Self-Association of Proteoglycan Subunits from Pig Laryngeal Cartilage

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Proteoglycans from pig laryngeal cartilage prepared by dissociative extraction in guanidine hydrochloride were studied in dilute solution by light-scattering and ultracentrifugation. In buffered 150 mM-NaCl, pH 7.4, the proteoglycan particle weights were about 5×10^6 daltons, but at 100 mM- and 300 mM-NaCl particle weights of 2.5×10^6 – 3.0×10^6 daltons were observed. These results, together with corroborative evidence from sedimentation-velocity experiments, were interpreted in terms of proteoglycans self-associating at physiological ionic strength. The data were examined by using a proteoglycan monomer–dimer model. Proteoglycan preparations that had thiol groups partially carboxymethylated gave particle weights of 3.2×10^6 – 3.5×10^6 daltons in 150 mM-NaCl, which suggested that carboxymethylation inhibited multimerization and hence that the protein core is implicated in the binding site. Further studies showed that the multimers were stable to 60°C, unlike the hyaluronate–proteoglycan complex.

Proteoglycans of cartilage are polydisperse and in any given cartilage exhibit a range of chemical composition and molecular size. The principal variation is in the ratio of chondroitin sulphate/protein, which generally increases with molecular size (for review see Muir & Hardingham, 1975). Proteoglycans of cartilage, compared with those of other tissues, appear to be unique in forming multi-molecular aggregates (Hascall & Sajdera, 1969) in which many proteoglycan molecules are bound to a chain of hyaluronate (Hardingham & Muir, 1972, 1973, 1974; Hascall & Heinegård, 1974*a,b*). The binding to hyaluronic acid involves a single specific site on the proteoglycan that has a high affinity for a decasaccharide unit of hyaluronic acid (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). This region, which lacks chondroitin sulphate, has been estimated to have a molecular weight of about 60000 (Heinegård, 1977) and has been isolated by mild proteolysis of aggregates (Hascall & Heinegård, 1974*a*; Heinegård & Hascall, 1974) and by CNBr cleavage of monomeric proteoglycans (Heinegård, 1977). Binding to hyaluronate is dependent on intramolecular disulphide bridges and on direct subsite interactions of arginine and ϵ -amino groups of lysine residues, and indirectly on tryptophan residues (Hardingham *et al.*, 1976).

When proteoglycans are fractionated according to buoyant density in CsCl under dissociative conditions in the presence of 4M-guanidine hydrochloride (Hascall & Sajdera, 1969), molecules of progressively decreasing size and buoyant density are distributed

up the gradient (Hardingham & Muir, 1974; Thyberg *et al.*, 1975; Hardingham *et al.*, 1976; Heinegård, 1977), although proteoglycans throughout the gradient are capable of binding to hyaluronate. It has been proposed (Heinegård & Hascall, 1974; Heinegård, 1977) that the hyaluronate-binding region forms an invariant part of the core protein that is present in molecules of all sizes, whereas the region bearing chondroitin sulphate chains is variable in length.

In the present experiments, a relatively homodisperse fraction was obtained from the polydisperse dissociated proteoglycans. By repeating the CsCl-density-gradient centrifugation at a higher starting density (Hardingham *et al.*, 1976) and taking the fraction of greatest density, the largest proteoglycans of high chondroitin sulphate content were selected.

Experimental

Materials

Proteoglycans were prepared from fresh pig laryngeal cartilage as described in detail by Hardingham *et al.* (1976), except that proteinase inhibitors were used (Oegema *et al.*, 1975) during the extraction, dialysis and in the associative density gradient. The second CsCl density gradient was run in 0.5M-guanidine hydrochloride and the fraction taken for the present experiments was equivalent to fraction D1-1 (Hardingham *et al.*, 1976) of density 1.9 g/ml.

Proteoglycan concentrations were determined from uronic acid contents determined by an automated procedure (Heinegård, 1973) of the modified (Bitter & Muir, 1962) carbazole reaction (Dische, 1947). Samples in 0.5M-guanidine hydrochloride, were dispersed in separate bottles, each lot containing about 20mg of uronic acid, and were kept frozen at -20°C until required. Each sample tested was thus frozen and thawed only once.

