Electron Microscopic Studies of Proteoglycan Aggregates from Bovine Articular Cartilage*

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SUMMARY

Proteoglycan aggregates from bovine articular cartilage have been visualized by electron microscopy of mixed proteoglycan-cytochrome c monolayers. The proteoglycan aggregates consist of proteoglycan subunits arising laterally at fairly regular intervals (20 to 30 nm) from the opposite sides of an elongated filamentous structure. The filamentous backbone in individual aggregates varies in length from 400 to 4060 nm. The individual proteoglycan subunits in the aggregate vary in length from 100 to 400 nm. However, there is no difference in the average size of the proteoglycan subunits associated with the largest or smallest aggregates. The sizes of the individual aggregates are determined mainly by the lengths of their filamentous backbones. The stoichiometry of binding of subunits to filament, calculated from the data reported here, is close to that for the binding of subunits to hyaluronic acid reported by others.

EXPERIMENTAL PROCEDURES

Isolation of Proteoglycan Aggregate—Proteoglycans were extracted from bovine proximal humeral articular cartilage in 3 mM MgCl₂, 0.15 mM potassium acetate, pH 6.3, at 5°C for 48 hours. Proteoglycan aggregate was reassociated by dialysis against 10 volumes of 0.15 M potassium acetate, pH 6.3, for 24 hours at 5°C. Extraneous matrix proteins were removed by equilibrium density gradient centrifugation under associative conditions, as described in detail previously (5). Fraction 8 from this gradient, recovered at densities greater than 1.69 g/ml, contained 16 S proteoglycan subunit and 70 S proteoglycan aggregate in approximately equal amounts (5). This preparation, referred to as proteoglycan complex, was used in the electron microscopic studies described in this report.

Preparation for Electron Microscopy—Proteoglycan complex was dissolved in phosphate buffer (0.10 M KCl, 0.02 M KH₂PO₄, and 0.03 M KH₂PO₄, pH 7) with intermittent stirring with a Vortex mixer over an 18-hour period at 5°C. Dilutions of this solution were made with 1 M ammonium acetate (Merek), adjusted to pH 5 by acetic acid, and cytochrome c (Nutritional Biochemical Corp.) was added. The final spreading solution contained 2 to 4 µg of proteoglycan per ml and 0.01% (w/v) cytochrome c. A Teflon-coated aluminum trough (Bikoin-Tec, Inc., White Plains, N. Y.) was filled with a hypophase of 0.3 M ammonium acetate, pH 5, at room temperature. The surface of the hypophase was swept clean with a Teflon-coated bar. A wetted glass ramp was partly immersed in the hypophase and supported by the side of the trough. Talcum powder was dusted onto the hypophase at the insertion area of the ramp to indicate the cytochrome c film boundaries; 0.1 ml of the spreading solution was released slowly onto the wetted ramp to produce a mixed protein film of 120 to 150 cm² area. After spreading, the film was slightly compressed by the bar so that the talcum particles became rather immobilized, indicating high surface viscosity and coherence of film material.

Electron Microscopy and Measurements—Samples of the proteoglycan complex monolayer were transferred to carbon-reinforced, collodion-coated platinum grids (Siemens type) by touching the film surface of the monolayer. The grids were rinsed with water for 15 s to remove the droplet of the hypophase, stained for 60 s with acetonitrile uranyl acetate (10⁻¹ m), then rinsed with 95% ethanol. The grids were blotted dry and heated in a hot oven at 180°C for 10 min to remove the colloidion (14).

Electron micrographs were taken at 80 kv in a Siemens Elmiskop IA equipped with a decontamination device. The two-stage magnification was repeatedly calibrated by a grating replica (Fullum, Inc.). Optimal contrast was obtained by dark field microscopy using the condenser tilt lens system, with the monolayer mounted on extremely thin carbon supports.

Measurements were made by projecting the electron micrographs on a Grafacon board (RAND tablet) as a screen. The enlarged images of the filamentous backbones of proteoglycan aggregates, and the long axis (protein core) of subunits, were traced with a stylus. Their contour lengths were then calculated with a digital computer (PDP-8 of the Digital Equipment Corp.).

