A study of equilibrium binding of link protein to hyaluronate

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(Received 9 March 1983/Accepted 22 April 1983)

Link protein was extracted from bovine femoral-head cartilage, radiolabelled while in the proteoglycan-aggregate stage, and then purified by density-gradient centrifugation and gel chromatography. The purity of the preparation was assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and two species with approx. mol.wts. 45000 and 48000 were observed. Sedimentation-velocity experiments were performed in 0.5 M-guanidinium chloride/5 mM-phosphate, pH 7.4, and yielded an s20, w of 4.75 S. The proportion of link protein unable to interact with hyaluronate was determined by chromatography on Sepharose CL-4B. The binding of link protein to high-molecular-weight hyaluronate was studied by frontal-gel chromatography on Sepharose CL-4B in 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4. Experiments were performed at 10, 17 and 25°C and the results were treated as described by Scatchard [(1949) Ann. N.Y. Acad. Sci. 51, 660–672]. Dissociation constants of approx. (1–4) × 10–8 M were obtained. The length of hyaluronate occupied per link-protein molecule was determined to be six to seven disaccharides.

The proteoglycans of cartilage occur in vivo as multimolecular aggregates, which may also form in vitro when proteoglycans are supplemented with a ‘glycoprotein link’ fraction isolated from a cartilage extract by density-gradient centrifugation (Hascall & Sajdera, 1969). Two major components have been identified in this fraction, namely hyaluronate (Hardingham & Muir, 1972) and link protein (Keiser et al., 1972; Gregory, 1973). Analysis of the link protein revealed that, in bovine nasal (Baker & Caterson, 1977, 1978; Bonnet et al., 1978; Tang et al., 1979), epiphyseal (Amadio et al., 1980) and articular cartilages (Treadwell et al., 1980), two major species, with apparent mol.wts. of 44000–46000 and 48000–50000, occur. However in the Swarm-rat chondrosarcoma (Oegema et al., 1975; Caterson & Baker, 1979) and rat growth cartilage (Pita et al., 1979) only a single species, comparable with the lower-molecular-weight form, is present. Amino acid analysis (Bonnet et al., 1978), peptide mapping (Baker & Caterson, 1979), CNBr fragmentation (Pépin et al., 1980) and trypsin digestion (Heinegård & Hascall, 1974) have indicated the similarity between the two species. It is suggested that they may possess identical protein structures with the difference residing in the presence of a carbohydrate-enriched region in the larger species (Baker & Caterson, 1979; Roughley et al., 1982).

Although proteoglycans will aggregate with hyaluronate in the absence of link protein, Hardingham (1979) has shown that the presence of link protein results in increased stabilization of the aggregate, and it is suggested that the function of link protein is to lock the proteoglycans, almost irreversibly, into the matrix. Link protein may also protect the proteoglycan core protein from proteolytic attack and thereby extend its functional life. Link protein also interacts with hyaluronate (Hascall & Heinegård, 1974) and proteoglycan (Caterson & Baker, 1980) individually, thus indicating the probability of two separate binding sites. In proteoglycan aggregates formed in vitro, the ratio of proteoglycan to link protein has been estimated by Heinegård & Hascall (1974) as approx. 1:1.

Of the three individual interactions occurring in the proteoglycan–hyaluronate–link-protein ternary complex, only that between hyaluronate and proteoglycan has been extensively investigated, and values for the dissociation constant (Kd) of 2.5 × 10–8 M (Nieduszynski et al., 1980; Nieduszynski & Sheehan, 1981), 2 × 10–8 M (Cleland, 1979) and 5.6 × 10–8 M (Christner et al., 1978) have been obtained. The interaction between link protein and hyaluronate has been studied by Tengblad (1981) by the use of affinity chromatography. The dissociation

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Experimental

Materials

Guanidinium chloride (practical grade) was obtained from Sigma (Poole, Dorset, U.K.) and further purified by treatment of 8 M stock solutions with activated charcoal. Benzamidine hydrochloride, dimethyl dichlorosilane, poly(ethylene glycol) (20,000 mol wt.), high-purity bovine serum albumin and umbilical-cord hyaluronate (grade III) were also obtained from Sigma. CsCl (Analar), 6-aminohexanoic acid and carbazole were from BDH (Poole, Dorset, U.K.). Sepharose CL-4B and Sephacrylis S-300 and S-1000 were from Pharmacia (Uppsala, Sweden). [3H]Acetic anhydride (500 mCi/mmole) was from Amersham International (Amersham, Bucks., U.K.).