To prepare carboxymethylated proteoglycans the proteoglycan stock solution was dialysed free of guanidine hydrochloride against 0.2M-sodium phosphate, pH 7.4, and then a 10-fold molar excess of iodoacetic acid was added to the proteoglycan solution, which was incubated in the dark for 16h, and then dialysed free of excess reagent against 150mM-NaCl/1mM-sodium phosphate/1mM-EGTA, pH 7.4.

Light-scattering

Buffers for light-scattering were made up from de-ionized water with NaCl as detailed below and, initially, 5mM-sodium phosphate, pH 7.4, with a trace of sodium azide. In early experiments those solutions that contained proteoglycan showed a slow precipitation after about 24h, which confused experimentation. This effect was attributed to calcium phosphate precipitation, and subsequent buffers contained NaCl/1mM-sodium phosphate/1mM-EGTA and a trace of sodium azide at pH 7.4.

All buffers were filtered through a Buchner funnel with $1.0\mu\text{m}$ pore size and the proteoglycan stock was dialysed free of guanidine hydrochloride against three changes of buffer. The refractive-index increment of this proteoglycan stock versus the buffer was determined with a Brice-Phoenix differential refractometer (Phoenix Division of VirTis, Gardiner, NY 12525, U.S.A.). Serial dilution of this stock yielded the solutions that were each dialysed with stirring against the buffer to achieve Donnan equilibrium. The solutions and buffer were further purified from dust by centrifugation at 20000g for 1h before being pipetted into the light-scattering cells.

All light-scattering cells and the wide-bore pipettes used were extensively washed and then cleaned in refluxing acetone and housed in a dust-free cabinet in which the cells were loaded.

The intensity of light scattered at angles between 30° and 150° was determined with a Sofica model 42000 photo-gonio-diffusometer (Société Française d'Instruments de Contrôle et d'Analyses, 78 Le Mesnil-Saint-Denis, France), with a light-source of 436nm wavelength. Calibration of the instrument was performed with re-distilled AnalaR benzene with a Rayleigh ratio of $45.6 \times 10^6 \text{ cm}^{-1}$, and additional calibration used the polymer solid standard provided with the instrument. The data were evaluated by the

reflection correction outlined by Tomimatsu *et al.* (1968).

Linear least-squares analysis yielded the extrapolates to $c = 0$ and $\theta = 0$; the latter values being obtained from the data at 30° , 34° and 37.5° . Radii of gyration were calculated from the slope of the $c = 0$ line by using the data at 30° , 34° and 37.5° .

Ultracentrifugation

Sedimentation rate measurements were performed in an MSE Centriscan 75 ultracentrifuge. Results were obtained from the same stock solutions of proteoglycan at 150mM- and 300mM-NaCl as were used in the preparation of the light-scattering solutions.

Results

Proteoglycan behaviour at different ionic strengths

The behaviour of proteoglycans in solution was studied as a function of ionic strength to determine conditions for ideality.

I 0.10. A light-scattering study of the proteoglycan in 100mM-NaCl/1mM-sodium phosphate/1mM-EGTA with a trace of sodium azide at pH 7.4 yielded a weight-average molecular weight, $\bar{M}_w$, of 2.7×10^6 and a radius of gyration, R_G , of 88 nm (see Table 1).

I 0.15. In a similar buffer, but at 150mM-NaCl, the Zimm plot shown in Fig. 1 was obtained. Three separate experiments were performed and yielded $\bar{M}_w = 4.5 \times 10^6$, 4.6×10^6 and 5.8×10^6 with $R_G = 130\text{nm}$. The solution concentrations were in the range 50–250 $\mu\text{g/ml}$ and $(\Delta n/\Delta c)_{436} = 0.146 \text{ ml/g}$.

