Binding of Oligosaccharides of Hyaluronic Acid to Proteoglycans

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Oligosaccharides derived from hyaluronic acid were shown to inhibit proteoglycan-hyaluronic acid interaction, as measured in a viscometer. The relative inhibition increased with the size of the oligosaccharide and the results suggested that deca-saccharides were the smallest fragments able to bind strongly to the proteoglycan.

In a previous report (Hardingham & Muir, 1973) hyaluronic acid was identified as a minor component of proteoglycan aggregates isolated from pig laryngeal cartilage, and it was demonstrated *in vitro* that it reversibly bound to proteoglycans derived from these aggregates (Hardingham & Muir, 1972a). Results from gel chromatography showed hyaluronic acid to produce a large increase in the hydrodynamic size of the proteoglycans that was detectable with an extremely low proportion of hyaluronic acid to proteoglycan (1:1000, w/w), and increased with hyaluronic acid content until a maximum was reached at 1:75 (w/w). Further addition of hyaluronic acid then resulted in a decrease in the proportion of material of largest size. Comparable results were also obtained from viscometry, where the interaction was evident as a large increase in viscosity of proteoglycan solutions after the addition of small amounts of hyaluronic acid. Not all proteoglycans appeared able to interact with hyaluronic acid, and this applied to about 40% of those extracted from pig laryngeal cartilage.

The results were interpreted as showing that a large number of proteoglycan molecules were binding to each hyaluronic acid chain. The existence of an optimum interacting ratio of the reactants and failure of the system to form a 'gel' at high hyaluronic acid concentrations was evidence that each proteoglycan molecule possessed only one binding site; otherwise the proteoglycans would have been able to cross-link hyaluronic acid chains when the latter were in excess.

The molecular weights of the hyaluronic acid used did not appear to affect its ability to interact. Moreover ascorbate-degraded hyaluronic acid also interacted (Hardingham & Muir, 1972b). The experiments described below were designed to examine the interaction of proteoglycans with small oligosaccharides derived from hyaluronic acid, in order to investigate further the hyaluronic acid-binding site.

Experimental

Proteoglycans were extracted from fresh pig laryngeal cartilage with 4 M-guanidinium chloride and

were purified and disaggregated by equilibrium density-gradient centrifugation as previously described (Hardingham & Muir, 1972a). This proteoglycan preparation contained less than 5% of material that was excluded by gel chromatography on Sepharose 2B, and about 60% was able to interact with hyaluronic acid (Hardingham & Muir, 1972a). Oligosaccharides were prepared from hyaluronic acid (glucosamine/galactosamine molar ratio 28:1; ex umbilical cord; BDH Chemicals Ltd., Poole, Dorset, U.K.) by partial digestion with testicular hyaluronidase (EC 3.2.1.35; 1000 units/mg; Boehringer, Mannheim, W. Germany). A 50mg portion of hyaluronic acid in 5ml of 0.1 M-sodium acetate buffer, pH 5.5, was digested with 2mg of hyaluronidase at 37°C for 16h. The digest was fractionated by gel chromatography on a column (130cm × 2.5cm) of Sephadex G-25 (fine grade) eluted with 0.1 M-NaCl in 50mm-sodium acetate buffer, pH 6.8, at 4°C, 2ml fractions being collected. The uronic acid contents of the fractions were determined by an automated modification (D. Heinegard, personal communication) of the carbazole reaction (Bitter & Muir, 1962) with glucuronolactone as standard. The three larger fractions were further purified by gel chromatography on Sephadex G-50 (superfine grade) by Dr. V. C. Hascall. Fractions were pooled and concentrated by pressure dialysis with an Amicon Diaflo with UM-05 membranes, and were analysed for uronic acid and then rechromatographed on the same column. Fractions isolated from the centres of all the rechromatographed peaks were analysed for uronic acid and reducing terminal hexosamine by the Good & Bessman (1964) modification of the Morgan-Elson reaction with glucosamine hydrochloride as standard. Samples were also treated with 0.1 M-sodium borohydride in 50mm-sodium tetraborate buffer, pH 9.0, at room temperature for 3h. Under these conditions glucosamine hydrochloride was quantitatively reduced to glucosaminitol, as determined by the loss of Morgan-Elson-positive material and ion-exchange separation of the products (R. J. F. Ewins & T. E. Hardingham, unpublished work). Glucosamine and glucosaminitol in the reducing oligosaccharides were determined after hydrolysis under N₂ in 8 M-HCl at 95°C for 3h

followed by ion-exchange chromatography in 0.2 M-boric acid–0.35 M-sodium citrate buffer, pH 5.0, at 50°C with a Locarte amino acid analyser.

