A comparative study of the binding of cartilage link protein and the hyaluronate-binding region of the cartilage proteoglycan to hyaluronate-substituted Sepharose gel

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The hyaluronate-binding proteins from bovine nasal cartilage, i.e. the hyaluronate-binding region of the proteoglycan and the link protein, were labelled with ^{125}I and separated from each other by gel chromatography. The proteins were characterized by molecular-weight determinations and their purity was established by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and immunodiffusion. The binding properties of the two proteins to hyaluronate-substituted Sepharose gel were compared. It was found that both proteins behaved similarly. They bound with the same efficiency to the gel, they showed the same time course of binding, had slightly different pH optima for binding and both proteins had a decreasing affinity for the gel with increasing ionic strength. The binding to the gel could be inhibited by soluble hyaluronate, and the minimum size of a hyaluronate oligosaccharide required for inhibition was in both cases a deca-saccharide (only even-numbered oligosaccharides were tested). The proteins did not show any co-operative binding in the system tested, which could be explained by the large number of binding sites in the hyaluronate-substituted gel. Binding constants for the protein–hyaluronate interaction were estimated. A value of $1.3 \times 10^7 \text{ M}^{-1}$ was obtained for the hyaluronate-binding region of the proteoglycan, in agreement with literature data. The corresponding value for the link protein was $0.7 \times 10^7 \text{ M}^{-1}$.

It has been shown that proteins with specific affinity for hyaluronate can be used in a sensitive assay for hyaluronate (Tengblad, 1980; Laurent & Tengblad, 1980). A mixture of the globular part of the cartilage proteoglycan and the cartilage link protein, which both have affinity for the polysaccharide, was labelled with ^{125}I and partitioned between free hyaluronate and hyaluronate substituted to an agarose gel. The labelled material adsorbed to the gel was a function of the concentration of free hyaluronate present. No attempt was made in the earlier studies (Tengblad, 1980; Laurent & Tengblad, 1980) to separate the two labelled cartilage components and investigate the usefulness of the individual proteins in the assay procedure. The present paper describes a comparative study of the binding properties of the two proteins to the hyaluronate gel.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Hyaluronate

Sodium hyaluronate from rooster comb (Healon) was kindly supplied by AB Pharmacia, Uppsala, Sweden. Oligosaccharides of hyaluronate were prepared by gel chromatography of a hyaluronidase digest essentially as described by Hascall & Heinegård (1974b) and by Cleland (1979).

Hyaluronate-substituted agarose gel

Hyaluronate with a molecular weight of 52 000 as measured by viscosity (Cleland, 1970; Cleland & Wang, 1970) was obtained by limited hyaluronidase digestion (Tengblad, 1979). It was immobilized on an AH-Sepharose (1,6-diaminohexane-Sepharose) gel (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (Tengblad, 1979). The gel contained 1.5 mg of hyaluronate/ml of wet gel as determined by trace incorporation of radio-

active hyaluronate (Tengblad, 1979). The volume of the gel given below corresponds to the volume of the gel phase after it had settled at unit gravity for 24 h at room temperature. Sepharose 4B (Pharmacia Fine Chemicals) was used as a control gel.

Proteins

Unlabelled cartilage proteins with affinity for hyaluronate were prepared as described by Tengblad (1979) by affinity chromatography and two gel-chromatographic steps. Fraction BI (Tengblad, 1979) will in the following text be called hyaluronate-binding region of the proteoglycan, and fraction CII (Tengblad, 1979) will be called link protein. The proteins were dialysed against water and freeze-dried. Before use, hyaluronate-binding region and link protein were dissolved in 4 M-guanidinium chloride/0.5 M-acetate, pH 5.7.

Bovine serum albumin (fraction V) was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of ^{125}I -labelled cartilage proteins with affinity for hyaluronate

A mixture of hyaluronate-binding region and link protein bound to hyaluronate was prepared and labelled essentially as described by Laurent & Tengblad (1980). Carrier-free Na^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.; 0.5 mCi) was made to react with protein (100 μg) as described by Greenwood *et al.* (1963).

