The Formation of a Stable Complex between Dissociated Proteoglycan and Hyaluronic Acid in the Absence of a Link Protein

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A proteoglycan fraction prepared from bovine articular cartilage under dissociative conditions was shown to interact with three purified hyaluronic acid preparations to form stable complexes in the analytical ultracentrifuge. It is concluded from these experiments that, although link proteins are associated with hyaluronic acid and proteoglycans in cartilage extracts, they are not an essential requirement for the formation of stable complexes between these macromolecular constituents.

Hyaluronic acid is a ubiquitous constituent of the extracellular matrices of connective tissues, and the finding (Hardingham & Muir, 1972) that this substance interacts specifically with cartilage proteoglycans indicates that it probably plays a unique role in the macromolecular organization of cartilage and possibly other connective tissues even when only small quantities are present (Hardingham & Muir, 1973).

The earlier studies (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969) concerned with the dissociative extraction of proteoglycans from cartilage with 4M-guanidinium chloride showed that, when the molarity of the guanidinium chloride in the extract was decreased to 0.4M by dialysis, stable complexes were formed. After separation of the complex from other apparently extraneous substances and re-fractionation in a density gradient under dissociative conditions, the complex yielded a proteoglycan fraction and a link-protein fraction. The interaction between these fractions could be manipulated by altering the concentration of the guanidinium chloride, and both fractions were required for re-formation of the complex under associative conditions. It was not realized at that time that hyaluronic acid was one of the essential components of the link-protein fraction. Gregory (1973), however, was able to separate the link-protein fraction into two subfractions, both of which were required when the analytical ultracentrifuge was used to detect the presence of complex. The buoyant densities of these subfractions indicated that one consisted of peptide and the second of hyaluronic acid. Subsequent work (Hascall & Heinegård, 1974a,b) confirmed that hyaluronic acid is a link

factor, and studies involving partial proteolysis of the proteoglycan fraction isolated under associative conditions have indicated that two link proteins are involved in the formation of complex (Heinegård & Hascall, 1974; Hascall & Heinegård, 1975). When viscosity measurements and gel chromatography are used as assay techniques, complex-formation can be demonstrated in the absence of link proteins (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a). The failure to detect the complex in the analytical centrifuge when only hyaluronic acid and proteoglycan are present has thus been thought to indicate that the link proteins serve to stabilize the complex formed by the interaction of these macromolecules.

On the basis of these observations a model has been proposed to describe the structure of the proteoglycan complex (Heinegård & Hascall, 1974; Hascall & Heinegård, 1975), which assumes that the re-formation of complex under associative conditions *in vitro* is a reversal of the dissociative extraction process. Connective-tissue extracts, however, contain aggregating protein constituents that are closely associated with hyaluronic acid (Swann *et al.*, 1975), and, whereas they appear to be distinct from the two link proteins described above, it is not clear whether these self-aggregating proteins play a role in proteoglycan complex-formation. It was decided therefore to re-examine the nature of the interaction between hyaluronic acid and articular-cartilage proteoglycans to determine whether link proteins are required for the formation of stable complexes when analysed in the analytical ultracentrifuge.

Materials and Methods

Articular cartilage was from the metacarpal-phalangeal joints of ox feet obtained from the slaughterhouse. Proteoglycans were prepared from

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articular cartilage by dissociative extraction [4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, containing 5mM-benzamide (750ml/100g wet wt. of tissue) for 72h at 4°C] followed by two consecutive dissociative density-gradient-fractionation procedures [gradient (a), initial density of CsCl solution 1.69g/cm³]. The fraction sedimenting at a density higher than 1.58g/cm³ was re-centrifuged in gradient (b) (initial density of CsCl solution 1.50g/cm³). Both procedures were performed in the presence of 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8. Centrifugation was at 170000g at 4°C for 64h. The dissociated proteoglycan fraction sedimenting at a density higher than 1.55g/cm³ in gradient (b) was dialysed against water and 1M-NaCl for 16h each, and then against water until Cl⁻ was not detectable; it was then freeze-dried and used for the experiments described below.

