The Link Protein in Proteoglycan Aggregates from the Swarm Rat Chondrosarcoma

(Received for publication, February 23, 1977)

Theodore R. Oegema, Jr.†

From the Departments of Orthopedic Surgery and Biochemistry, The University of Minnesota, Minneapolis, Minnesota 55455

Martin Brown and Dominic D. Dziewiatkowski

From the Departments of Oral Biology and Biological Chemistry and the Dental Research Institute, The University of Michigan, Ann Arbor, Michigan 48109

Aggregated proteoglycans (70% aggregated), isolated from the Swarm rat chondrosarcoma by extraction with 4 M guanidinium chloride in the presence of protease inhibitors and purified by centrifugation in an associative cesium chloride gradient, were separated into the component parts by centrifugation in a dissociative cesium chloride gradient. The gradient was cut into five equal fractions. The bottom fraction contained 98% of the chondroitin sulfate and 84% of the protein of the aggregate preparation. Sedimentation equilibrium studies on the protein core of this fraction, isolated by column chromatography from chondroitinase ABC digests, suggest that its molecular weight is $2.0 \times 10^4$ to $2.2 \times 10^4$. The intermediate fractions of lower buoyant densities contained hyaluronic acid (0.8% of the total weight of the aggregated preparations) and proteoglycan monomers with fewer chondroitin sulfate chains relative to the protein core than in the bottom fraction. The top fraction, in addition to proteoglycans, contained a link protein. The link protein was separated from the proteoglycans by chromatography on Sephadex G-200 in the presence of 4 M guanidinium chloride. Its molecular weight was estimated to be $40,000$. It stabilized complexes of proteoglycan monomers and hyaluronic acid so that they could be seen in the analytical ultracentrifuge at pH 5.8.

The amino acid composition of the link protein differs significantly from that of the protein core of the proteoglycan monomers and from that of the hyaluronic acid binding region of the protein core of the proteoglycans.

In hyaline cartilages, proteoglycans (glycosaminoglycans covalently linked to protein) are the structural components primarily responsible for the characteristic elasticity and reversible resistance to compressive forces (1). These molecules exist as aggregate structures in the extracellular matrix (2).

The aggregates are effectively dissociated and their noncovalent interactions are minimized in solvents with high concentrations of electrolytes; they are readily extracted from hyaline cartilages by such solvents (3, 4). When the salt concentration in the extracts is lowered to about 0.5 M by dilution or dialysis, the proteoglycans reaggregate. Subsequently, isopycnic eucine chloride gradient procedures can be used to separate the proteoglycan aggregates from other materials in the extracts. In turn, the components of the proteoglycan aggregates can be separated from each other by centrifugation in a dissociative cesium chloride gradient. The chemical and physical properties of proteoglycan aggregates and proteoglycan monomers from a variety of cartilages, including the Swarm rat chondrosarcoma, have been described recently (5-16).

Hascall and Sajdera (6) showed that aggregated proteoglycans could be dissociated and separated into at least two fractions, a proteoglycan monomer fraction and a link protein(s) fraction, by gradient density centrifugation in the presence of 4 M guanidinium chloride. Hardingham and Muir (17) found that proteoglycan monomers formed complexes with hyaluronic acid which could be detected by viscometry and gel filtration. However, these complexes were not demonstrable in the analytical ultracentrifuge under conditions used for the demonstration of aggregates. The seeming discrepancy was resolved by Gregory (18), who showed that both hyaluronic acid and the protein-rich top fraction of a dissociative gradient were required for a demonstration of proteoglycan aggregates with an analytical ultracentrifuge at pH 5.8. The protein-rich top fraction contains link protein(s) and proteoglycans of low buoyant density.

On the basis of work in several laboratories, a model for the structure of proteoglycan aggregates in hyaline cartilages has been proposed (19, 20). The proteoglycan monomer is polydisperse, average molecular weight of $2.5 \times 10^4$ and a range of 1.0 to $4.0 \times 10^4$ (8, 9). It contains about 100 chondroitin sulfate chains, each with an average molecular weight of $2.0 \times 10^4$, and 35 to 50 keratan sulfate chains, each with an average molecular weight of about $5 \times 10^4$ (11-13). The polysaccharide chains are covalently linked to a protein core, which has a weight average molecular weight of $1.8 \times 10^5$ to $2.0 \times 10^5$ (21). One end of the protein core interacts with hyaluronic acid (22). This
end is relatively free of polysaccharide chains. The remainder of the protein core provides the attachment points through the hydroxyls of serine and threonine, for the chondroitin sulfate and keratan sulfate chains (22-29). These polysaccharide chains are not uniformly distributed over the protein core. On the protein core of the proteoglycans of bovine nasal septum and trachea, about 60 to 70% of the keratan sulfate chains and less than 10% of the chondroitin sulfate chains are attached to a segment of the protein core immediately adjacent to the segment of the protein core involved in binding to hyaluronic acid (19).