Hyaluronate and degraded proteins were then removed by affinity chromatography (Tengblad, 1979) and intact iodinated proteins were eluted from the gel with 4 M-guanidinium chloride/0.5 M-sodium acetate, pH 5.7. The specific radioactivity was approx. 1.1×10^6 c.p.m./ μg of protein. A portion of the labelled protein mixture was diluted to suitable concentrations with the eluent + 0.1% albumin. The remaining material was chromatographed at 7°C on a column (2.6 cm $\times$ 95 cm) of Sepharose CL-6B (Pharmacia Fine Chemicals) in the same solvent. The flow rate was 11 ml/h, and 1 ml fractions were collected. The chromatogram showed two distinct peaks (Fig. 1), and materials from the two peaks were pooled as indicated in Fig. 1 and stored in the elution buffer until used.

Chemical analyses

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and hexuronic acid was determined by the modification of the carbazole method described by Bitter & Muir (1962), with glucuronolactone as standard.

SDS/polyacrylamide-gel electrophoresis

Reduced samples were analysed as described by Neville (1971) and the necessary reagents were from

Eastman Kodak (Rochester, NY, U.S.A.). Bovine serum albumin and ovalbumin (Sigma) were used as reference proteins. The protein bands were either stained with Coomassie Brilliant Blue R 250 (Merck, Darmstadt, Germany) (Korn & Wright, 1973) or detected by scintillation counting after the gels had been sliced in 1 mm-thick slices.

Radioactivity

^{125}I was determined in a Packard Autogamma scintillation spectrometer.

Immunodiffusion

Antisera were raised in rabbits against the unlabelled hyaluronate-binding region and link protein, respectively. The proteins (1 mg/ml) were mixed with equal volumes of Freund's complete adjuvant and 0.1 ml of the solutions were injected into one lymph node on the back of each thigh. Booster doses of the incomplete adjuvant were given twice at 14-day intervals via the same route. The rabbits were bled after the last injection and the serum was stored at -20°C until further use.

Unlabelled and radioactive proteins were mixed before double immunodiffusion (Ouchterlony, 1958) was carried out in 1% agarose gels containing 0.4 M-veronal buffer, pH 8.6. About 10–15 μl of the antisera and protein solutions were used in the wells. The protein concentrations and radioactivities in the samples analysed are given in the legend of Fig. 3. After 2 days the gels were stained by Coomassie Brilliant Blue and radioautography was done with No-screen Film NS-2T (Eastman Kodak). The films were exposed for 12 days.

Ultracentrifugation

Molecular weights were determined by sedimentation equilibrium at 20°C in a Spinco model E analytical ultracentrifuge. Interference optics and the long-column meniscus-depletion method of Chervenka (1970) were employed. The runs were made in 12 mm double-sector capillary synthetic-boundary cells at speeds of 15 000 rev./min (hyaluronate-binding region) and 24 000 rev./min (link protein). The solvent was either 1 M-NaCl/0.5 M-sodium acetate, pH 5.7, or 4 M-guanidinium chloride/0.5 M-sodium acetate, pH 5.7. The proteins were analysed at concentrations between 0.5 and 3 mg/ml. Equilibrium conditions were obtained after 20 h. The interference pattern was analysed in a micro-comparator (Nikon model 6 CT2; Nippon Kogaku K.K., Japan). The fringe displacement was measured on at least two separate fringes in each run. Apparent molecular weights were calculated from plots of log (fringe displacement) versus r^2 (r is the distance from the centre of rotation) (Schachman, 1957).

The values of the partial specific volumes used in

the calculations were estimated from the amino acid and hexosamine contents (Tengblad, 1979). Partial specific volumes of individual amino acid residues were taken from Cohn & Edsall (1943). The glucosamine and galactosamine contents in the hyaluronate-binding region were assumed to represent keratan sulphate and chondroitin sulphate respectively. (This treatment does not take into account that some hexosamine is derived from *N*-linked oligosaccharides.) A value for the partial specific volume of 0.54 ml/g was used for chondroitin sulphate (Luscombe & Phelps, 1967), and the same value was assumed for keratan sulphate (Hascall & Riolo, 1972). The contribution of sugar residues to the partial specific volume of the link protein was neglected. The values calculated finally were for the hyaluronate-binding region 0.65 ml/g and for the link protein 0.72 ml/g.