Three different hyaluronic acid samples were used. Hyaluronic acid (I) from adult bovine vitreous humour obtained as described by Swann & Constable (1972) was purified by chloroform treatment, ethanol precipitation and ultracentrifugation (Swann, 1968), followed by a single CsCl-density-gradient-fractionation procedure (Swann & Caulfield, 1975). Hyaluronic acid (III) from rooster comb dermis, obtained and treated as described by Swann (1968), was purified similarly to hyaluronic acid (I), except that the CsCl gradient was omitted. Hyaluronic acid (II), also from rooster comb dermis, was purified by two consecutive CsCl gradients (Swann & Caulfield, 1975).

Aggregation experiments were performed by dissolving the dissociated proteoglycan and hyaluronic acid preparations in 0.5M-guanidinium chloride/0.05M-Tris/HCl, pH 8.0, and incubating the mixtures at 4°C for 16h. The specific viscosities of dissociated proteoglycan and hyaluronic acid solutions and the complex formed after mixing were determined in a Cannon-Ubbelohde (Cannon Instrument Co., State College, PA, U.S.A.) capillary viscometer (solvent flow time 98s). The specific-viscosity increment is the value obtained for the complex mixture minus the sum of the dissociated proteoglycan and hyaluronic acid values measured at equivalent concentrations before mixing. After performing viscosity measurements, samples were then examined in the analytical ultracentrifuge. Chemical analyses of freeze-dried samples were performed as previously described (Swann & Radin, 1972; Swann & Caulfield, 1975).

Results and Discussion

The analyses of the dissociated proteoglycan sample used in this study are given in Table 1. The composition is similar to that of a bovine articular-cartilage proteoglycan preparation isolated previously by Strider *et al.* (1975). All three hyaluronic

acid preparations contain small quantities of amino acids (I contained 0.5%, w/w; II, 0.7%, w/w; III, 0.4%, w/w), but no peptides were detected by polyacrylamide-gel electrophoresis (Furthmayr & Timpl, 1971). The amino acid composition of hyaluronic acid (II) has been reported previously (Swann & Caulfield, 1975; preparation HA CsCl-II). The intrinsic viscosities of these hyaluronic acids in 0.15M-NaCl/0.01M-Tris/HCl, pH 7.5, were 400, 2600 and 4200ml/g for hyaluronic acids (I), (II) and (III) respectively. After mixing proteoglycan and hyaluronic acid, the viscosity increment was found to depend on both the concentration and intrinsic viscosity of the hyaluronic acid (Fig. 1). The sedimentation patterns obtained with several of the mixtures are shown in Plate 1. With each hyaluronic acid a bimodal sedimentation pattern was obtained. For the hyaluronic acid (I)/dissociated proteoglycan

Table 1. *Composition of the dissociated proteoglycan fraction*

	Amino acid content (residues/1000 residues)
Hydroxyproline	0
Aspartic acid	65
Threonine	62
Serine	116
Glutamic acid	145
Proline	106
Glycine	115
Alanine	71
Cystine (half)	11
Valine	67
Methionine	4
Isoleucine	37
Leucine	76
Tyrosine	21
Phenylalanine	33
Hydroxylysine	0
Lysine	19
Histidine	19
Arginine	30
Total amino acids (% w/w)	16.1
	Carbohydrate content (% w/w)
Hexuronic acid	14.9
Glucosamine	6.6
Galactosamine	15.1
Xylose	1.9
Fucose	1.2
Mannose	0.5
Galactose	8.3
N-Acetylneuraminic acid	2.2
Sulphate content (% w/w)	14.0
Intrinsic viscosity (ml/g)	200
$s_{20,w}^0$ (S)	12.2

EXPLANATION OF PLATE I

Sedimentation patterns obtained with solutions containing 2.5 mg of dissociated proteoglycan/ml