Such proteoglycan monomers can complex with hyaluronic acid (17). The complexes are stabilized by link proteins, which can interact noncovalently with both the protein core of the proteoglycan monomers and with hyaluronic acid (24). Hoffman et al. (20, 27) have suggested an alternative model for proteoglycan aggregates, in which a specific link protein is not required. They suggest that formation of proteoglycan aggregates is an expression of the polydispersity of the proteoglycans. In their model, proteoglycans of low buoyant density (high protein content) help stabilize aggregate structures of high buoyant density proteoglycans and hyaluronic acid.

In general, the proteoglycan aggregate preparations from the Swarm rat chondrosarcoma have properties very similar to those reported for proteoglycan aggregates from bovine nasal septum and trachea (7) with respect to the following: (a) monomer and aggregate size; (b) the presence of a large proportion of aggregate; (c) the ability of the proteoglycan monomer to interact with hyaluronic acid; and (d) the presence of a hyaluronic acid-protein complex which is resistant to digestion with chondroitinase and trypsin. They are different in those reported for proteoglycan aggregates from bovine nasal septum and keratan sulfate chains (22-25). These polysaccharide chains in the fraction were separated and quantified by the use of the micellar column procedure of Antonopoulos et al. (28). The amount of glucosamine and galactosamine in each fraction was determined. After digestion of the Al-D1a through Al-D3 fractions with papain or treatment with 0.5 M sodium hydroxide for 24 h at 4°C, the relative sizes of the chondroitin sulfate chains in the fractions were estimated by gel permeation, using analytical columns of Sepharose 6B.

In the electrophoretic analyses, samples were applied to 3 or 7% polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol and run as described by Weber and Osborn (29). The gels were stained with Coomassie blue (29) or periodic acid-Schiff reagent for carbohydrate (30). The molecular weights of the separated components were estimated by comparing their positions in the gels with those of proteins of known molecular weights (29, 31).

Column Chromatography
Analytical columns (0.9 x 165 cm) were packed with either Sepharose 2B, Sepharose 4B, or Sepharose 6B. Elution and analyses of the effluent by automated procedures were as previously described (7).

For preparative purposes, columns (0.9 x 100 cm) were packed with either Sepharose G-200 (medium) or Sephadex G-150 (medium) in 4 M GuanCl, 0.05 M sodium acetate, pH 5.8. The samples were applied in and then eluted with the above solvent. The flow rate was 1.4 to 1.5 ml/h at a hydrostatic pressure of about 10 cm. Fractions of 0.7 ml were collected.

Preparation of Dissociative Gradient Fractions of Proteoglycan Aggregates
Proteoglycan aggregates (Al) were isolated from 4 M GuanCl extracts of the Swarm rat chondrosarcoma as previously described (7). The associative gradients were cut into two fractions. The bottom two-fifths or the Al fraction (5) was diluted 1:1 with water and GuanCl was added to increase the concentration of GuanCl to 4 M. CsCl was then added to adjust the density of the solution to 1.50. Dissociative gradients were established by centrifugation in a Beckman Ti-50 rotor at 40,000 rpm for 40 to 48 h at 10°C. The gradients were cut into 4 parts, labeled A1-D1 to A1-D4, from bottom to top (5). In most of the experiments, to obtain a finer fractionation, the gradient was cut into 5 equal parts. labeled A1-D1a, A1-D1b to A1-D4.

Comparison of Size of Proteins in Al and in A1-D Fractions
Chondroitin sulfate chains were removed from the core proteins by incubation with chondroitinase ABC for 60 min at 37°C, as previously described (7). The digests were reduced with 5-mercaptoethanol and subjected to electrophoresis on 7% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate.