The apparent molecular weights determined at at least three different concentrations for each protein were used to calculate a value at infinite dilution by extrapolation.

Binding experiments

Binding studies were performed in 3 ml glass tubes that had been pre-washed with 0.1% albumin in 0.5 M-sodium acetate buffer, pH 5.7. Hyaluronate-substituted gel (0–50 μ l) suspended in the appropriate buffer (containing 0.1% albumin) in a total volume of 500 μ l was mixed with 50 μ l of labelled protein [dissolved in 4 M guanidinium chloride/0.5 M-sodium acetate (pH 5.7)/0.1% albumin] and the tubes were agitated at room temperature, usually for 16 h. In some experiments various concentrations of soluble hyaluronate or hyaluronate oligosaccharides were included in the incubations. After termination of the incubations the gel was pelleted by centrifugation at 900 *g* for 5 min and the supernatant was removed. The gel was washed twice with 3 ml of buffer and pelleted before its radioactivity was measured.

In experiments to investigate the effect of pH on the binding, a universal buffer containing diethylbarbituric acid, citric acid, potassium phosphate and boric acid (approx. 0.03 M of each) and NaOH (0.02–0.1 M) was used (Britton & Welford, 1937). The pH of the incubation mixture after addition of the protein solution was determined in a PHM 62 standard pH-meter (Radiometer, Copenhagen, Denmark) in parallel experiments with larger volumes. In all other experiments, 0.5 M-sodium acetate buffer, pH 5.7, was employed with additions of various amounts of NaCl.

Results

Characterization of unlabelled and 125 I-labelled hyaluronate-binding region and link protein

The purification of 125 I-labelled hyaluronate-

binding region and link protein was achieved by a slightly modified technique as compared with the preparation of unlabelled material (Tengblad, 1979). Because the radioactive protein had to be handled in smaller quantities, only one gel-chromatographic step was used (Fig. 1). The peaks in the gel chromatogram indicate that the iodination did not lead to the formation of aggregates or degradation products. The two peaks were not completely separated. SDS/polyacrylamide-gel electrophoresis combined with radioactivity measurements was used to test each fraction in the chromatogram, and fractions where only single bands were seen were pooled (as indicated in Fig. 1). This procedure could select populations of the hyaluronate-binding region and the link protein different from those present in the unlabelled material. However, in the SDS/polyacrylamide-gel electrophoresis the radioactive material migrated as unlabelled proteins (Tengblad, 1979). The mobilities correspond to molecular weights of 90 000 and 45 000 for the hyaluronate-binding region and the link protein respectively.

The molecular weights of the unlabelled proteins were determined by sedimentation-equilibrium centrifugation in 4 M-guanidinium chloride/0.5 M-sodium acetate, pH 5.7. Hyaluronate-binding region was also studied in 1.0 M-NaCl in the same acetate buffer. Both proteins gave linear plots of log concentration versus r^2 (Fig. 2), indicating homogeneity. Extrapolation of the apparent molecular weights to infinite dilution gave values of 91 000 and 98 000 for the hyaluronate-binding region in 4 M-guanidinium chloride and 1 M-NaCl respectively (the

Fig. 1. Gel chromatography of 125 I-labelled hyaluronate-binding proteins

Labelled proteins, purified by affinity chromatography on a hyaluronate-substituted gel, were chromatographed at 7°C on a column (2.6 cm $\times$ 95 cm) of Sepharose CL-6B in 4 M-guanidinium chloride/0.5 M-sodium acetate/0.1% albumin, pH 5.7. Material from the two peaks was pooled as indicated: I, hyaluronate-binding region; II, link protein.