Preparation of link protein

Cartilage from heifer femoral heads (obtained from a local abattoir) was shaved off and extracted for 48 h at 6°C in 10 vol. of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, containing the proteinase inhibitors 0.1 M-6-aminohexanoic acid, 0.01 M-EDTA and 0.005 M-benzamidine hydrochloride (Oegema et al., 1975). The extract was filtered through nylon mesh and dialysed against 7 vol. of 0.05 M-sodium acetate, pH 5.8 (containing proteinase inhibitors). After adjustment to a density of 1.60 g/ml with solid CsCl the extract was subjected to 'associative' density-gradient centrifugation at 87000 gav, for 70 h at 15°C (MSE Prepspin 50 centrifuge, AI angle head rotor). The bottom one-thirds of the tubes, containing the proteoglycan aggregates, were pooled (Al fraction). A sample of the Al fraction was dialysed against 1 M-NaCl/5 mM-phosphate/0.01 M-EDTA, pH 7.4, and then radiolabelled by the addition of 25 mCi of [3H]acetic anhydride (Riordan & Vallee, 1972). After being stirred for 1 h the solution was extensively dialysed against 1 M-NaCl/5 mM-phosphate/0.01 M-EDTA, pH 7.4, followed by 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4. The density was adjusted to 1.50 g/ml with solid CsCl and the solution was subjected to 'dissociative' density-gradient centrifugation at 87000 gav, for 72 h at 15°C. The top one-fifths (AI-[3H]-D5 fraction) were pooled and concentrated to 2 ml by dialysis against concentrated poly(ethylene glycol) in 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4. This was then chromatographed on a column (0.8 cm x 147 cm) of Sephacryl S-300 and eluted with 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4. The link-protein-containing fractions were pooled and stored at 4°C in a silicone-treated glass vial. The concentration was determined by using an absorption coefficient (A450nm) at 280 nm in 4 M-guanidinium chloride of 1.39 (Tang et al., 1979). The level of [3H] incorporation was 25.4 x 10⁶ c.p.m./mg, which corresponds to an incorporation of approx. 1 acetyl group per molecule.

A minority of the data in the present paper was obtained with a similarly prepared [3H]-labelled link-protein preparation with a 14C specific radioactivity of 5.7 x 10⁵ c.p.m./mg (corresponding to an incorporation of approx. 0.4 acetyl group per molecule).

The proportion of the link-protein preparation able to interact with hyaluronate was assessed by a gel-chromatographic procedure. A sample containing 1.4 μM-link protein and 26 μM-hyaluronate (expressed as disaccharide) in 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4 (containing proteinase inhibitors) was equilibrated at 6°C for 24 h. Under these conditions virtually all of the link protein capable of interacting with hyaluronate would do so, assuming a Kd ≤ 10⁻⁷ M. A portion was then chromatographed at 6°C on a column (0.6 cm x 13.5 cm) of Sephacryl CL-4B and eluted with 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4.

Preparation of hyaluronate

Hyaluronate (sodium salt) was further purified by fractionation with cetylpyridinium chloride (Scott, 1960). Purified hyaluronate (2 mg/ml) dissolved in 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4, was chromatographed on a column (2.6 cm x 109 cm) of Sephacryl S-1000 and eluted with the above solvent at a flow rate of 20 ml/h. The column was calibrated with large proteoglycan aggregates and glucuronolactone. A broad profile was obtained with a peak Kav, of 0.63. Fractions containing the higher-molecular-weight hyaluronate, with a Kav < 0.68, were pooled, dialysed and freeze-dried. A stock solution was prepared in 0.5 M-guanidinium chloride/5 mM-phosphate, pH 7.4, and the concentration was determined by the carbazole assay (Bitter & Muir, 1962) with glucuronolactone as the standard.

