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BINDING OF HYALURONIC ACID OLIGOSACCHARIDES BY CARTILAGE PROTEOGLYCAN

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SUMMARY. Hyaluronic acid oligosaccharides containing five or more disaccharide units form 1:1 complexes with proteoglycan, binding strongly with an association constant K estimated to be at least 50 μM^{-1}. Smaller oligosaccharides appear also to bind, K being about 1 to 2 μM^{-1}. These results are consistent with the observed competitive displacement of macromolecular hyaluronic acid from proteoglycan binding sites by oligosaccharides containing no fewer than five disaccharide units.

"Synthetic" aggregates are formed under "associative" solvent conditions (ionic strengths below about 2 and pH above 3) by complex formation between purified hyaluronic acid from any source and proteoglycan from pig laryngeal (1) and bovine nasal septum (2) cartilages. Such complexes, probably involving in addition bound "link" glycoproteins (3,4), are evidently responsible for the large aggregates observed (3) in cartilage extracts under associative conditions. On the basis of gel chromatography and viscometry, hyaluronic acid oligosaccharides containing no fewer than five disaccharide units appeared to displace macromolecular hyaluronic acid from complexes with proteoglycan from both of the above sources (1,2). In the present work complex formation of oligosaccharides containing from two to seven disaccharide units with calf nasal septum proteoglycan was investigated by means of equilibrium dialysis, as part of a program of physical characterization of this complex.

MATERIALS AND METHODS

Proteoglycan Preparation. Frozen cleaned whole calf bovine nasal septa were shredded followed by extraction (24 h) and washing by resuspension (each in 10 ml/g of frozen tissue) . Extractions were performed twice in each of a) 0.15 M NaCl containing protease inhibitors (5): 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine hydrochloride; pH ca 6 and b) 4 M Gdn HCl[1]

[1] Abbreviations: GdnHCl, guanidine hydrochloride; HA_i is used to represent hyaluronic acid components, where i is the number of monosaccharides in a given oligosaccharide.

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containing the same inhbitors. Yields at 5°C: 21 mg in a) and 52 mg in b) of proteoglycan per g of tissue. The first extract in b) (or third serial extract) was fractionated as described previously (6) (except at 10°) in a dissociative density gradient. The densest fraction (Dl_1, see (6)) of density 1.68 g/ml, and containing 45 % of the uronic acid in the gradient, was dialyzed thoroughly against deionized water. The dialyzed solution was kept frozen at -20° between intervals of use.

Fraction Dl_1 contained 0.24 g of protein per g of uronic acid by uv analysis (7). The viscosity and sedimentation velocity were measured as described previously (6) in 0.2 M NaCl, pH 6.5, to give the following properties: limiting viscosity number $[\eta]$ 271 cm^3/g; sedimentation coefficient s^o at 20°, 29 × 10^{-13} s. Solution concentrations of uronic acid were determined by a modification (8) of the standard carbazole method; the proteoglycan concentration c_o (mg/ml) was taken to be 3.25 times the uronic acid concentration [see Eq. (1), ref. (6)].

Oligosaccharide Preparation. A sample of hyaluronic acid with ^3H-labelled N-acetyl groups was prepared by Lindahl et al[2]. Labelling involved limited N-deacetylation by the method of Dimitriev et al (9) with lower temperature and shorter reaction time followed by reacetylation with [^3H]acetic anhydride by a modification of the N-acetylation method of Danishefsky and Steiner (10). The resulting sample (1.3 mg) was dissolved in 1 ml of 0.15 M NaCl, 0.1 M acetate buffer, pH 5.0 and hydrolyzed enzymatically with 2.4 µg of testicular hyaluronidase (AB Leo, Hälsingborg, Sweden) at 37° for 3 h followed by enzyme deactivation at 100° for 5 min. The digest was chromatographed in a 1.5 × 195 cm column packed with Sephadex G-50 Superfine gel and eluted with 0.25 M pyridine-acetate buffer, pH 6, to produce a series of oligosaccharide fractions, as described by Hascall and Heinegård (2), except that fractions were not rechromatographed[3].

