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Dermatan sulphate proteoglycans from sclera examined by rotary shadowing and electron microscopy

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Two dermatan sulphate-containing proteoglycans from bovine sclera were examined by rotary shadowing and electron microscopy, and the results were compared with previous biochemical findings. Both the large iduronate-poor proteoglycan (PGI) and the small iduronate-rich proteoglycan (PGII) possessed a globular proteinaceous region. Whereas PGI had a branched extension from the globular region, with five to eight side chains attached to it, PGII had only a single tail, which was of glycosaminoglycuronan. PGII aggregated via globular-region interactions, which were much diminished by reduction and alkylation. PGI aggregated via side chains and globular-region interactions. Although a few PGI aggregates were large, and similar to the hyaluronan–cartilage proteoglycan aggregates [Weidemann, Paulsson, Timpl, Engel & Heinegård (1984) Biochem. J. 224, 331–333], hyaluronan did not cause enhanced aggregation. PGII is very similar in shape to the small cartilage chondroitin sulphate proteoglycan, whereas PGI somewhat resembles the large cartilage chondroitin sulphate proteoglycan, although with many fewer glycosaminoglycan side chains, and probably only one globular region as opposed to two in the cartilage proteoglycan.

INTRODUCTION

Multicelled organisms are held together by tissues which sustain mechanical stress of a kind which could cause disaggregation, e.g. during active movement. These tissues, the connective tissues, are made up of fibrils, mainly collagen and elastin, that resist tensile forces. The fibrils are surrounded by a gel or concentrated sol, structured by the presence of water-soluble protein–carbohydrate polymers, the proteoglycans (Scott, 1974, 1986; Scott & Hughes, 1986). Proteoglycans consist of a polypeptide 'core', with up to 100 glycosaminoglycan (GAG) side chains comprised of repeating disaccharide units (see, e.g., Poole, 1986, for a review).

There are three families of GAG polymers, the chondroitins (including the dermatans), the keratans and the heparans, each of which contains, in principle, a very large number of possible GAG chains. Although one can talk of 'typical' chondroitin 4- or 6-sulphate, in which the main heterogeneity is variable chain length, and isolate the substance from cartilage, it is difficult to consider the corresponding dermatan sulphate, in which both iduronate and glucuronate are present, as a 'typical' structure. The ratio of glucuronate to iduronate varies from tissue to tissue, and within the tissue. The same is true of the heparans.

Recent work on the structure of proteoglycans has brought some simplification, to the chondroitin–dermatan group at least. There is usually an inverse correlation between the size of the proteoglycan (largely determined by the number of GAG side chains attached to the protein core) and the iduronate/glucuronate ratio in the side chains. Thus the 'large' cartilage proteoglycan, M_r (1–2) $\times 10^6$ (up to 100 side chains) contains no measurable iduronate, whereas the 'small' dermatan sulphate proteoglycans (one or two side chains) of M_r $\sim 10^5$ from skin, tendon etc. may contain up to 90% of their hexuronate as iduronate. An intermediate size of proteoglycan, of M_r (2–3) $\times 10^5$ (five to ten side chains) contains $\sim$ 10% iduronate (Cöster *et al.*, 1979). The protein content of the large cartilage proteoglycans is much less ($\sim$ 10%) than that of the small proteoglycans (30–50%), reflecting the greater number of polysaccharide chains in the former.

Similarly, a pattern has begun to emerge from studies of the ultrastructural localization of proteoglycans in tissues (Scott & Haigh, 1985*b*). The larger chondroitin sulphate-rich proteoglycans are present in the interfibrillar space, with the probable function of keeping the fibrils apart, which in the case of cartilage endows the tissue with elasticity (Scott, 1974). As yet, no specific interaction with fibrils in the tissue has been unequivocally demonstrated. The small dermatan proteoglycans, on the other hand, are found specifically located at the gap zone ('d' and 'e' bands) of collagen fibrils (Scott & Orford, 1981; Scott & Haigh, 1985*a*). The implications of this specific interaction to biological events of fundamental importance, such as fibril growth and calcification, have been discussed (Scott & Haigh, 1985*a*). The mechanism of this interaction in the tissue is not known, but available evidence suggests that proteoglycan-protein-to-collagen binding is important (Scott & Orford, 1981), and a similar mechanism may account for the specific binding of the small protein-rich keratan sulphate proteoglycan to the 'a' and 'c' bands of corneal collagen fibrils (Scott & Haigh, 1985*b*). It is thus important to know the broad details of the shape and structure of dermatan sulphate proteoglycans in order to

Abbreviations used: PGI, large iduronate-poor, glucuronate-rich, proteoglycan; PGII, small iduronate-rich proteoglycan; GAG, glycosaminoglycan.