Proteoglycan solutions (1.60mg/ml) in the same buffer were examined in the ultracentrifuge in a sedimentation-velocity experiment at 50000rev./min and 20°C and the concentration profile obtained, shown in Fig. 2(a), has a bimodal distribution with the majority of the material (approx. 65%) in the leading peak. The leading peak was interpreted as

Table 1. Light-scattering data from proteoglycan at various ionic strengths

Sample	[NaCl] (mM)	$10^{-6} \times \bar{M}_w$ (daltons)	R_G (nm)
Proteoglycan subunit	100	2.7	88
	150	4.5	—
	150	4.6	132
	150	5.8	129
	200	2.8	96
	300	2.5	88
	300	2.7	68
Carboxymethylated proteoglycan subunit	300	3.0	—
	150	3.2	—
	150	3.5	147

Fig. 1. Zimm plot of the light-scattering data from proteoglycan solutions at concentrations of 50, 100, 150, 200 and 250 $\mu\text{g/ml}$ in 150 mM-NaCl/1 mM-phosphate/1 mM-EGTA with a trace of sodium azide at pH 7.4. The optical constant $K = 3.47 \times 10^{-7} \text{ ml}^2 \cdot \text{g}^{-2} \cdot \text{cm}^{-4}$.

Fig. 2. Sedimentation-velocity experiments in the ultracentrifuge demonstrating concentration profile after (a) 16 min of proteoglycan (1.60 mg/ml) in 150 mM-NaCl/1 mM-phosphate/1 mM-EGTA with a trace of sodium azide at pH 7.4 and (b) 42 min of proteoglycan (2.57 mg/ml) in 300 mM-NaCl/1 mM-phosphate/1 mM-EGTA with a trace of sodium azide at pH 7.4.

Both were run at 50 000 rev./min and 20°C.

corresponding to proteoglycan multimer and the trailing peak to monomer.

I 0.20. At 200 mM-NaCl, the light-scattering results gave $\bar{M}_w = 2.8 \times 10^6$ and $R_G = 96 \text{ nm}$ (see Table 1).

I 0.30. For 300 mM-NaCl the Zimm plot is shown in Fig. 3 and yields $\bar{M}_w = 2.5 \times 10^6$ and $R_G = 88 \text{ nm}$ [the refractive index increment $(\Delta n/\Delta c)_{436} = 0.145 \text{ ml/g}$]. This particle weight is very similar to the 2.3×10^6 obtained by Pasternack *et al.* (1974) on proteoglycans extracted from bovine nasal septum. Proteoglycan solutions (2.57 mg/ml) were also examined in the ultracentrifuge in sedimentation-velocity mode at 50 000 rev./min and 20°C (see Fig. 2b), and again a bimodal distribution is observed in the concentration profile, indicating that only approx. 15% of the material is in the leading peak. Hence at 300 mM-NaCl the majority of the preparation is in monomer form.

Two further experiments were performed to study the nature of the multimer. First, with the 150 mM-NaCl buffer, which most favoured multimer, the solutions were gradually heated up to 60°C in the light-scattering instrument, but no change in scattering was observed. This effect is in sharp contrast with that observed on the hyaluronate-proteoglycan complex in the same solvent conditions. The latter complex is completely dissociated at 60°C (see also Hardingham & Muir, 1974).

Some proteoglycan was also treated with sodium iodoacetate to partially carboxymethylate any thiol groups, and this preparation was then studied in 150 mM-NaCl buffered to pH 7.4. The Zimm plot of this material is shown in Fig. 4 and yields $\bar{M}_w = 3.5 \times 10^6$ and $R_G = 147 \text{ nm}$. Comparing this molecular weight with that obtained for native proteoglycan in 150 mM-NaCl, it is clear that carboxymethylation significantly decreases the ability to multimerize.

Discussion

The variation in particle weight in proteoglycan solutions as a function of ionic strength can only

Fig. 3. Zimm plot of the light-scattering data from proteoglycan solutions at concentrations of 50, 100, 150, 200 and 250 $\mu\text{g/ml}$ in 300 mM-NaCl/1 mM-phosphate/1 mM-EGTA with a trace of sodium azide at pH 7.4. The optical constant $K = 3.43 \times 10^{-7} \text{ ml}^2 \cdot \text{g}^{-2} \cdot \text{cm}^{-4}$.