The basic structural unit of cartilage ground substance is the proteoglycan species of lowest average molecular weight, not further dissociable into smaller units without breaking covalent bonds. This proteoglycan species has been called the proteoglycan subunit (1, 2). In cartilage ground substance, most of the proteoglycan molecules are present as aggregates of much higher molecular weight, formed by the noncovalent association of subunits with other macromolecular species (1-5). Several recent studies indicate that hyaluronic acid is one of the additional species required for aggregation (6-12). Two low molecular weight proteins are also involved in aggregate formation (9-13).

In the studies reported here, electron microscopy of monolayers (14) prepared from mixtures of proteoglycans with cytochrome c has been used to visualize directly the molecular architecture of proteoglycan aggregates from bovine proximal humeral articular cartilage (5).

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RESULTS AND DISCUSSION

Electron Microscopic Appearance of Proteoglycan Aggregates—Proteoglycan complex containing roughly equal amounts of 16 S proteoglycan subunit and 70 S proteoglycan aggregate was isolated by equilibrium density gradient centrifugation under associative conditions, following dissociative extraction of bovine proximal humeral articular cartilage with 3 mM MgCl₂ as previously described (5). The appearance of proteoglycan aggregates invariably found in preparations of proteoglycan complex is illustrated in Figs. 1 to 3. The aggregates consist of proteoglycan subunits of varying length that arise laterally at fairly regular intervals from the opposite sides of an elongated, filamentous, thread-like structure. From a large number of electron micrographs of many preparations, electron micrographs of seven proteoglycan aggregates were selected in which the molecules were spread on the monolayer without kinking, entanglement, or overlapping of the filamentous backbone or proteoglycan subunits. In these molecules, measurements were made of the contour lengths of the filamentous backbone, and of the long axis (core protein) of the proteoglycan subunits, and the number of subunits per aggregate were counted (Table I). Fig. 1 shows the structure of one of the largest proteoglycan aggregates encountered. It contains 140 proteoglycan subunits of varying length, which arise in roughly perpendicular fashion from the opposite sides of a filamentous backbone approximately 4200 nm in length. Fig. 2, at higher magnification, shows an aggregate of intermediate size, consisting of 77 subunits arising from a filament approximately 1700 nm in length. In this case, both subunits and filament were unusually well extended on the monolayer and clearly defined. Fig. 3 shows two small aggregates surrounded by free proteoglycan subunits, which are also in the variable present in proteoglycan complex preparations. The conditions of spreading and staining employed (described under "Experimental Procedures") represent the best set of conditions so far identified for demonstrating the molecular architecture of the proteoglycan aggregate, specifically the filamentous backbone of the aggregate and the numbers of subunits per aggregate. Under these conditions, the chondroitin sulfate and keratan sulfate chains of proteoglycan subunits are usually not extended but appear to be clumped alongside the protein core. A proteoglycan subunit is occasionally found in which the mucopolysaccharide side chains are extended (Fig. 3, inset). Under other conditions (spreading solution 0.05 M ammonium acetate, hypophase 0.01 M ammonium acetate), the mucopolysaccharide chains of the proteoglycan subunits are more uniformly extended. In this case, proteoglycan subunits and the filamentous backbone of proteoglycan aggregates are entangled and overlapping, and the molecular architecture of the aggregate is obscured.

Measurements of the length of the filamentous backbone of each aggregate, of the average length of the long axis (protein core) of proteoglycan subunits, and of the number of proteoglycan subunits per aggregate are presented in Table I. These measurements indicate that the size of the proteoglycan aggregate is determined mainly by the length of its filamentous backbone. Consider first the size of the proteoglycan subunits associated with individual aggregates (Table I, Column 1). Measurements of the average lengths of the long axis (protein core) of the subunits indicate that there is no difference in the average size of the subunits associated with the largest or smallest aggregates. The long axis of the subunits associated with each aggregate are all very close to an over-all average length of 226 nm. Consider next the relationship between the number of subunits per aggregate and the length of the filamentous backbone of each aggregate (Table I, Columns 2 and 3). The number of subunits per aggregate increases roughly in proportion to the length of the filamentous backbone. The calculated average interval between subunits is approximately 26 nm. Direct measurements of the intervals between sites of attachment of the subunits to the filament gave an average value of 29 nm.