Fig. 2. Sedimentation equilibrium of hyaluronate-binding proteins

The hyaluronate-binding region of proteoglycan (a) and the link protein (b) were centrifuged for 20 h at various concentrations (0.4–3 mg/ml) in 0.5 M-sodium acetate, pH 5.7, containing either 1 M-NaCl (●) or 4 M-guanidinium chloride (○, ■). The rotor speeds were 15 000 rev./min for the hyaluronate-binding region of proteoglycan and 24 000 rev./min for the link protein. Figs. 2(a) and 2(b) show plots of $\ln$ (fringe displacement) versus r^2 (r = distance from the centre of rotation). The apparent molecular weights obtained from the slopes of these plots were used to extrapolate the values of the molecular weights at infinite dilution (c). These are 98 000 and 91 000 for the hyaluronate-binding region in NaCl (●) and guanidinium chloride (○) respectively, and 44 000 for the link protein in guanidinium chloride (■).

difference is not regarded as significant), and 44 000 for the link protein.

Immunodiffusion was carried out on hyaluronate-binding region and link protein (carrier + labelled protein). Fig. 3 demonstrates that the hyaluronate-binding region reacted with antibodies raised against itself, but not with antibodies raised against the link protein. Radioautography shows that the radioactive hyaluronate-binding region precipitated together with the unlabelled material. Similarly, but less clearly, the labelled and unlabelled link protein

Fig. 3. Immunodiffusion of hyaluronate-binding proteins

Double immunodiffusion of labelled + unlabelled hyaluronate-binding proteins against antibodies raised against hyaluronate binding region (I) and link protein (II). The proteins in the wells were as follows. (I) Link protein: (a) 0.1 μ g, 1000 c.p.m.; (b) 1.6 μ g, 4000 c.p.m.; (c) 0.8 μ g, 2000 c.p.m. Hyaluronate-binding region: (1) 0.75 μ g, 4000 c.p.m.; (2) 1.5 μ g, 1000 c.p.m.; (3) 0.75 μ g, 500 c.p.m. (II) Link protein: (a) 0.1 μ g, 2000 c.p.m.; (b) 1.6 μ g, 2000 c.p.m.; (c) 1.0 μ g, 2000 c.p.m. Hyaluronate-binding region: (1) 0.75 μ g, 4000 c.p.m.; (2) 1.5 μ g, 1000 c.p.m.; (3) 0.75 μ g, 500 c.p.m. The upper pictures were stained by Coomassie Brilliant Blue and the lower pictures are radioautographs.

only reacted with antibodies against itself. A considerable amount of labelled and unlabelled link protein precipitated unspecifically around the wells, which obscures the analysis. Both the hyaluronate-binding region and the link protein gave rise to one single precipitation line.

Time course of binding

The time course of the binding of the radioactive proteins to hyaluronate-substituted gel, in 0.5 M-sodium acetate/0.36 M guanidinium chloride/0.1% albumin, pH 5.7, with or without 1 M-NaCl, is shown in Fig. 4. The equilibration required 10–20 h for both the hyaluronate-binding region and the link protein. Both proteins had a higher affinity for the gel at the lower ionic strength. The equilibration time was, however, approximately the same. An equilibration time of 16 h has been used in subsequent experiments.

Binding efficiency of the protein

The total binding of the two proteins was determined in experiments with increasing gel concentration. As shown in Fig. 5, the binding

Fig. 4. Time course of binding to hyaluronate-substituted gel

Hyaluronate-substituted gel ($4\mu\text{l}$) was mixed with ^{125}I -labelled proteins and the binding to the gel was measured after various time intervals at room temperature. The proteins studied were: (a) the hyaluronate-binding region of proteoglycan (O, ●); (b) link protein (□, ■); (c) a mixture of the two proteins (△, ▲). The experiments were carried out in 0.5 M-sodium acetate/0.36 M-guanidinium chloride/0.1% albumin, pH 5.7 with (closed symbols) or without (open symbols) 1 M-NaCl. The binding ($< 4\%$ for hyaluronate-binding region and $< 6\%$ for link protein) to control gel (Sephacel CL-4B) has been subtracted.

reached a plateau. At the lower ionic strength approx. 60% of either protein was bound at high gel concentrations, whereas at the higher ionic strength only 30 and 40% of the hyaluronate-binding region and link protein, respectively, were bound.