Frontal-gel chromatography

Samples (22 ml) containing hyaluronate (244 nm or 478 nm, expressed as disaccharide) and several concentrations of radiolabelled link protein (in the range 26-260 nm) were prepared in 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4 (containing proteinase
inhibitors). The link protein was added directly from a 4 m-guanidinium chloride stock solution. The samples were equilibrated for 24h and then pumped on to a column (1 cm x 20 cm) of Sepharose CL-4B and eluted with the same solvent at a flow rate of 2.7 ml/h. Fractions (0.43 ml) were collected and counted for radiolabel. The column, sample container and solvent reservoir were maintained at a constant temperature and experiments were performed at 10, 17 or 25°C.

Other analytical methods
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of link protein was performed on 10% (w/v) gels by the method of Weber & Osborn (1969). Sedimentation-velocity experiments on link protein (0.25, 0.35, 0.5 and 0.6 mg/ml) in 0.5 M-guanidinium chloride/5 mM-phosphate, pH 7.4, were performed on an MSE Centriscan 75 analytical ultracentrifuge at 230 000 g, and 20°C. A value of 0.72 ml/g (Tengblad, 1981) was used as the partial specific volume (ρ) of link protein. Radiolabelled link protein was counted for radioactivity in a Packard Tri-Carb 300 liquid-scintillation counter by the method of Nieduszynski et al. (1980). All glass columns and vessels used with link protein were treated with 1% (v/v) dimethylchlorosilane in diethyl ether (Schwartz & Zabin, 1966) to reduce losses by adsorption.

Results and discussion
Purified link protein was prepared from bovine femoral cartilage by 'associative' and 'dissociative' density-gradient centrifugations, followed by Sephacryl S-300 chromatography (Fig. 1). The preparation was radiolabelled while in the proteoglycan-aggregate stage with relatively little chemical modification of the link protein. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the link protein revealed two species with apparent mol.wts. 45 000 and 48 000 (approx.). Since both species are capable of interacting with hyaluronate (Hascall & Heinegård, 1974) an average molecular weight of approx. 46 000 was used for determination of molar concentrations.

Preliminary experiments were undertaken to determine the degree of self-association of the link protein in 0.5 M-guanidinium chloride/5 mM-phosphate, pH 7.4 (the solvent chosen for the binding studies). Sedimentation-velocity experiments yielded an s20, w of 4.75 S for link protein in this solvent, which may indicate a link-protein dimer, as a value of approx. 35 may be expected for a monomer from the empirical equation of Halsall (1967). In contrast, Tang et al. (1979) obtained an s20, w of 7.8 S in 1 M-NaCl, pH 7.0, a value corresponding to a larger oligomer. However, the elution position of link protein on Sepharose CL-4B (e.g. Fig. 2), at concentrations lower than used in the ultracentrifugal experiments, appears to indicate a monomeric species. It is probable that self-association is not significant at the very low concentrations (approx. 10–100 nM) used for frontal-gel chromatography, and that link protein occurs as a monomer.

Previous attempts at using equilibrium dialysis and ultrafiltration for investigating link protein binding to hyaluronate were beset by high losses of link protein, which were attributed to adsorption on to the surface of the Millipore membranes, as also found by Tang et al. (1979). The losses resulted when either 0.5 M-guanidinium chloride or 1 M-NaCl were used as solvent.

An alternative technique for studying such an interaction is frontal-gel chromatography, which does allow a continuous monitoring of link-protein concentration, and can also reveal possible link-protein self-association. The gel medium chosen for this investigation was Sepharose CL-4B, as high-molecular-weight hyaluronate and hyaluronate–link-protein complex are eluted in the void volume, and link protein is eluted near the total volume, thereby maximizing the resolution between the complex and the unbound link protein and simplifying the interpretation (Nichol & Winzor, 1964). A high-molecular-weight hyaluronate was prepared by fractionation on Sephacryl S-1000 in 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4. The solvent used for the binding studies was 0.5 M-

Fig. 1. Gel chromatography of Al-[3H]-D5 fraction (see the text) on Sephacryl S-300
A column (0.8 cm x 147 cm) of Sephacryl S-300 was eluted with 4 M-guanidinium chloride/5 mM-phosphate, pH 7.4, at 2.6 ml/h. Fractions (1.3 ml) were collected and counted for 3H radioactivity. V0 and Vt were determined with Dextran Blue and glucuronolactone respectively. Link-protein-containing fractions were pooled as shown (I—I).
Hyaluronate (26 μM expressed as disaccharide) and [3H]link protein (1.4 μM) were chromatographed on a column (0.6 cm x 13.5 cm) of Sepharose CL-4B and eluted with 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4, at 6°C. The flow rate was 0.6 ml/h and fractions (0.11 ml) were counted for 3H radioactivity. V₀ and Vₑ were determined with Dextran Blue and glucuronolactone respectively. Bound and non-bound (i.e. inactive) link protein were estimated as 42% and 58% of the radiolabel respectively.