Recent studies by Hardingham and Muir (6-8), Gregory (9), and Hascall and Heinegård (10-12) indicate that proteoglycan subunits bind noncovalently to hyaluronic acid in the formation of proteoglycan aggregates. The latter studies, viewed in context with the work reported here, strongly suggest that the filamentous backbone of the proteoglycan aggregate demonstrated by electron microscopy is hyaluronic acid.

Hardingham and Muir (6) demonstrated that the addition of small amounts of hyaluronic acid (0.7%) to proteoglycan subunit from pig laryngeal cartilage resulted in a large increase in the hydrodynamic size of the subunit, demonstrated by gel chromatography or viscosity studies. They postulated that the interaction was a cooperative binding of many proteoglycan subunits with a single hyaluronic acid chain. The complex formed between subunits and hyaluronic acid was dissociated under the same conditions as proteoglycan aggregate. Hyaluronic acid was directly isolated from pig laryngeal cartilage and found to bind proteoglycan subunits isolated from the same cartilage (7).

Hascall and Heinegård (10-12) have confirmed and greatly extended the observations of Hardingham and Muir. Working with proteoglycans from bovine nasal cartilage, Hascall and Heinegård found that 0.4 to 0.8% hyaluronic acid (w/w) was present in proteoglycan aggregate (10). Studies of the binding of proteoglycan subunit to hyaluronic acid preparations of different molecular weights suggested that the sizes of proteoglycan aggregates are determined mainly by the sizes (lengths) of the hyaluronic acid molecules, in accord with the results reported here. Hascall and Heinegård also studied the specificity of the interaction of proteoglycan subunit, and of proteoglycan subunit core preparations in which chondroitin sulfate was removed with chondroitinase, with hyaluronic acid. It was found that hyaluronic acid decasaccharides or nonasaccharides interacted strongly with proteoglycan subunit, whereas smaller hyaluronic acid oli-

### Table I

Dimensions of seven proteoglycan aggregates from bovine proximal humeral articular cartilage

| Length | Subunits per aggregate | Filament length per subunit | Molecular weights |
|--------|-------------|-----------------|-----------------|
| Subunit | Filament   | nm               | ×10⁴ | Aggregates |
| nm     |             |                 |      |           |
| 228    | 447         | 19               | 24   | 1.9       | 20 |
| 208    | 973         | 44               | 22   | 4.1       | 66 |
| 212    | 1242        | 40               | 31   | 5.2       | 60 |
| 199    | 1404        | 75               | 20   | 6.2       | 113 |
| 255    | 1723        | 77               | 22   | 7.2       | 116 |
| 245    | 2250        | 70               | 32   | 9.4       | 106 |
| 235    | 4165        | 140              | 30   | 17.4      | 212 |
| Average | 220         | 20               |      |            |   |

*a* Calculated assuming that the filament is hyaluronic acid, using 418 as the molecular weight and 1 nm as the length of the disaccharide repeating unit.

*b* Calculated from the sum of the molecular weights of the filament and the total number of subunits per aggregate, using 1.5 × 10⁴ as the average molecular weight of subunit.
Fig. 1. Dark field electron micrograph of a large proteoglycan aggregate from bovine proximal humeral articular cartilage. Proteoglycan subunits of varying length arise laterally from the opposite sides of an elongated filament approximately 4200 nm in length (× 71,000). The bar at the lower right equals 1 μm.
Fig. 2. Dark field electron micrograph of a proteoglycan aggregate of intermediate size in which the proteoglycan subunits and filamentous backbone are particularly well extended and clearly defined (× 120,000). The bar at the lower right equals 0.5 μm.
Fig. 3. Electron micrograph showing two smaller aggregates surrounded by free proteoglycan subunits, not associated with aggregates (× 70,000). The inset (upper right, × 89,000) shows a proteoglycan subunit in which some of the mucopolysaccharide chains are extended. The bar at the lower right equals 1 μm.
TABLE II
Stoichiometry of binding of proteoglycan subunits to hyaluronic acid