Equilibrium Dialysis. Standard seamless cellulose dialysis tubing (Fisher Scientific), 1 cm flat width, was cleaned essentially by the method described by Brewer et al (11) and stored in the cold with a little formaldehyde as preservative. Solutions were made up by weighing into 1 × 7.5 cm glass culture tubes with tight-fitting polyethylene stoppers. The ingredients of the solutions included proteoglycan (Fraction Dl_1) neutralized to pH 6.5, ^3H-labelled hyaluronic acid oligosaccharide, concentrated solutions to give 0.2 M NaCl, 0.03 M phosphate buffer, pH 6.5, and water. Typically, 1.5 ml of this solution served as the outer dialysis liquid, and the dialysis bag was filled with a similar volume of 0.2 M NaCl, 0.03 M phosphate buffer, pH 6.5. The bags were closed by knotting, wiped dry, and weighed with the tube. All equilibrium experiments were performed at 10° (±0.2°). Tubes were inverted once or twice each day to homogenize contents of inner and outer solutions. The time required to reach equilibrium was determined by similar separate experiments containing no proteoglycan. About ten days sufficed for HA_4 and about one week more for each additional disaccharide unit. (In an early experiment continuous agitation with a mechanical tumbler caused some denaturation of the proteoglycan with consequent low binding of oligosaccharide.) Equilibrated bags were weighed after wiping dry to permit determination of weight change (thus concentration change of proteoglycan) and were cut open into another 1 × 7.5 cm glass culture tube. Radioactivity was assayed in 6 ml plastic liquid scintillation vials (Wheaton Scientific) with weighed aliquots of the equilibrium solutions, the total assay volume brought to 0.5 ml with water, and 4 ml of Instagel liquid scintillation cocktail (Packard Instrument Co.) added. Samples were counted for from five to ten periods of 10 min each in a liquid scintillation counter (Searle Model Mark II).

[2]Lindahl, U., Höök, M. and Riesenfeld, J., personal communication.

[3]Laurent, T. C., personal communication.

Equilibrium Calculations. Equilibrium concentrations (all as μM) were obtained as follows. The total proteoglycan weight concentration c_o (mg/ml) was calculated from the stock solution concentration and the volume dilution factor determined in making up the solution and its subsequent volume change during dialysis. The corresponding micromolar concentration P_o is then given by $10^6 \times c_o/\bar{M}_n$, where $\bar{M}_n$ is the number-average molecular weight (see below). The hyaluronic acid concentrations H_o of oligosaccharide in mixtures with proteoglycan and H in the equilibrium dialysates were calculated from the radioactivity (as cpm per ml of solution) and the micromolar activity (cpm per μmol), as determined from the specific activity and the molecular weight (400 per disaccharide unit) of the oligosaccharide fraction. The value of the specific activity (7.0×10^4 cpm per μg of hyaluronic acid, Na^+ form) was obtained from measurements of radioactivity and uronic acid content of solutions of the unhydrolyzed sample of ^3H-labelled hyaluronic acid. All oligosaccharide fractions were thus assumed to have equal specific activity.

RESULTS AND DISCUSSION

Proteoglycan Molecular Weight. A molecular weight of $3.2(\pm 0.8) \times 10^6$ was calculated for Fraction DI_1 in 0.2 M NaCl from $[\eta]$ and s^o by the relation of Mandelkern and Flory (12). In this calculation their constant β was taken to be $2.5\ (\pm 0.4) \times 10^6$, where the rather large limits of error assigned to β reflect uncertainty in the appropriate experimental value of this constant for proteoglycan (6). Since equilibrium calculations are based on numbers of moles per unit volume, the number-average molecular weight $\bar{M}_n$ should be used, while the above calculation gives more nearly the weight-average value $\bar{M}_r$. The ratio $\bar{M}_r/\bar{M}_n \approx 1.2$ may be estimated from fractionation data for a similar preparation (6), provided the assumption is made that sharp fractions were obtained. This estimate gives $\bar{M}_n = 2.7\ (\pm 0.8) \times 10^6$. The equilibrium data presented below provide an approximate independent check of this result.

Equilibrium Dialysis Data. The data were treated in the form of the familiar plot suggested by Scatchard (13). The number ν of oligosaccharides complexed per proteoglycan was calculated from $\nu = (H_o - H)/P_o$ and plotted against ν/H as shown in Fig. 1. Within experimental error plots of this type were linear for HA_{10}, HA_{12} and HA_{14}, while the lower oligosaccharides gave ν values only to about 0.1 in these experiments, and no significant variation in ν/H was observed. The linear plot of Fig. 1 corresponds to the equation

$$\nu/H = K(n - \nu) \qquad (1)$$

where K is a site association constant and n the number of binding sites per

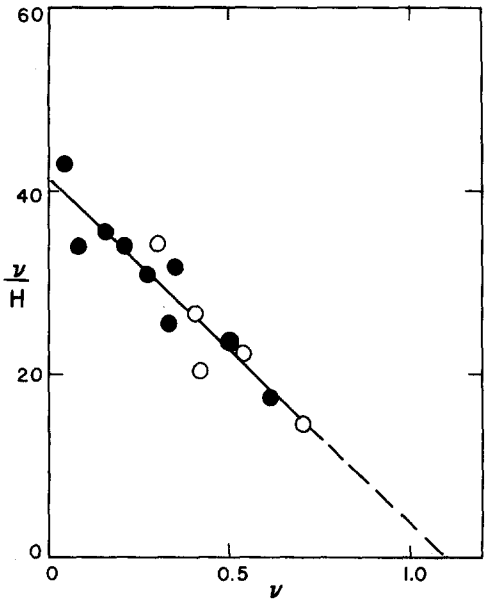

Fig. 1. Equilibrium data at 10°C in the form of a Scatchard plot for the HA_{12}-proteoglycan complex in 0.2 M NaCl, 0.03 M phosphate buffer, pH 6.5 for two equilibrium concentrations of proteoglycan: ○, 0.073±0.03 μM; ●, 0.18±0.01 μM. The solid line represents the least-squares fit to the data at the higher proteoglycan concentration.