Preparation of Protein Core of A1-D1a
The protein core of the proteoglycan monomer (A1-D1a) was prepared by chondroitinase ABC digestion and it was purified as previously described (7). The material eluted from the Sepharose 4B column at K, = 0.60 was examined in the analytical centrifuge. At this end, this fraction was dialyzed three times, for 12 h each time, against 100 volumes of 0.5 M sodium acetate, pH 7.0, at 4°C. The final dialysate was used for the preparation of reagent blanks and for determinations of the retentate for analysis in the analytical ultracentrifuge.

A1-D1a indicates the bottom two-fifths of an associative gradient, with A2, A3, and A4 each being one-fifth portions of gradient going from bottom to top. Sequential treatment of a sample is indicated by a hyphen and the next steps. The term A1-D1 indicates the bottom fraction from a dissociative gradient when the A1 fraction is used.

1 The systematic nomenclature of Heinigard is used throughout this report (5). Sequential steps are indicated by letters; A indicates associative (low salt) and D, dissociative (4 M GuanCl) gradient steps, with a number to indicate the gradient fraction. A1 indicates the bottom two-fifths of an associative gradient, with A2, A3, and A4 each being one-fifth portions of gradient going from bottom to top. Sequential treatment of a sample is indicated by a hyphen and the next steps. A1-D1 indicates the bottom fraction from a dissociative gradient when the A1 fraction is used.

3 Beckman Instruments has recently cautioned against the use of high concentrations of salts that may form crystals in lower parts of gradients. For alternate procedure see Ref. 13 or contact Beckman Instruments, Palo Alto, Ca.
Link Protein in Proteoglycan Aggregates

Table I
Composition of fractions of dissociative gradient when AI preparation of proteoglycans extracted from Swarm rat chondrosarcoma was used

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Density</th>
<th>Total protein mg</th>
<th>Total GalN μmol</th>
<th>Total uronic acid μmol</th>
<th>GalN (μmol)/protein (μg)</th>
<th>GluN/GalN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-D1a</td>
<td>1.609</td>
<td>13.80</td>
<td>198</td>
<td>229</td>
<td>14.30</td>
<td>0.144</td>
</tr>
<tr>
<td>A1-D1b</td>
<td>1.541</td>
<td>0.53</td>
<td>2.42</td>
<td>3.24</td>
<td>4.55</td>
<td>0.149</td>
</tr>
<tr>
<td>A1-D2</td>
<td>1.485</td>
<td>0.40</td>
<td>1.10</td>
<td>2.24</td>
<td>2.75</td>
<td>0.178</td>
</tr>
<tr>
<td>A1-D3</td>
<td>1.409</td>
<td>0.27</td>
<td>0.57</td>
<td>1.32</td>
<td>2.42</td>
<td>0.197</td>
</tr>
<tr>
<td>A1-D4</td>
<td>1.395</td>
<td>1.52</td>
<td>0.40</td>
<td>0.65</td>
<td>0.26</td>
<td>0.100</td>
</tr>
<tr>
<td>A1-D4-G300</td>
<td>0.86</td>
<td>0.113</td>
<td>0.57</td>
<td>0.65</td>
<td>0.26</td>
<td>0.100</td>
</tr>
<tr>
<td>A1-D4-G2000</td>
<td>0.89</td>
<td>0.013</td>
<td>0.57</td>
<td>0.65</td>
<td>0.26</td>
<td>0.100</td>
</tr>
</tbody>
</table>

* A portion of the material in each fraction (A1-D1a through A1-D4) was digested with papain and the glycosaminoglycans were separated according to the method of Antonopoulos et al. (28). The ratios of GluN/GalN were calculated, using the values for glucosamine in the material eluted with 0.75 M MgCl₂ in 0.05% cetylpyridinium chloride.

* These are ratios of GluN to GalN found in hydrolysates of the materials without prior separation by the procedure of Antonopoulos et al. (28).

Preparation of Hyaluronic Acid Binding Region of Protein Core of Proteoglycan Monomer

An AI preparation (200 mg, 70% aggregated) was treated with chondroitinase ABC to achieve 80% digestion of the chondroitin sulfate chains and then with trypsin (22). The hyaluronic acid binding region of the protein core was isolated in association with the glycosaminoglycan, as previously described (17). The effluent fractions in which this material (AI-CB-T-2B) was present were pooled. Following extensive dialysis against water, 12 mg of material was recovered by lyophilization. The AI-CB-T-2B preparation was dissolved in 1 ml of 4 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, and placed on a column of Sephadex G-150. Elution was with 4 M GdmCl, 0.05 M sodium acetate, pH 5.8. The effluent fractions were checked for absorbance at 280 nm and for hexuronic acid, protein, and neutral sugar contents of the effluent fractions were determined.