Binding capacity of the gel

At low protein concentrations the amount of protein bound to the hyaluronate-substituted gel was directly proportional to the concentration of protein in the system. There was no marked difference in the behaviour of the hyaluronate-binding region and the

Fig. 5. Determination of the fractional amount of protein capable of binding to hyaluronate-substituted gel

Increasing amounts of hyaluronate-substituted Sepharose gel (0–50 μl) were incubated with approx. 35 ng of labelled hyaluronate-binding region of proteoglycan (O, ●) or approx. 30 ng of link protein (□, ■) in 0.5 M-sodium acetate/0.36 M-guanidinium chloride/0.1% albumin, pH 5.7 with (closed symbols) or without (open symbols) 1 M-NaCl. The radioactivity bound to the gel after 16 h at 20°C was measured and plotted, after correction for unspecific binding, as a function of added gel.

link protein. It was also apparent that the unspecific binding to an unsubstituted agarose gel was smaller for the hyaluronate-binding region than for the link protein.

Experiments were performed to estimate the total binding capacity of the gel. A constant amount of labelled protein was mixed with increasing amounts of unlabelled protein until the unlabelled protein inhibited the binding of the labelled one. These experiments are presented in Fig. 6. Assuming that the unlabelled proteins have the same binding ability as the labelled ones, one finds that more than $1\mu\text{g}$ of unlabelled protein is required to affect significantly the binding of labelled material to $4\mu\text{l}$ of gel.

The data may be used to calculate approximate association constants for the binding of the proteins to the hyaluronate in the gel. The following equation describes the binding (see, e.g., Steward & Petty, 1972):

$$\frac{1}{[P_b]} = \frac{1}{K \times [\text{TBS}]} \cdot \frac{1}{[P_f]} + \frac{1}{[\text{TBS}]} \quad (1)$$

where $[P_b]$ = concentration of bound protein, $[P_f]$ = concentration of free protein, $[\text{TBS}]$ = concentration of total binding sites on the gel, and K = association constant.

The data presented in Fig. 6(a) in the interval where unlabelled protein inhibits the binding of labelled protein have been replotted according to eqn. (1) in Fig. 6(b). The molar concentrations of the proteins were calculated from the values of molecular weights given above (98 000 and 44 000,

Fig. 6. *Binding capacity of hyaluronate-substituted gel* Increasing amounts (0–400 μg) of unlabelled hyaluronate-binding region of proteoglycan and link protein were mixed with ¹²⁵I-labelled hyaluronate-binding region (approx. 80 ng) or link protein (approx. 40 ng), respectively, and equilibrated with 4 μl of hyaluronate-substituted gel at 20°C in 0.5 M-sodium acetate/0.36 M-guanidinium chloride/0.1% albumin, pH 5.7. (a) The fraction of radioactive hyaluronate binding region (●) and link protein (■) bound to the gel was registered. Between 1 and 10 μg of either unlabelled protein must be added before it starts to compete with the labelled protein for binding sites. (b) The data in the range of binding-site saturation have been replotted according to eqn. (1). The intercept on the ordinate gives the reciprocal value of the total number of binding sites on the gel per litre of incubation mixture. From this value and the slope of the line one can calculate the association constant (see the text).

respectively, for hyaluronate-binding region and link protein). The binding efficiency of the unlabelled proteins was assumed to be the same as that of the labelled counterparts. The concentrations of total binding sites obtained from the intercepts on the ordinate are 0.08 and 0.27 μmol/l respectively, and can be recalculated as 10 and 37 μl of the gel. Thus the larger hyaluronate-binding region can occupy less sites in the gel than does the link protein. From the slopes of the plots and the values of the total binding sites one can calculate association constants for hyaluronate-binding region and link protein of $1.3 \times 10^7 \text{ M}^{-1}$ and $0.7 \times 10^7 \text{ M}^{-1}$ respectively.