| Method                                                                 | Molecular weights | Maximum binding subunit:hyaluronic acid | Spacing between subunits | References         |
|-----------------------------------------------------------------------|-------------------|----------------------------------------|--------------------------|-------------------|
| 1. Inhibition of binding by hyaluronic acid oligosaccharides.          | 2.5 × 10^6        | 1200:1                                 | 5                        | Hardingham and Muir (8) |
| 2. Same as above, using proteoglycan core.                             | 4.5 × 10^6        | 216:1                                  | 5                        | Hascall and Heinegård (11) |
| 3. Binding of proteoglycan core to hyaluronic acid.                    | 4.5 × 10^5        | 135:1                                  | 8-10                     | Hascall and Heinegård (11) |
| 4. Determined hyaluronic acid content of proteoglycan aggregate.       | 2.5 × 10^6        | 250:1-125:1                            | 24-48                    | Hascall and Heinegård (11) |
| 5. Binding of proteoglycan subunit to hyaluronic acid.                 | 2.5 × 10^6        | 250:1                                  | 24                       | Hardingham and Muir (8) |
| 6. Measurement of electron micrographs of proteoglycan aggregates.     | 1.5 × 10^6        | 5 × 10^3                               | 20-30                    | This paper         |

* Calculated assuming that the filamentous backbone of the proteoglycan aggregate is hyaluronic acid, using a molecular weight of 416 and a length of 1 nm for the hyaluronic acid disaccharide repeating unit.

In Table II, the stoichiometry of binding of proteoglycan subunits to hyaluronic acid derived from chemical studies is compared with that calculated from the measurements of the electron micrographs of the proteoglycan aggregates. Both the studies of Hardingham and Muir, and those of Hascall and Heinegård indicate that the shortest chain length of hyaluronic acid to which a single proteoglycan subunit can bind strongly is approximately 5 nm in length (Table II, Lines 1 and 2). However, when many proteoglycan subunits bind to a single hyaluronic acid chain of high molecular weight (Table II, Lines 4 and 5), they bind at intervals far greater than 5 nm. The spacing between neighboring subunits bound to the same hyaluronic acid chain appears to be related to the lengths of the mucopolysaccharide chains arising from the proteoglycan subunit core protein, and to the resulting steric effects between adjacent subunits (8, 11).

Thus, core molecules from which chondroitin sulfate chains have been removed, consisting of keratan sulfate chains ~6 nm in length arising from core protein, bind to high molecular weight hyaluronic acid chains at 8- to 10-um intervals (Table II, Line 3). Native proteoglycan subunits with chondroitin sulfate chains, 40 to 50 nm in length, are spaced at a minimum of 24 nm. Consider finally the actual intervals at which proteoglycan subunits arise from the filamentous backbone of the proteoglycan aggregates, based on direct measurements of the electron micrographs (Table II, Line 6). The ratio of the length of the filamentous backbone of each aggregate to the number of subunits per aggregate indicates that the average spacing between subunits is from 20 to 30 nm. This correspondence between the spacing of subunits calculated from binding studies with that demonstrated by electron microscopy strongly suggests that the filamentous backbone of the proteoglycan aggregate is hyaluronic acid.

**Length of Long Axis (Protein Core) of Proteoglycan Subunits—** The individual lengths of the long axis of 465 proteoglycan subunits associated with the seven proteoglycan aggregates whose dimensions were presented in Table I were also measured. The length distribution of the subunits is presented in Fig. 4. The long axes of the subunits vary greatly in length from 100 to 400 nm. There is no indication of the existence of a population of monomers and dimers. These results are in accord with, and support the suggestion of Heinegård and Hascall (12), that the core protein of proteoglycan subunit contains a polysaccharide attachment region of variable length.

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