the antigen-antibody complexes, which were then centrifuged, washed and counted for radioactivity as described above. The results were expressed as percentage inhibition of ¹²⁵I-labelled-antigen binding to *S. aureus* versus concentration [$\log(\text{mol} \cdot \text{ml}^{-1})$] of non-radioactive antigen. For unknown samples, serial dilutions were prepared for the assay in incubation buffer A or B. From the results the concentration of antigen was calculated by averaging the values obtained for dilution giving between 20 and 60% inhibition (at least five values).

The concentration of standards was calculated by assuming the following molecular weights: link protein, trypsin-prepared, 41 000, and gradient-prepared, 48 000; binding region, 80 000; proteoglycan, 2×10^6 ; binding region-link protein-hyaluronate complex, 131 000 (Hardingham, 1981). The concentration of proteoglycan was determined by assuming a hexuronate content of 25% (w/w) (Hardingham *et al.*, 1976).

Iodination of antigens

[¹²⁵I]Iodination of the antigens was performed by the method of Greenwood *et al.* (1963), with chloramine-T as the oxidizing agent. A portion (15 μg) of antigen was iodinated with 0.5 mCi of

Na¹²⁵I, and the iodinated protein was separated from iodide that had not reacted by gel filtration on a column (10 cm × 0.7 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated with incubation buffer A.

Results

The antisera raised to link protein were able to precipitate up to 85% of the ¹²⁵I-labelled link protein when a 1:32 dilution was used, whereas no binding was observed when the anti-(link protein) antiserum were titrated against ¹²⁵I-labelled binding region (Fig. 1). Similarly, the antisera raised to binding region were shown by radioimmunoassay to precipitate up to 90% of the ¹²⁵I-labelled binding region, with a 1:16 dilution of the antisera (Fig. 1). No binding to ¹²⁵I-labelled link protein was detected at any antiserum dilution after adsorption of the antiserum with link protein.

Dilutions of antisera giving 50% of maximum ¹²⁵I-labelled-antigen binding were used for radioimmunoassays, and standard inhibition curves were produced by using known amounts of unlabelled antigen (see Fig. 4). For link protein the minimum concentration detectable was 50 fmol. For binding region the minimum was 100 fmol. These sensitivities are similar to those of other immunoassays previously reported for proteoglycan components (Caterson *et al.*, 1979; Wieslander & Heinegård, 1980). The s.d. of the measurements of inhibition was only 1.3%, which corresponds to a s.d. of the concentration (log scale) of unknown samples of approx. 4%.

Link protein of three different molecular forms can be obtained from pig laryngeal cartilage. That of lowest molecular weight prepared by trypsin

digestion of proteoglycan aggregate was chosen as the antigen to which the anti-(link protein) antiserum was raised. As this is derived from the larger forms of link protein by proteolytic cleavage, the antigenic determinants that it carries are likely to be present on the larger forms, and the antisera raised against it should therefore recognize all the forms of link protein. Comparison of different preparations of pig laryngeal-cartilage link protein showed that link protein prepared by equilibrium density-gradient centrifugation and consisting mainly of a link protein of mol.wt. 48 000 and the smaller trypsin-prepared link protein (mol.wt. 41 000) gave equal inhibition in the link-protein radioimmunoassay (Fig. 2). The antibodies therefore detected the large and small forms of the same link protein equally well.

Detection of link protein and binding region in aggregates

The ability of the antibodies to detect link protein and binding region in aggregates was assessed by using a purified link-stabilized proteoglycan-aggregate preparation that was depleted of monomer. Detection of the link protein was found to be severely restricted (Fig. 3), as a high concentration range was required to produce an inhibition curve. Comparison of the inhibition curves produced by purified link protein and proteoglycan aggregate suggested that the availability of the antigenic sites of link protein within the aggregate were decreased to only 0.45% of that of purified link protein (Fig. 4a). Methods of dissociating the aggregate were therefore tested in order to reveal the antigenic determinants of link protein (Fig. 3). Heat treatment of the aggregate at 80°C for 15 min improved the

Fig. 1. Titration of (a) anti-(link protein) antiserum and (b) anti-(binding region) antiserum

Titration was performed as described in the Materials and methods section with ¹²⁵I-labelled link protein (O) and ¹²⁵I-labelled binding region (□). The decrease in the binding of labelled antigen at high antibody concentration was due to the binding capacity of *S. aureus* being exceeded. However, the maximum percentage bound remained the same when extra *S. aureus* was used at the higher antibody concentrations. The dilution of antiserum used in the competitive-inhibition assay (Fig. 2) is that which precipitates 50% of the maximum ¹²⁵I-labelled antigen bound by the antiserum (*).

Fig. 2. *Radioimmunoassay of link protein*
Determinations of link protein prepared without trypsin (A1A1D4-S-300 preparation) (O) and that prepared with trypsin (●) were compared by the radioimmunoassay.