The lower lines of (a), (b) and (e) were obtained with dissociated proteoglycan alone. The upper line of (a) was obtained with a solution containing 11 μ g of hyaluronic acid (I)/ml; the upper line of (b) and the lower and upper lines of (c) were obtained with 16, 27 and 32 μ g of hyaluronic acid (II)/ml respectively; the lower and upper lines of (d) were obtained with 12 and 37 μ g of hyaluronic acid (III)/ml respectively. The upper line of (e) was obtained with 150 μ g of hyaluronic acid (II)/ml. The centrifuge was operated at 20°C and 42000 rev./min; double-sector cells, one of which was fitted with a 1° window, were used. Photographs were taken at a bar angle of 60° at 19 min (c and d), 20 min (b), 23 min (a) and 41 min (e) after reaching two-thirds full speed.

mixture (Plate 1a) the sedimentation coefficients of the slow- and fast-moving peaks were 10.7S and 16.7S respectively. For hyaluronic acids (II) (Plate 1b, upper line) and (III) (Plate 1d, lower line) the values were 11.4S and 25.7S, and 11.4S and 26.2S respectively. At low hyaluronic acid concentrations the area of the fast-sedimenting peak increased with increasing concentration of hyaluronic acid (Plate 1c). At higher hyaluronic acid concentrations, however, the area of the fast peak decreased, as did the S value of the fraction (Plate 1d, upper line). At still higher hyaluronic acid concentrations (Plate 1e, upper line) the complex was not detected, although the sedimenting boundary showed much less

diffusion than when the dissociated proteoglycan was run in the absence of hyaluronic acid (Plate 1e, lower line). As was found previously (Hardingham & Muir, 1972; Hascall & Heinegård, 1974), only a portion of the dissociated proteoglycan formed a complex, and the question arose as to whether the interaction with hyaluronic acid was a selective process. After chromatography on a Bio-Gel A5M (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column (4.5 cm × 120 cm) eluted with 1 M-guanidinium chloride/0.05 M-Tris/HCl, pH 7.8, the dissociated proteoglycan was distributed throughout the included volume of the column and six arbitrary fractions were obtained with increasing K_{av} values (Laurent & Killander, 1964). When these fractions were mixed with hyaluronic acid (II) at ratios of approx. 30:1 (w/w), there was an increase in the viscosity and each mixture showed a bimodal distribution in the centrifuge (Table 2).

The sedimentation and viscosity data show that the ability to interact with hyaluronic acid was not restricted to one particular size range of proteoglycans and that the size of the complex depended largely on the molecular weight of hyaluronic acid. These results conform to the current model of the structure of proteoglycan complexes (Hascall & Heinegård, 1975), but it is clear that link proteins are not an absolute requirement for the formation of stable complexes. The articular cartilage used in this study, however, appears to be similar to other cartilaginous tissues in that two link proteins are preferentially retained in the proteoglycan fraction isolated under associative conditions (D. A. Swann, S. Powell & S. Sotman, unpublished work). This implies, as suggested previously (Heinegård & Hascall, 1974; Hascall & Heinegård, 1975), that these constituents are involved in complex-formation. The specific nature of their role, however, is not clear at the present time.

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Fig. 1. Specific-viscosity increments observed when solutions of dissociated proteoglycan (5 mg/ml) were mixed with equal volumes of hyaluronic acids (I) (Δ), (II) ($\circ$) and (III) ($\bullet$).

The values shown for hyaluronic acid are the final concentrations in the mixtures. The sedimentation patterns obtained with samples (a), (b), (c), (d) and (e) are shown in Plate 1.

Table 2. Aggregation of dissociated proteoglycan fractions with hyaluronic acid (II)

The elution position of the dissociated proteoglycan fractions (K_{av}) was calculated as described by Laurent & Killander (1964). The concentration of hyaluronic acid (II) after mixing was 0.085 mg/ml.

Dissociated proteoglycan fraction		Specific-viscosity increment	Sedimentation coefficient (S)	
K_{av}	Concentration (mg/ml)		Slow peak	Fast peak
0.11	2.70	0.62	9.3	17.0
0.27	2.55	0.77	9.2	17.8
0.41	2.60	0.64	8.6	17.2
0.53	2.36	0.55	8.5	15.9
0.66	2.50	0.37	7.6	14.7
0.81	2.08	0.21	8.5	18.0

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