Fig. 7. *pH-dependence of the binding of proteins to hyaluronate-substituted gel*

Hyaluronate-substituted Sepharose gel (4 μl) was mixed with labelled hyaluronate-binding region of proteoglycan (○), link protein (□), or a mixture of both (Δ) in a universal buffer (pH 2–12) of *I* approx. 0.15 mol/l, containing 0.1% albumin and 0.36 M-guanidinium chloride. The amount of radioactivity bound to the gel was measured after 16 h at 20°C. The binding is given on the ordinate as percentage of the maximal binding to 30 μl of gel at pH 6.5. The binding to control gel has been subtracted from the data.

pH-dependence of binding

The pH-dependence of the binding is shown in Fig. 7. Both proteins bound over a large pH range. Maximal binding was found between pH 5 and 7. The link protein showed maximal activity at a lower pH than the hyaluronate-binding region, whereas the latter showed a higher binding activity in the alkaline range. A mixture of the two proteins showed an intermediate behaviour.

Inhibition of binding with hyaluronate oligosaccharides

Oligosaccharides prepared by digestion of hyaluronate with testicular hyaluronidase were tested for their inhibitory effect on the binding of hyaluronate-binding region and link protein to the hyaluronate-substituted gel. As shown in Table 1, decasaccharides and longer oligosaccharides inhibited the binding of both proteins.

Inhibition of binding with high-molecular-weight soluble hyaluronate

Curves corresponding to the calibration curves for the hyaluronate assay (Tengblad, 1980) were constructed with both hyaluronate-binding region and link protein. Such curves are shown in Fig. 8. Both proteins demonstrated very similar behaviour, and so did a mixture of the two proteins. Both proteins apparently interacted with high-molecular-weight hyaluronate to a similar degree.

Table 1. Inhibition of protein binding to hyaluronate-substituted Sepharose by oligosaccharides prepared from hyaluronate

¹²⁵I-labelled hyaluronate-binding proteins (approx. 20 ng), hyaluronate-substituted gel (4 μl) and hyaluronate-derived oligosaccharides (560 ng, 1 μg or 10 μg) were incubated for 16 h in 0.5 M-sodium acetate/0.36 M-guanidinium chloride/0.1% albumin, pH 5.7 at 20°C. The amount of protein bound is given as percentage of the binding in the absence of soluble hyaluronate.

Protein	Number of monosaccharides in oligosaccharide	Binding (%) in the presence of the following amounts of oligosaccharides:		
		560 ng	1 μg	10 μg
Hyaluronate-binding region	4	100	100	100
	6	100	100	100
	8	100	100	100
	10	90	60	2
	12	76	50	4
	14	74	60	8
	16	76	60	8
	425	50	35	8
Link protein	4	100	100	100
	6	100	100	100
	8	100	100	100
	10	90	76	20
	12	64	45	13
	14	61	42	4
	16	58	42	6
	425	51	40	7
Hyaluronate-binding region + link protein	4	100	100	100
	6	100	100	100
	8	100	100	100
	10	67	53	25
	12	53	42	10
	14	50	33	10
	16	52	42	5
	425	47	33	3

Fig. 8. Inhibition of protein binding to hyaluronate-substituted gel by soluble hyaluronate. Approx. 20 ng of ¹²⁵I-labelled hyaluronate-binding region of the proteoglycan (○), or link protein (□), or a mixture of 20 ng of both (△), was incubated with 4 μl of hyaluronate-substituted gel and various amounts of soluble hyaluronate (0–100 μg) for 16 h at 20°C in 0.5 M-sodium acetate/0.36 M-guanidinium chloride/0.1% albumin, pH 5.7. The ordinate gives the amount of protein bound as percentage of the binding in the absence of soluble hyaluronate.

Discussion

The preparation method used in this investigation includes a trypsin treatment of the proteoglycan-hyaluronate aggregates. We should therefore expect that the hyaluronate-binding region also contains part of the keratan sulphate-rich region of the proteoglycan, as described by Heinegård and co-workers (Heinegård & Hascall, 1974a; Heinegård & Axelsson, 1977; Heinegård, 1977).