Preparation of High Molecular Weight Hyaluronic Acid

The preparation of the hyaluronic acid from umbilical cords (2 mg in 1 ml of 0.2 M sodium acetate buffer, pH 7.0) was fractionated by the use of a Sepharose 2B column. Elution was with 0.5 M sodium acetate buffer, pH 7.0. The fraction with Kᵥ of 0.30 was designated as the high molecular weight fraction (HAᵥ). The other hyaluronic acid sample (HAᵥ), estimated average molecular weight of 120,000, was more polydisperse than the preparation from umbilical cords. On the same column its Kᵥ was 0.61.

Interaction of AI-Dia with Hyaluronic Acid and Link Protein

Solutions of the materials were prepared as follows: A1-D1a was dissolved in 4 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, at a concentration of 3.58 mg/ml; the hyaluronic acid (HAᵥ) was dissolved in 0.5 M sodium acetate, pH 7.0, at a concentration of 360 μg/ml; and the purified link protein (AI-D1-D4-G2000) in 4 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, at 227 μg/ml. The solutions were mixed in varying proportions and diluted to 1.1 ml with 4 M GdmCl, 0.05 M sodium acetate buffer. The mixtures were dialyzed at 4 °C against three changes of 0.50 M GdmCl, 0.05 M sodium acetate buffer, pH 5.8, each for 12 h. The final dialysate was used as the reference blank in the ultracentrifugal studies. Aliquots of the retentates were also chromatographed on Sepharose 2B columns.

---

* An equivalent fraction could be obtained if the chondroitinase treatment was omitted and the trypsin digest centrifuged in a cesium chloride gradient ρ = 1.62: 40,000 rpm, Ti-50 rotor, 12°, 40 h, and the top third of gradient recovered; also see Ref. 30.

Sedimentation Velocity - To determine the effect of the purified link protein on the stabilization of complexes of proteoglycan monomer (AI-D1a) and hyaluronic acid, samples were centrifuged at 40,000 rpm in a 12-mm double-sector cell with sapphire windows. The temperature was 20°. Schlieren patterns were photographed with a Nikon comparator.

Sedimentation to Equilibrium - Sedimentation equilibrium experiments, using the meniscus depletion method of Yphantis (32), were performed on the protein core of the proteoglycan (AI-D1a) of the rat chondrosarcoma, as described by Hasall and Riolo for chondroitinase-treated proteoglycan of bovine nasal septa (31). Protein concentrations were 164, 112, and 88 μg/ml. An Aj rotor was used at speeds of 9,000, 10,000, and 11,000 rpm and at a temperature of 20°. A partial specific volume of 0.67 g/ml was calculated from a dry weight protein content of 51.2%, assuming a partial specific volume of 0.72 g/ml for protein and 0.52 g/ml for the carbohydrate portion. The data were collected and calculated as described by Hasall and Riolo (21). Apparent weight average molecular weights were obtained by extrapolation to zero protein concentration.

**RESULTS**

Characteristics of AI-D Fractions - A typical distribution of protein, hexuronic acid, and galactosamine in a dissociative cesium chloride gradient of AI preparations from the Swarm rat chondrosarcoma is shown in Table I. About 84% of the protein and about 98% of the galactosamine and hexuronic acid are present in the bottom fifth of the gradient. In this fraction (AI-D1a), the ratio of galactosamine to protein is higher than in any of the other fractions; the ratio of galactosamine to protein decreases as the buoyant density of the fractions decreases. On the other hand, the ratio of glucosamine to galactosamine in the proteoglycans increases as the buoyant density of these decreases. Realizing that hyaluronic acid was present in fractions of intermediate buoyancy, the glucosamine of the hyaluronic acid was eliminated from the estimate of the ratio of glucosamine to galactosamine by the use of the procedure of Antonopoulos et al. (28) for the estimation of hyaluronic acid in the fractions.