Fig. 4. Zimm plot of the light-scattering data from carboxymethylated proteoglycan at concentrations of 50, 100, 150, 200 and 250 $\mu\text{g/ml}$ in 150 mM-NaCl/5 mM-phosphate with a trace of azide at pH 7.4. The optical constant $K = 3.47 \times 10^{-7} \text{ ml}^2 \cdot \text{g}^{-2} \cdot \text{cm}^{-4}$.

be interpreted in terms of self-association of proteoglycan subunits. But what is the size of the multimer or multimers formed? Extrapolated particle weights obtained by light-scattering for self-associating systems depend on both the pro-

portional concentrations and particle weights of the component species so this technique does not uniquely specify the multimer size. The ultracentrifugal data (1.6 mg/ml of proteoglycan) in 150 mM-NaCl show both that the majority of the material is in the

multimeric form and that the two peak breadths are similar. The latter indicates that only a small distribution of particle weights is present in the multimer and thus that a preferred multimer size exists. The light-scattering data in 150mM-NaCl at proteoglycan concentrations between 50 and 250 $\mu\text{g/ml}$ extrapolate without significant deviation from linearity and thus with little apparent dilution of the multimeric species to particle weights corresponding to a dimer. Since light-scattering yields a weight-average particle weight it emphasizes the largest species present, so that if the majority of the material is in the multimeric form and yet the observed particle weight only corresponds to a dimer it is apparent that the preferred multimer size is small, with dimer as the most probable multimer.

Rigorous evaluation of light-scattering data depends on extrapolation to infinite dilution, but dilution of a system in equilibrium changes the proportions of individual components. Nevertheless, examination of the $\theta \rightarrow 0$ line of the Zimm plot for proteoglycan concentrations in the range 50–250 $\mu\text{g/ml}$ shows no deviation from linearity, which indicates that at these concentrations the multimer is not significantly dissociated. If these data are evaluated by using a monomer–dimer hypothesis the apparent dissociation constant K_D must be $<10\text{nm}$. The fact that two sedimenting peaks are observed in the ultracentrifuge experiments in 150mM-NaCl would indicate that the equilibrium between a monomer and a dimer is attained slowly. This finding is consistent with the strong binding reflected in the dissociation constant above.

It is noteworthy that the ability to multimerize is optimum at physiological ionic strength ($I 0.15$) and it seems probable that lower ionic strength ($I 0.10$) results in severe repulsions between the charged glycan chains on the proteoglycans, whereas higher ionic strengths inhibit the binding at the protein site(s). Since carboxymethylation of thiol groups inhibits multimer formation it is clear that the protein core is implicated in at least one of the proteoglycans. If, as seems probable, dimer is the highest multimer formed, then it is clear that a protein site is involved on both of the interacting monomers.

The binding of proteoglycan to proteoglycan parallels that of proteoglycan to hyaluronate, in that carboxymethylation of the thiol groups on the protein core inhibits association, although the proteoglycan–hyaluronate interaction is only inhibited after both reduction and carboxymethylation (Hardingham *et al.*, 1976), suggesting perhaps that similar sites are involved; however, the former system is quite stable to 60°C , whereas the latter dissociates.

It is noteworthy that the radius of gyration for the carboxymethylated proteoglycan is larger than that observed for multimer. This is interpreted as

indicating a substantial unfolding of the protein ‘foot’, and indicates considerable structural re-arrangement in this zone.

The results suggest that at physiological ionic strength proteoglycans and hyaluronate may compete for the protein sites on the proteoglycan core. It is important to note that the observation of multimers in the ultracentrifuge is facilitated by having homodisperse proteoglycan preparations.

Conclusion

The particle weights of fractionated proteoglycan in solution at pH 7.4 were examined by light-scattering as a function of ionic strength. In 150mM-NaCl, the particle weights were about 5×10^6 , but at 100mM-, 200mM- and 300mM-NaCl particle weights of 2.5×10^6 – 3.0×10^6 daltons were observed. These results, together with corroborative evidence from sedimentation-velocity experiments, were interpreted in terms of proteoglycans self-associating to form multimers that occur at physiological ionic strength.

Proteoglycan preparations that had thiol groups partially carboxymethylated gave particle weights of 3.2×10^6 – 3.5×10^6 daltons in 150mM-NaCl, which suggested that carboxymethylation inhibited multimerization and hence that the protein core is implicated in the binding site. Further studies showed that the multimer was stable to 60°C , unlike the hyaluronate–proteoglycan complex.

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