The analyses were carried out on a fraction that was pure on gel chromatography on Sephadex G-25. From its elution position it appeared to consist of tetrasaccharides (Flodin *et al.*, 1964), and gave a uronic acid:*N*-acetylglucosamine molar ratio of 1.67 by the Good & Bessman (1964) method. This implied that the reducing glucosamine gave 20% more colour than did free glucosamine, in agreement with the results obtained by Flodin *et al.* (1964), who also used a borate modification of the Morgan–Elson reaction (Reissig *et al.*, 1955). The results were thus corrected by a factor of 0.835 in order to calculate the amount of terminal reducing *N*-acetylglucosamine in the oligosaccharide fractions. The results of borohydride reduction were in agreement with those obtained by the Morgan–Elson reaction and showed a molar ratio of glucosamine to glucosaminitol of 1:0.94 for this 'tetrasaccharide' fraction.

For measurements of viscosity a standard solution of proteoglycan was prepared containing 585 µg of uronic acid/ml in 0.14 M-NaCl–10 mM-sodium phosphate buffer, pH 7.4. For inhibition experiments hyaluronic acid (15.1 µg/ml) was added to this solution. Various amounts of the oligosaccharide fractions were added to 3 ml samples of the proteoglycan–hyaluronic acid solution. After equilibration overnight at room temperature the viscosity was measured in an Ostwald capillary viscometer (M-2; Baird and Tatlock, Chadwell Heath, Essex, U.K.) at 30°C and readings were taken until they were constant, which was usually after 1 h. No allowance was made for the dilution of the proteoglycan solution by the addition of the oligosaccharide, but in no case was this more than 1%.

Results and discussion

Hyaluronic acid is a single-chain unbranched polysaccharide containing a repeating disaccharide unit of β -(1→4)-D-glucuronic acid- β -(1→3)-D-*N*-acetylglucosamine. Testicular hyaluronidase cleaves the hyaluronic acid chain, producing even-numbered oligosaccharides containing terminal reducing *N*-acetylhexosamine residues (Brimacombe & Webber, 1964). Fractionation and rechromatography of the hyaluronic acid digest produced five resolved peaks. Assuming that the released oligosaccharides contained one terminal reducing *N*-acetylhexosamine/molecule (Brimacombe & Webber, 1964), the chain lengths of the oligosaccharides were calculated from the molar ratio of reducing *N*-acetylhexosamine/uronic acid, and from the molar ratio of glucosamine/glucosaminitol after borohydride reduction (Table 1). Both methods produced similar results and showed the fractions of increasing size to contain the even-numbered oligosaccharides from tetrasaccharide to duodecasaccharide in length respectively.

The addition of oligosaccharides alone had no effect on the viscosity of proteoglycans. However, when the oligosaccharides were added to the proteoglycan–hyaluronic acid mixture there was a decrease in viscosity that was proportional to the amount of oligosaccharide added. The results appeared to reflect the ability of the oligosaccharides to compete with hyaluronic acid in binding to the proteoglycan, and the degree of inhibition produced by the different oligosaccharide fractions was taken as a measure of their affinity for the binding site on the proteoglycan.

The results of adding various amounts of different oligosaccharides to the standard proteoglycan–hyaluronic acid solution was plotted on a weight basis (Fig. 1a) and on a molar-concentration basis

Table 1. Analysis of oligosaccharides from hyaluronic acid

The chain lengths were calculated as described in the text from (a) the molar ratio of uronic acid to terminal reducing *N*-acetylhexosamine, corrected by a factor of 0.835, and (b) from the molar ratio of glucosamine to glucosaminitol after borohydride reduction.