As the buoyant densities of the proteoglycans in a dissociative gradient decrease, their molecular weights apparently decrease; they are retarded to a progressively greater extent on a column of Sephrase 2B (Table II). This, however, is not a reflection of shorter chondroitin sulfate chains. It appears, Table II, that the average length of the chondroitin sulfate chains prepared by either digestion with papain or treatment with 0.5 M sodium hydroxide, is the same in the proteoglycans at all levels of the dissociative gradient. These data in conjunction with the data in Table I, which clearly indicate a decrease...
ing amount of chondroitin sulfate per unit weight of protein as the buoyant density of the fractions decreases, clearly indicate that there is a decrease in the number of chondroitin sulfate chains attached to the protein core.

The protein core of the proteoglycans decreases in size as the buoyant density of the proteoglycan decreases. The proteoglycan fractions were treated with chondroitinase ABC to remove the chondroitin sulfate chains (95% digestion). Such a treatment produced protein cores which still carried the linkage region of neutral sugars and an average of 1.5 repeat disaccharides of the chondroitin sulfate chains. When these samples, after reduction with \( \beta \)-mercaptoethanol, were electrophoresed in 3\% polyacrylamide gels in the presence of sodium dodecyl sulfate, it was found (Fig. 1) that the mobility of the protein cores increased as the buoyant density of the proteoglycan fractions decreased. This decrease in the size of the protein cores occurred in discrete steps, as seen in the electrophoretograms, the protein cores in any given fraction contained one or more clearly separated protein entities. In A-D1a only a single protein component was seen. Two components were readily discerned in A-D1b; a slower migrating component was in higher concentration than a faster migrating component (Fig. 1B). The reverse was seen in the case of A-D2 (Fig. 1C). A third protein component was additionally present in the A-D3 fraction; its mobility (Fig. 1D) was even greater than that of the two protein components seen in A-D1b and A-D2. The protein core of the proteoglycans in the A-D4 is primarily of the size of the fastest migrating protein component in the A-D3 fraction. The A-D4 fraction, however, also contains two other protein components, whose mobility is less than that of the major protein component, but decidedly faster than that of the two protein components seen in A-D1b and A-D2. Each of the fractions was recentrifuged in a dissociative isopycnic cesium chloride gradient at the same starting density. The buoyant density of the fractions was not altered. Nor were the protein/uronic acid ratios changed thereby. Moreover, the electrophoretograms of the recentrifuged fractions were indistinguishable from the electrophoretograms of the fractions before recentrifugation.

For comparison, the protein components in A1 preparations are shown in Fig. 1F. It is apparent from such electrophoretograms that in A1 preparations there is a mixture of proteoglycans, since their protein cores vary in size. It is further apparent that in such preparations a protein core of lowest mobility is present in highest concentration.

To obtain an estimate of the molecular weight of the protein core in the A-D1a fraction (Fig. 1A), the A-D1a fraction was treated with chondroitinase ABC, as above. The resultant protein core was examined in the analytical ultracentrifuge, using the techniques of sedimentation to equilibrium. On extrapolation of the apparent molecular weights to zero protein concentration, it was found that the molecular weight of the protein core was in the range of \( 2.0 \times 10^6 \) to \( 2.2 \times 10^6 \).

The amino acid profiles of the proteoglycan fractions, expressed in residues per 1000 amino acid residues are given in Table III. Certain progressions are apparent. As the buoyant density of the fractions decreases, the protein core contains progressively more lysine and arginine and less threonine and serine. There is also an increase in the ratio of glucosamine of oligosaccharides (1\% cetylpyridinium chloride fraction) to galactosamine of chondroitin sulfate as the buoyant density of the proteoglycan fractions decreases (Table I). Since the proteoglycan of the Swarm rat chondrosarcoma lacks keratan...
Link Protein in Proteoglycan Aggregates

Table III

Amino acid compositions of materials separated by centrifugation of Al in dissociative gradient compared with that of link protein and of hyaluronic acid (HA) binding region of proteoglycan monomers from Swarm rat chondrosarcoma