In order to quantify the interaction between hyaluronate and link protein it is necessary to know the molar concentrations of the interacting species, but in the case of link protein it cannot be assumed that all of the molecules are capable of binding to hyaluronate. The active proportion of the link-protein preparation was determined (as radiolabel) by chromatography of link protein with excess hyaluronate, on Sepharose CL-4B, under conditions optimized for link-protein complexation. Total recoveries of link protein from the column were >95%. That proportion of the preparation which eluted in the position of 'free' link protein was assumed to be inactive, with respect to binding to hyaluronate. The proportion of 'active' [3H]link protein was initially found to be approx. 42% (as a percentage of the total 3H radioactivity; see Fig. 2). This was monitored throughout the duration of the binding studies, and approx. 20% of the hyaluronate-binding activity was lost during 3 months storage. The active proportion is relatively low, although, interestingly, a higher proportion is active towards proteoglycan (results not shown). It is not clear whether this degree of inactivity is due to the radiolabelling procedure, a denaturation process or an inherent characteristic of the link protein population from articular cartilage. In the latter context the study by Poole et al. (1982) of the distribution of link protein in articular cartilage is noteworthy. This study revealed significant variation in the co-distribution of link protein with other macromolecules between different regions of the articular cartilage. This may reflect differing abilities of link-protein molecules within the population to interact with these macromolecules.

Frontal-gel-chromatography experiments were performed on samples containing a fixed hyaluronate concentration and several concentrations of radiolabelled link protein, at temperatures of 10, 17 or 25°C. Samples were pre-equilibrated for 24 h at

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**Fig. 2. Chromatography of hyaluronate/link protein on Sepharose CL-4B**

Hyaluronate (26 μM expressed as disaccharide) and [3H]link protein (1.4 μM) were chromatographed on a column (0.6 cm x 13.5 cm) of Sepharose CL-4B and eluted with 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4, at 6°C. The flow rate was 0.6 ml/h and fractions (0.11 ml) were counted for 3H radioactivity. V₀ and Vₑ were determined with Dextran Blue and glucuronolactone respectively. Bound and non-bound (i.e. inactive) link protein were estimated as 42% and 58% of the radiolabel respectively.

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**Fig. 3. Frontal-gel chromatography of (a) [3H]link protein and (b) hyaluronate [3H]-link protein on Sepharose CL-4B**

Samples (22 ml) were chromatographed on a column (1 cm x 20 cm) of Sepharose CL-4B and eluted with 0.5 M-guanidinium chloride/5 mM-phosphate/0.1% bovine serum albumin, pH 7.4. The flow rate was 2.7 ml/h and fractions (0.43 ml) were counted for 3H radioactivity. LPₗ represents bound link protein and LPₘₐₜ represents apparently free link protein (including both free and inactive components).
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the required temperature, as Tengblad (1981) found that approx. 16h was necessary at room temperature. Preliminary frontal chromatography of link protein alone gave an enantiographic profile (Fig. 3a), indicating no apparent self-association within the resolution of the gel. An example of a hyaluronate/link-protein frontal-chromatography profile, with link protein as the monitored species, is shown in Fig. 3(b). The ascending limb of the elution profile represents a reaction boundary and is followed by a plateau corresponding to the total link protein concentration. This plateau ends in the descending limb of the hyaluronate–link-protein complex, which leads into a second plateau corresponding to the apparently free link protein (\(F_{\text{app}}\)). The latter yields a value for the free link protein concentration (\(F\)), after correction for the independently determined concentration of 'inactive' link protein. The difference between the two plateaux represents the bound-link-protein concentration (\(B\)). The total recoveries of link protein from such experiments were more than 95%.