Fig. 3. *Determination of link protein in proteoglycan aggregate*

Link protein was determined by radioimmunoassay in preparations of proteoglycan aggregate after the following treatments: (i) no treatment (curve 1); (ii) heating at 80°C for 15 min (curve 2); (iii) incubation in 0.5% SDS for 2 h at room temperature (curve 3); (iv) heating at 80°C for 15 min in the presence of 0.5% SDS (curve 4); (v) heating at 80°C for 15 min in the presence of 0.025% SDS (curve 6). The inhibition curves produced were compared with that produced by link protein (curve 7) and link protein after incubation with 0.5% SDS for 2 h at room temperature (curve 5).

availability of the antigenic sites of link protein, as did incubation of the aggregate in the presence of 0.5% SDS for 2 h before the immunoassay. However, the inhibition curves produced showed that it was still not quantitatively detected. A combination of these two methods, involving heating the aggregate at 80°C for 15 min in the presence of 0.5% SDS, improved the detection so that the inhibition curve was very similar to that obtained by using standard link protein with the same treatment. However, these inhibition curves were somewhat suppressed, which was probably due to the high concentration of SDS present (Dimitriadis, 1979). By using a range of SDS concentrations, a protocol of heating the aggregate at 80°C for 15 min in the presence of 0.025% SDS was found to be sufficient to reveal the antigenic sites of the link protein, and the inhibition curve produced by proteoglycan aggregate after this treatment was very similar to that produced by standard link protein, except at the highest concentrations of aggregate.

The availability of the antigenic determinants of the binding region within the proteoglycan monomer (A1A1D1) was found to be unrestricted, the inhibition curve produced in the binding-region radioimmunoassay being very similar to that produced by purified binding region. However, comparison of the inhibition curves produced by the proteoglycan aggregate and purified binding region showed that there was significant masking of the binding-region antigenic sites in the aggregate (Fig. 4b). The binding of the antibodies to the aggregate was only 55% efficient. Application of the protocol of heat treatment in the presence of SDS was again successful in restoring the quantitative measurement

of binding region in all but the highest concentrations used. The results of both assays showed that the molar ratio of link protein to binding region in the aggregate preparation was approx. 1:1 and the detection of both antigens was insensitive to the time of heating between 5 and 30 min.

Further analysis of the masking of the antigenic sites of link protein and binding region within proteoglycan aggregate was performed by using the binding-region-link-protein-hyaluronate complex (prepared by chondroitinase ABC/trypsin digestion of aggregate) as inhibitor in the radioimmunoassays (Figs. 4a and 4b). The availability of the link-protein antigenic sites within the complex was found to be only 1.2% compared with that for purified link protein, a result very similar to that obtained with proteoglycan aggregate. Testing the binding-region-link-protein-hyaluronate complex in the binding-region radioimmunoassay also produced similar results to those obtained with the proteoglycan aggregate. The availability of the binding region within the complex to the antibodies was decreased to 49% of that of the purified binding region, but this was fully restored after SDS and heat treatment to dissociate the complex before the assay.

Fig. 4. Comparison of the availability of the antigenic sites of (a) link protein and (b) binding region in proteoglycan aggregate and the binding-region-link-protein-hyaluronate complex

Inhibition in the radioimmunoassays was measured in incubation buffer A for proteoglycan aggregate (Δ) and the complex (∇), and after heating at 80°C for 15 min in incubation buffer B (containing SDS) for proteoglycan aggregate ($\blacktriangle$) and complex ($\blacktriangledown$). Standard link protein ($\circ$) and binding region ($\square$) were in buffer A.

These results show that the extended region of the protein core bearing all the glycosaminoglycan chains makes little contribution to the masking of the link-protein and binding-region antigenic sites.

The ability of the modified radioimmunoassays to measure binding region and link protein in aggregates, as well as in isolated components, was applied to the measurement of the antigens in impure mixtures, including 4M-guanidinium chloride extracts of pig laryngeal cartilage. Additive experiments with purified proteoglycan aggregate and 4M-guanidinium chloride extracts have shown that heat treatment of the diluted samples in an incubation buffer B with SDS before the normal assay procedure permitted quantitative measurements of both components in impure proteoglycan mixtures. The antisera did not appear to contain antibodies to other extractable cartilage proteins when tested by SDS/polyacrylamide-gel electrophoresis and Western blotting (D. G. Dunham & T. E. Hardingham, unpublished work).

Immunochemical analysis of proteoglycan preparations

The assays allowed critical assessment of the purity of isolated proteoglycan preparations. Purified link protein (A1A1D4-S300) was found to give a small but significant amount of inhibition in the binding-region assay. This was shown to result from contamination with binding region (0.017 mol/mol), as it was successfully removed by adsorbing the link protein with specific anti-(binding region) antibodies (see the Materials and methods section). A similar contamination of link protein previously purified by chromatography on Sepharose CL-6B was noted by Caterson *et al.* (1979). Analysis of a preparation of binding region showed that only trace

amounts of contaminating link protein were present (0.004 mol/mol).