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Al-Dla</th>
<th>Al-Dlb</th>
<th>Al-D2</th>
<th>Al-D3</th>
<th>Al-D4-G200,</th>
<th>HA binding region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>18</td>
<td>27</td>
<td>37</td>
<td>48</td>
<td>52</td>
<td>49</td>
</tr>
<tr>
<td>Histidine</td>
<td>24</td>
<td>17</td>
<td>17</td>
<td>22</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Arginine</td>
<td>29</td>
<td>38</td>
<td>38</td>
<td>48</td>
<td>48</td>
<td>58</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>73</td>
<td>82</td>
<td>84</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Threonine</td>
<td>91</td>
<td>84</td>
<td>76</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Serine</td>
<td>135</td>
<td>123</td>
<td>116</td>
<td>99</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>133</td>
<td>142</td>
<td>139</td>
<td>128</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>Proline</td>
<td>83</td>
<td>79</td>
<td>77</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Glycine</td>
<td>138</td>
<td>126</td>
<td>127</td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Alanine</td>
<td>64</td>
<td>71</td>
<td>73</td>
<td>77</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>1/2-Cystine</td>
<td>7</td>
<td>11</td>
<td>14</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Valine</td>
<td>54</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Methionine</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Leucine</td>
<td>74</td>
<td>65</td>
<td>69</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>16</td>
<td>25</td>
<td>18</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Phenyllalanine</td>
<td>27</td>
<td>29</td>
<td>28</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>residues/1000 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>1/2-Cystine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenyllalanine</td>
</tr>
</tbody>
</table>

- Al-D4-G200, and Al-D4-G200, (link protein) are the major components isolated from Al-D4 by gel permeation through Sephadex G-200 in the presence of 4 M GdmCl.
- HA binding region or Al-CB-T-2B,-G150,. See text for further explanation.

sulfate chains (7, 33) but does have oligosaccharides in which glucosamine and galactosamine are present (7), the data suggest that these oligosaccharides are clustered near the hyaluronic acid binding region of the protein core, much as the keratan sulfate chains are clustered on the protein core of proteoglycans in hyaline cartilages (19, 25, 34, 35).

The amino acid composition of the hyaluronic acid binding region of the proteoglycan monomer, designated in this report as Al-DB-T-2B,-G150, approaches that of the protein core of the proteoglycans in the Al-D4 fraction (Table III). This region of the protein core, in terms of residues per 1000 amino acid residues, also has more lysine and arginine and less threonine and serine than the protein core of the proteoglycans in the Al-Dla fraction. Additionally, it has more aspartic acid, alanine, tyrosine, and phenylalanine and less proline and glycine than the protein core in the Al-Dla fraction.

Examination of the papain digests of the Al-D fractions for the glycosaminoglycans in them by the method of Antonopoulos et al. (26) indicated that most of the hyaluronic acid was present in Fractions Al-D2 and Al-D3 and accounted for 0.8% of the weight of the Al preparations. It was previously reported (7) that when hyaluronic acid is at about this concentration in a mix of hyaluronic acid and proteoglycan monomer (Al-Dla), the complex formation of these two macromolecules is maximal, as indicated by the exclusion of the complexes from columns of Sepharose 2B.

Nature of Link Protein – The separation of the link protein (Al-D4-G200, from the proteoglycan monomers in the Al-D4 fraction of a dissociative gradient on a column of Sephadex G-200 with 4 M GdmCl as the eluent was as shown in Fig. 2. The fractions indicated by the bar above Peak II were pooled. When such pools of the fractions were examined by electrophoresis in polyacrylamide gels, one protein component was found (Fig. 3, A and B). Its mobility was greater than that of the hyaluronic acid-binding peptide derived from the protein core of the Al-Dla fraction (Fig. 3C). The amino acid composition of the link protein was different than that of the hyaluronic acid-binding peptide and that of the protein cores of the proteoglycans in the Al-D fractions (Table III). In terms of residues per 1000 amino acid residues, there was significantly more lysine, arginine, aspartic acid, glycine, half-cystine, tyrosine, and phenylalanine and less threonine, serine, glutamic acid, alanine, valine, and methionine in the link protein than in the hyaluronic acid-binding peptide. Significantly more aspartic acid, alanine, half-cystine, tyrosine, and phenylalanine and less threonine, serine, and methionine were found in the link protein than in the protein core of the proteoglycans in all of the Al-D fractions.