Fraction	(a) Morgan–Elson procedure		(b) Borohydride reduction procedure	
	Molar ratio: uronic acid terminal reducing <i>N</i> -acetylhexosamine	Calc. no. of monosaccharides per chain	Molar ratio: glucosamine glucosaminitol	Calc. no. of monosaccharides per chain
Tetrasaccharide	1.81	3.62	0.90	3.60
Hexasaccharide	2.84	5.68	1.88	5.64
Octasaccharide	3.95	7.90	2.89	7.72
Decasaccharide	4.85	9.70	4.05	10.12
Duodecasaccharide	5.93	11.86	4.95	11.87

Fig. 1. Effect of various concentrations of oligosaccharides on the viscosity of a proteoglycan-hyaluronic acid solution

Conditions were as described in the text. A 100% interaction was the relative viscosity of the proteoglycan-hyaluronic acid solution and 0% was that of the proteoglycan solution alone. The fractions used were tetrasaccharides (●), hexasaccharides (■), octasaccharides (▲), decasaccharide (○) and duodecasaccharide (□). HA marks the functional molar concentration of hyaluronic acid in the proteoglycan solution calculated as described in the text, i.e. $1.51 \mu\text{M}$. Results are plotted on a weight basis (a) and a molar basis (b).

(Fig. 1b) from the molecular sizes calculated in Table 1. The tetrasaccharide, hexasaccharide and octasaccharide fractions were relatively poor inhibitors, suggesting that their affinities for the proteoglycan were low. In contrast, deca- and duodeca-saccharide-fractions were strong inhibitors of the proteoglycan-hyaluronic acid interaction. The decasaccharide fraction appeared to bind to the proteoglycan about 35 times more strongly than did the octasaccharide fraction, and duodecasaccharide fraction bound more strongly than decasaccharide, an effect observed particularly when considered on a molar basis (Fig. 1b).

The abrupt increase in the affinity of proteoglycan for decasaccharides compared with tetra-, hexa- and octa-saccharides suggested that a decasaccharide was the minimum length of the hyaluronic acid chain able to bind strongly with the interacting site on the proteoglycan. The greater affinity of the duodecasaccharides for proteoglycan compared with the decasaccharide suggested some additional binding of sugar residues beyond the decasaccharide region.

Results of gel chromatography (Hardingham & Muir, 1972a) suggested that when the amount of hyaluronic acid was limiting the weight ratio of interaction of proteoglycan with hyaluronic acid was about 250:1. The stoichiometry of interaction under

these conditions when hyaluronic acid chains would be saturated with proteoglycans was a measure of the maximum degree of packing of proteoglycans along the hyaluronic acid chain. Assuming a molecular weight of the proteoglycan of 1×10^6 – 4×10^6 (Luscombe & Phelps, 1967; Eyring & Yang, 1968; Rosenberg *et al.*, 1970a; Hascall & Sajdera, 1970) it was calculated that about 50 proteoglycan molecules could bind to a hyaluronic acid chain of 5×10^5 molecular weight. Thus each proteoglycan would occupy a region of hyaluronic acid of about 10000 molecular weight. If this estimate of the minimum length of hyaluronic acid occupied by a proteoglycan was assumed to be its equivalent weight in binding to proteoglycan, its effective concentration in the proteoglycan-hyaluronic acid solution was $1.51 \mu\text{M}$ (Fig. 1b). The inhibition produced by the oligosaccharides at this concentration suggested that deca- and duodeca-saccharides (about 60% inhibition) were binding as effectively as the undegraded hyaluronic acid, octasaccharides much less effectively (4% inhibition) and hexa- and tetra-saccharides very poorly (less than 2% inhibition). Thus most of the binding of hyaluronic acid with proteoglycan would appear to involve a region about the size of a decasaccharide unit, 4–5 nm (40–50 Å) long. This was considerably shorter than the 10000-

molecular-weight unit (24 nm long) occupied by each proteoglycan. However, as the proteoglycan is a very large highly hydrated molecule, which by electron microscopy appears to be about 340 nm (3400 Å) long (Rosenberg *et al.*, 1970b), it is perhaps not surprising that there is a limit to the proximity with which adjacent proteoglycan molecules can approach each other when bound along the same hyaluronic acid chain. This would depend, not only on the size of the proteoglycan, but also on the location and geometry of the binding site on the proteoglycan molecule.

The relatively small size of the binding site and its specificity for hyaluronic acid (Hardingham & Muir, 1972a) suggests that it is analogous to the binding site of an enzyme for its substrate. Moreover, it also strongly suggests that there is some tertiary structure in the proteoglycan molecule and implies that the simple 'bottle brush' model of the molecule originally proposed by Mathews & Lozaityte (1958) and by Partridge *et al.* (1961) may need to be revised.

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