The abundance of link protein and binding region was measured in equilibrium-density-gradient-purified proteoglycan preparations. The aggregated proteoglycan fraction (A1) contained most (>90%) of the binding region and link protein in a 4M-guanidinium chloride extract of pig laryngeal cartilage. Fractionation of aggregated proteoglycan in a dissociative gradient separated most of the binding region with proteoglycan monomer in the A1D1 fraction, whereas link protein separated into the A1D4 fraction. Link protein was present in the A1D1 fraction (0.017 mol of link protein/mol of binding region), but this was successfully removed (<0.005 mol/mol) by further dissociative density-gradient centrifugation of the A1D1 fraction to give an A1D1D1 fraction. The link-protein fraction A1D4 contained a significant amount of binding region (0.05 mol of binding region/mol of link protein), and required further purification by gel filtration on Sephacryl S-300 (as described above) to decrease the contamination.

Species specificity

The cross-reactivity of proteoglycans from different species was compared by using the radioimmunoassays. Binding region was measured in aggregated proteoglycan preparations of pig, dog, rabbit and cow articular cartilage, rat chondrosarcoma (Swarm) and a monomer preparation from human articular cartilage (a gift from Dr. M. Bayliss, Royal National Orthopaedic Hospital, Stanmore, Middx., U.K.). The inhibition curves obtained showed decreased immunoreactivity with the proteoglycans from other species, but approximately equal reactivity for proteoglycans from pig

laryngeal and articular cartilages. These results assume a similar molecular weight for the different proteoglycan preparations, which may not be correct, but any error this introduces will be small compared with the major differences observed between the species. Comparison of the results indicates that the binding regions of cow (89%) and dog (78%) show a high degree of cross-reactivity with that of pig, with rat (37%) and human (22%) giving lower values and with rabbit (15%) being the lowest. Comparable results for antisera to proteoglycans from bovine nasal cartilage have been reported by Wieslander & Heinegård (1981).

Discussion

The inhibition radioimmunoassays described provide specific methods to determine the binding region of proteoglycan monomer and link protein. Application of the assays to proteoglycan aggregates showed that detection of antigenic determinants was incomplete and, furthermore, that neither the detergent buffer used in the assays nor the presence of specific antibodies dissociated the aggregates. Since similar masking of the antigenic sites was also seen in the binding-region-link-protein-hyaluronate complex, it was not due to the large chondroitin sulphate-bearing part of the proteoglycan monomer and therefore must be a consequence of the interaction of the three components. This ternary complex is more resistant to tryptic digestion (Heinegård & Hascall, 1974) than are the free components, which may indicate a compact structure. The antigenic determinants of link protein, and, to a lesser extent, binding region, may thus be buried on protein-protein and protein-carbohydrate contact surfaces.

The masking of antigenic determinants in aggregates made it necessary to devise a procedure to permit their detection. Most of the conditions that dissociate aggregates, such as 4M-guanidinium chloride, 2M-KSCN and 6M-urea, are incompatible with antibody-antigen or antibody-protein A interaction. However, dissociation in SDS (0.025%, w/v) at 80°C, which showed complete recovery of antigenicity, had no apparent effects on the assay, where the final concentration of SDS was 0.0125% (w/v). A low concentration of SDS ($\leq 0.05\%$, w/v) has been reported to decrease non-specific adsorption in antibody-antigen precipitation in the presence of other non-ionic detergents (Dimitriadis, 1979). Caterson *et al.* (1979) showed that antisera raised against link protein prepared by using SDS also detected link protein prepared without SDS, and recommended the use of SDS in link-protein assays. The present study confirms this finding and shows that binding region, after SDS treatment, is also detected well. Franzén *et al.* (1981) have also shown that treatment of samples containing aggregates with

SDS (0.8%, w/v) led to the quantitative detection of link protein in an enzyme-linked immunoabsorbent assay, and SDS has been used in the presence of Triton X-100 in a radioimmunoassay of 148 000-mol.wt. cartilage protein by using double-antibody precipitation (Paulsson & Heinegård, 1982).

With the antisera produced in the present study, the treatment with SDS and heat was essential for the quantitative detection of link protein and binding region in aggregates. By using this procedure, molar ratios of link protein to binding region in purified aggregate were close to 1:1 and the concentrations of the detected antigens were similar to the calculated concentration of the proteoglycan (Figs. 4a and 4b). Similar agreement between molar ratios and between concentrations was also obtained for the binding-region-link-protein-hyaluronate complex. These results are in agreement with the proposed stoichiometry of aggregation (see Hardingham, 1981) and support the assumptions made for the molecular weights of proteoglycan and the complex.

As well as dissociating aggregates, heating in SDS may be of importance in preventing other interactions and conformational changes that may affect antigenicity. Complex effects of masking and enhancement of proteoglycan-related determinants, which made quantification more difficult, have been reported when an enzyme-linked immunoabsorbent assay (Thonar *et al.*, 1982) was used. The procedure described here is thus a simple and versatile method for the determination of both link protein and binding region in pure and impure samples. The method may also be applicable to other proteins if their antigenicity is not adversely affected by the SDS treatment.

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