proteoglycan molecule, when these sites are assumed to be equivalent (13). The values of K×n corresponding to average values of ν/H at $\nu < 0.1$ for the weakly-binding oligosaccharides (HA_4 to HA_8) and values of both K and n from least-squares fits of data for strongly-binding oligosaccharides (HA_{10} to HA_{14}) are listed in Table I.

The most striking aspect of these data is the abrupt increase in binding strength at HA_{10}. This result exactly parallels the observed dissociating effects of these oligosaccharides on complexes of proteoglycan with hyaluronic acid of high molecular weight, as observed by viscometry and gel chromatography (1,2). The dramatic effect of the strongly-binding oligosaccharides in the latter experiments undoubtedly reflects the displacement of macromolecular hyaluronic acid from the complex by oligosaccharide due to the competitive equilibrium under the conditions used.

TABLE I

Complexation of Hyaluronic Acid Oligosaccharides with Proteoglycan

Oligosaccharide	P_o (μM)	Kn (μM^{-1})	K (μM^{-1})	n
HA_4	0.075-0.10	1.7 (±0.3)		
HA_6	0.069-0.075	1.0 (±0.2)		
HA_8	0.070-0.076	1.3 (±0.3)		
HA_{10}	0.070-0.078		59 (±6)	0.9 (±0.1)
HA_{12}	0.070-0.076		43 (±13)	1.0 (±0.3)
HA_{12}	0.17-0.20		38 (±6)	1.1 (±0.2)
HA_{14}	0.08-0.90		59 (±12)	0.9 (±0.2)

Data refer to solvent conditions: 0.2 M NaCl, 0.03 M phosphate buffer, pH 6.5 and to 10°C. Except for HA_{14}, data represent isotherms at essentially a single value of P_o, as shown. Error limits are estimated standard deviations for average values of Kn (HA_4 to HA_8) and least-squares linear fits of Scatchard plots (HA_{10} to HA_{14}).

Stoichiometry. The experimental value of n in Table I is about 1 for each of the strongly-binding oligosaccharides, which strongly suggests that a 1:1 complex is formed between proteoglycan and these oligosaccharides. The reasonable agreement of this result with the stoichiometry expected, provided one binding site per proteoglycan molecule is assumed, furnishes independent support for the value of $\overline{M}_n$ used. Estimates of n were also obtained from preliminary experiments at nearly saturating oligosaccharide concentrations. When H was about 6 μM, and from 2 to 10 times P_o, experimental values of ν for HA_{10} and HA_{12}, corrected to saturating conditions, gave n of 0.85±0.05 and 0.8±0.1, respectively, where error limits do not allow for probable error in the $\overline{M}_n$ value. Similar experiments for the weakly-binding oligosaccharides suggest that n may be greater than one in those cases. However, contamination with only a small percentage of strong-binding oligosaccharide would also lead to this apparent result, so that the data are not decisive on this point. In fact, the binding observed for the weakly-binding oligomers may conceivably be due entirely to such an effect, although this seems improbable. It should also

be noted that $n < 1$ is suggested by gel chromatographic data for whole proteoglycan (1) and cores (2) complexed with hyaluronic acid of high molecular weight. The tissue extraction method used in this work should minimize the content of proteoglycan lacking binding sites, however.

Equilibrium Constant. The numerical values of K reported in Table I must be regarded as semiquantitative. This is due, in part, to the uncertainties discussed above in the "effective" site concentration nP_o of proteoglycan and to possible contamination of oligosaccharide fractions, especially of weakly-binding fractions with oligosaccharides of strong-binding character and vice versa. Experiments with HA_{12}, for example, at low P_o (0.01 to 0.05 μM) and low H (< 0.005 μM) tended to give values of ν/H higher than those shown in Fig. 1. A qualitative explanation of such behavior is provided by the assumption of some contamination of this fraction with weakly-binding oligosaccharides. In this case a higher proportion of the unbound oligosaccharides (observed as H) would be weakly binding at high P_o than at low, thus leading to lower apparent K values at high P_o. Further data are required to establish more accurate values of the binding constants.

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