Small amounts of glucosamine and galactosamine were found in preparations of the link protein. In view of the identity of the ratio of these hexosamines in the preparations of the link protein and the ratio of these hexosamines in the proteoglycans (Al-D4-G200, in the Al-D4 fraction, it is possible that the link protein preparations (Al-D4-G200,) may still contain a small amount of proteoglycan. Caterson and Baker (36) also found these amino sugars to be present in link protein(s) isolated from bovine nasal septa. Attempts to determine whether other sugars were present in the link protein preparations have been unsuccessful, possibly because of the small amounts of material used. If there are other sugars, the sum of these is probably less than 2% of the weight of the link protein, as suggested by the use of phenolsulfuric acid assay (7) and by the use of the electrophoretic procedure of Segrest and Jackson (31). That the carbohydrate content may be very low is also suggested by the virtual absence of periodic acid-Schiff-positive staining of the link protein in acrylamide gels after electrophoresis. In line with this is the virtual absence of neutral sugars in the effluent fractions in which the link protein appeared when columns of Sephadex G-200 were used for separating it from proteoglycans in the Al-D4 fraction (Fig. 2).

Interaction of Link Protein with Hyaluronic Acid and with Proteoglycan Monomers – In the absence of hyaluronic acid or of proteoglycans, the link protein was retarded on a column of Sepharose 2B; it appeared in the effluent near the V, of the
FIG. 2. Gel filtration of the A1-D4 fraction on Sephadex G-200 in the presence of 4 M GdmCl. The materials in the peaks were pooled as indicated by the bars above the peaks. The material in Peak I and Peak II were designated A1-D4-G200, and A1-D4-G200, (link protein), respectively, on the basis of absorbance at 280 nm and content of hexuronic acid. The column was prepared and eluted as described in the text. Absorbance at 280 nm, A; protein, C; hexuronic acid, △; total sugar as glucose by phenolsulfuric acid, △. Samples were dialyzed against 0.5 M sodium acetate, pH 7.0, before protein and total sugar were determined.

FIG. 3. Disc electrophoresis of link protein (A1-D4-G200,) and of the hyaluronic acid-binding peptide of the protein core of an A1 preparation. A, 10 μg of A1-D4-G200, subjected to discontinuous electrophoresis. The arrow indicates the tracking dye. B, 10 μg of A1-D4-G200, in a 7% polyacrylamide gel in the presence of sodium dodecyl sulfate. C, densitometric tracing of a 7% polyacrylamide gel when 10 μg of the hyaluronic acid-binding peptide was electrophoresed under the same conditions used in B.

The elution profile of the A1-D1a preparation of proteoglycan monomers from a column of Sepharose 2B was as shown in column (Fig. 4). When the link protein was mixed with hyaluronic acid, the $K_{AV}$ for the hyaluronic acid was lowered, protein was present in association with the hyaluronic acid, and there was a decreased amount of the link protein in the $V_b$ of the column (Fig. 4).

The elution profile of the A1-D1a preparation of proteoglycan monomers from a column of Sepharose 2B as was shown in...
Link Protein in Proteoglycan Aggregates

Fig. 5. Elution profiles from an analytical column of Sepharose 2B of proteoglycan monomer, Al-Dia, singly and in combination with link protein, or hyaluronic acid, or both, are related to schlieren patterns seen in the analytical centrifuge. Samples were prepared and analyzed as described in the text. A, elution profile of hexuronic acid when 2 mg of Al-Dia were used; B, elution profile when 9 mg of Al-Dia and 20 pg of hyaluronic acid were used; C, elution profile when 2 mg of Al-Dia and 70 pg of link protein (Al-D4-G200,) were used; D, elution profile when 2 mg of Al-Dia, 20 pg of hyaluronic acid (HA,) and 70 pg of link protein were used; E, elution profile when 2 mg of Al-Dia, 20 pg of hyaluronic acid (HA,), and 70 pg of link protein were used. The schlieren patterns when only hexuronic acid or link protein was mixed with the Al-Dia preparation were as that for the Al-Dia preparation alone. F, at 2556 s; G, schlieren pattern at 1758 s, when a mixture of 2 mg of Al-Dia, 2 mg of hyaluronic acid (HA,), and 70 pg of link protein were used; H, schlieren pattern at 1630 s, when a mixture of 2 mg of Al-Dia, 20 pg of hyaluronic acid (HA,), and 70 pg of link protein were used.

DISCUSSION

Link protein, which stabilizes the complex of proteoglycan monomers and hyaluronic acid, is separable from the latter two components of aggregate preparations by centrifugation in a dissociative cesium chloride gradient. It is found, as is a small amount of proteoglycans of relatively low molecular weight, at the top of the gradient. The bulk of the proteoglycan monomers are at the bottom of the gradient and the hyaluronic acid is in the middle of the gradient.