In terms of the measured parameters \(B\) and \(F\), the dissociation constant (\(K_d\)) can be represented as:

\[
K_d = \frac{F}{B} (n - B)
\]

where \(n\) is the concentration of link-protein binding sites on the hyaluronate [see Nieduszynski et al. (1980) for a comparable treatment of hyaluronate–proteoglycan binding]. The results were thus plotted (Fig. 4) as \(B/F\) against \(B/[\text{HA}_2]\) in the manner described by Scatchard (1949) (where \([\text{HA}_2]\) is the concentration of hyaluronate expressed as disaccharide units). \(K_d\) was determined from the slope of the plot, and the reciprocal of the intercept on the \(B/[\text{HA}_2]\) axis is the number of hyaluronate disaccharides occupied per link-protein molecule at saturation (see Table 1).

A shortcoming of this analysis is an uncertainty in the true 'active'-link-protein concentration, arising from a likely difference in the level of incorporation of radiolabel between the 'active' and 'inactive' link-protein pools (the latter probably being higher). Although this should not affect the ratio \(B/F\), it will affect the absolute concentrations, since the latter are derived from measured radiolabel concentrations converted by using a specific radioactivity for the whole link-protein preparation. This would lead to an underestimation of \(B\) and therefore also \(K_d\), whereas the apparent binding-site size would be over-estimated. However, as the minimum link-protein binding site corresponds to approximately a decasaccharide (Tengblad, 1981), any such over-estimation must be relatively small.

The dissociation constant for link-protein binding to hyaluronate at 20°C is estimated to be approx.

![Fig. 4. Scatchard (1949)-type plots of link-protein binding to hyaluronate](image)

Experiments using fixed concentrations of hyaluronate and several concentrations of radiolabelled link protein (see the Experimental section) were performed at (a) 10°C, (b) 17°C and (c) 25°C. \(B\) is the concentration of bound link protein, \(B/F\) is the ratio of bound to free link protein and \([\text{HA}_2]\) is the concentration of hyaluronate expressed as disaccharide.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Dissociation constant, (K_d) (M)</th>
<th>Length of hyaluronate occupied per link-protein molecule at saturation (no. of monosaccharides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.2 × 10^{-8}</td>
<td>13.1</td>
</tr>
<tr>
<td>17</td>
<td>2.8 × 10^{-8}</td>
<td>12.1</td>
</tr>
<tr>
<td>25</td>
<td>3.5 × 10^{-8}</td>
<td>14.6</td>
</tr>
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All experiments were performed in 0.5 M-guanidinium chloride/5 mm-phosphate/0.1% bovine serum albumin, pH 7.4.
3×10⁻⁸M, which is significantly lower than the value of approx. 1.4×10⁻⁷M obtained by Tengblad (1981) at room temperature from affinity chromatography. Extrapolation of the data presented here would suggest an increase in $K_d$ (i.e. a decrease in affinity) from approx. 10⁻⁸ to 10⁻⁷M between 4°C and 37°C. It is noteworthy that the magnitude of the $K_d$ at physiological temperature and pH is essentially the same as that for the binding of proteoglycans to hyaluronate (Nieduszynski et al., 1980; Cleland, 1981). The value of six to seven disaccharides for the length of hyaluronate occupied per link-protein molecule is consistent both with the upper limit of approximately ten disaccharides, as estimated by Faltz et al. (1979) from enzyme digests of bound hyaluronate, and the lower limit of approx. five disaccharides corresponding to the smallest inhibitory oligosaccharide (Tengblad, 1981). Also, the apparently comparable binding site for the hyaluronate-binding region of proteoglycan (mol.wt. approx. 90000) is approx. eight disaccharides (Heinegård & Hascall, 1974). The binding data presented here supplements that already obtained for the proteoglycan–hyaluronate interaction; however, as proteoglycan aggregation occurs via a ternary complex, a complete understanding of the strength of aggregate formation still requires an analysis of the linker–proteoglycan interaction. This is also of particular relevance as a proteoglycan–link-protein complex may be the first to form in vivo (Kimura et al., 1979, 1980) and subsequently interacts with hyaluronate (Kimura et al., 1982).

We thank Ms. Joy Greenwood and Mr. Haydn Morris for invaluable technical assistance, Dr. John K. Sheehan for helpful discussions, and the Medical Research Council and the Arthritis and Rheumatism Council for support.

References


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