Similarly, we should expect that our link protein corresponds to the *a* protein described by Hascall & Heinegård (1974a), the *A* link protein described by Bonnet *et al.* (1978), and the link protein 2 described by Baker & Caterson (1979). Our analytical data, including molecular weights determined by SDS/polyacrylamide-gel electrophoresis and ultracentrifugation, amino acid composition (Tengblad, 1979), affinity for hyaluronate and lack of immunological cross-reactivity, corroborate this expectation.

The present investigation provides new information especially about the binding properties of the link protein. This protein has a low solubility in ordinary salt solutions and it is necessary to work in very dilute solution. Our data indicate that the link protein has binding properties very similar to those of the hyaluronate-binding region. Both proteins show a similar time course of binding (Fig. 4), approximately the same binding efficiency to hyaluronate gel (Fig. 5) and similar ionic-strength dependence of the binding (Figs. 4 and 5). There are small variations in the pH optimum of binding for the two proteins. The link protein has a slightly more acid pH optimum than the hyaluronate-binding region of the proteoglycan (Fig. 7). The binding to the gel is inhibited to approximately the same extent by soluble hyaluronate (Fig. 8), and the same length of oligosaccharides (decasaccharides and longer) is required for the inhibition (Table 1). This result is in agreement with the observation by Kimura *et al.* (1979) that 24 monosaccharides are sufficient to bind both a link protein and a proteoglycan monomer. The binding sites could be slightly shorter than decasaccharides, as only even-numbered oligosaccharides, formed by testicular hyaluronidase, were used in the inhibition studies.

It is somewhat surprising that in this and earlier studies (Tengblad, 1980; Laurent & Tengblad, 1980) only about 50% of the proteins were able to bind to the hyaluronate gel. There is, however, a marked ionic-strength dependence (Tengblad, 1980; Figs. 4 and 5), and the binding ability is presumably higher at physiological ionic strength than at the high ionic strengths used in the present investigation. An explanation of the salt effect could be a change in conformation in part of the protein molecules leading to a loss of affinity for hyaluron-

ate. The reasons for choosing a high ionic strength have been to avoid non-specific binding (Laurent & Tengblad, 1980) and to suppress hyaluronidase activity (Laurent, 1981) in the hyaluronate assay.

Experiments with mixed hyaluronate-binding region and link protein show that the mixtures behave similarly to the single proteins without any significant co-operative effects (Figs. 4 and 8). This was initially an unexpected observation, since it is known that the presence of the link protein stabilizes the binding of the proteoglycan to hyaluronate (Gregory, 1973; Heinegård & Hascall, 1974b; Hascall & Heinegård, 1974a, 1975; Muir & Hardingham, 1975; Hardingham, 1979). The lack of co-operativity can be explained by a modification of either or both proteins during the trypsin treatment or the radioactive labelling. The most probable reason is, however, the large excess of binding sites for the two proteins on the hyaluronate-substituted gel (Fig. 6a). Even if a triple complex of hyaluronate-binding region, link protein and hyaluronate is stronger than the individual protein-hyaluronate bonds, the latter are favoured, owing to the large amount of hyaluronate in the system. In accordance with this suggestion, preliminary results at higher concentrations of proteins show an increase in binding of one protein in the presence of the other protein. The association constants obtained for hyaluronate binding region of the proteoglycan and link protein were $1.3 \times 10^7 \text{ M}^{-1}$ and $0.7 \times 10^7 \text{ M}^{-1}$ respectively. The value for the proteoglycan fragment is of the same order as that previously presented for the proteoglycan monomer (Christner *et al.*, 1978; Cleland, 1979; Nieduszynski *et al.*, 1980).

The present investigation demonstrates that it is rational to use a mixture of hyaluronate-binding region and link proteins for the hyaluronate assay described previously (Tengblad, 1980; Laurent & Tengblad, 1980). Both proteins bind similarly to the hyaluronate-substituted gel and do not interact with each other under the conditions used in the assay. Separation of the two proteins and use of only one of them is therefore an unnecessary step.

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