In the upper portions (Al-D4 fraction) of dissociative gradients of proteoglycan aggregates from adult hyaline cartilages (38) and from a human chondrosarcoma, two proteins have been found, with molecular weights estimated by polyacrylamide gel electrophoresis as 40,000 and 45,000. Proteoglycan aggregates from cultures of chick limb-bud chondrocytes contain only a link protein of 45,000 molecular weight (13). Interestingly, proteoglycan aggregates isolated from chick limb buds at stage 25 also contain a protein of M, = 45,000, but by stage 35 a link protein of M, = 40,000 is additionally present (39). Pita et al. (31) using ultramicro methods, found only a link protein of M, = 45,000 in the intercellular fluid from epiphyseal growth cartilages of young rats. In the proteoglycan aggregates from the Swarm rat chondrosarcoma only one link protein, molecular weight of 40,000, was found (7). Moreover, it was shown that the Al-D4 fractions in which it was present could substitute for Al-D4 fractions from bovine nasal septa, which contain two link proteins, in the stabilization of proteoglycan aggregates of the bovine nasal septa. In turn, the Al-D4 fraction of the bovine nasal septa could substitute for the Al-D4 fraction of the Swarm rat chondrosarcoma in the stabilization of aggregates of the proteoglycans from the rat chondrosarcoma.

The link protein of the Swarm rat chondrosarcoma has been isolated in highly purified form and has been partially characterized. It has been shown to be capable of stabilizing complexes of well characterized proteoglycan monomers with hyaluronic acid. Recently, Caterson and Baker (36), using more complicated procedures, isolated a mixture of two link proteins from bovine nasal septa. From their brief report, it would seem that the material isolated by them is comparable in many respects to the link protein of the rat chondrosarcoma, but due to the reagents employed during isolation may be inactive.

The molecular weight of the link protein is 40,000, as previously reported (7). Its amino acid composition differs significantly from that of the hyaluronic acid binding region of the protein core of the proteoglycan monomers. Its amino acid composition is also significantly different from that of the intact protein cores of even the most buoyant proteoglycan fractions, i.e. Al-D3 and Al-D4-G200. The link protein differs in yet another way from the proteoglycan monomers: it is insoluble in water.

In vitro, the differences between complexes of proteoglycans and hyaluronic acid and proteoglycan aggregates are 2-fold. The latter is more stable and it is less sensitive to treatment with trypsin (29). The nonequivalent interactions of the link protein with both the hyaluronic acid and the protein core probably bring these three components into a spatial relationship with each other such that the link protein and a portion of the protein core of the proteoglycans are less accessible to trypsin. The hyaluronic acid of the proteoglycan aggregates is also protected. Faltz et al. (30) have reported that even after limit digestion of proteoglycan aggregates with trypsin and chondroitinase ABC the hyaluronic acid of the complex yielded fragments the molecular weights of which were 7,000 to 10,000 (18 to 25 repeat disaccharides).

Proteoglycan aggregates have been shown to have a greater thermal stability than complexes of hyaluronic acid and proteoglycan monomers (40). Additionally, both Hardingham and Muir (32) and Swann et al. (41) have recently shown that complexes of proteoglycans and hyaluronic acid can be detected by

1. T. Oegema, unpublished data.
the use of an ultracentrifuge, but only if the pH of the solution is above 7 and the concentration of the hyaluronic acid is greater than 1%. Such complexes were nearly completely dissociated at pH 5.8, the value used in the present study to demonstrate the stabilization of proteoglycan aggregates by the link protein.

The results of the present studies reaffirm the link protein as an entity different from proteoglycans of low buoyant density in which the protein to carbohydrate ratio is high. It is probable that in aggregates of hyaluronic acid, proteoglycan monomers and link protein the latter two components are in a 1:1 relationship. It may be that it promotes and then stabilizes complexes of proteoglycan monomers with hyaluronic acid in cartilages so that they function as they do.

**REFERENCES**

15. Pita, J. C., and Muller, F. J. (1973) Biochemistry 12, 2655-2665
The link protein in proteoglycan aggregates from the Swarm rat chondrosarcoma.
T R Oegema, Jr, M Brown and D D Dziewiatkowski


Access the most updated version of this article at http://www.jbc.org/content/252/18/6470.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/18/6470.citation.full.html#ref-list-1