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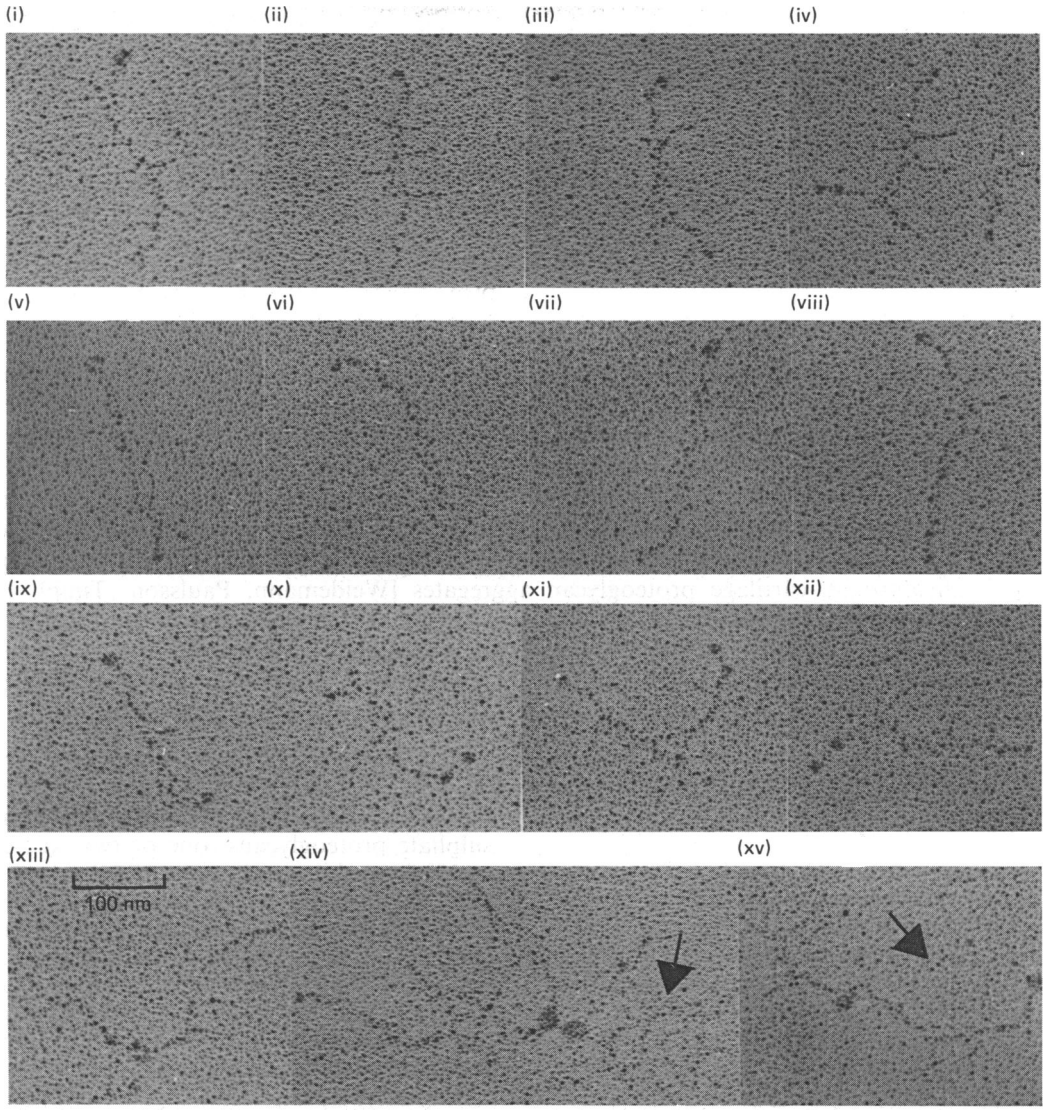

Fig. 1. Rotary-shadowed images of the large scleral proteoglycan (PGI), showing aggregates of increasing size from (i) to (xv)

(i) and (ii) are typical, showing a single globular head, with a single central extension, and about eight side chains at right angles to this extension. (ix) Shows a similarly sized 'molecule', but with two globular regions, one at each end of the 'molecule'. These may be dimers of (i)-type molecules. The globules and central extension are probably proteinaceous, whereas the side chains are GAG (see the text). The aggregates in (xiii)–(xv) show larger globular regions, probably attributable to the association of several monomer globules. (xiv) and (xv) show side-chain–side-chain interactions (arrowed).

assess the potential for interaction with other molecules and tissue elements, and perhaps to identify the regions of the molecules which are responsible for their specific interactions. The technique of rotary shadowing, followed by electron microscopy, has had success in delineating the structures of biopolymers (Furthmayr & Madri, 1982; Wiedemann *et al.*, 1984; Mould *et al.*, 1985). In the present paper we describe results obtained with the technique on the large and small dermatan sulphate proteoglycans from bovine sclera.

EXPERIMENTAL

Isolation and properties of dermatan sulphate proteoglycans

Dermatan sulphate proteoglycans were extracted from bovine sclera by the use of 4 M-guanidinium chloride supplemented with proteinase inhibitors. Density-gradient centrifugation in CsCl/4 M-guanidinium chloride, gel chromatography, ion-exchange chromatography and density-gradient centrifugation in Cs_2SO_4 separated them into two species (Cöster & Fransson, 1981; Sheehan *et al.*, 1981): one large glucuronic acid-rich (PGI) and one small iduronic acid-rich (PGII). The large proteoglycan (PGI) had an M_r of 200000–400000, a large core protein and five to seven galactosaminoglycan side chains, whereas the small proteoglycan (PGII) had an M_r of 89000, a small core protein of M_r 46000 and one or two galactosaminoglycan side chains (Cöster *et al.*, 1981).

PGII protein core

This was prepared by treating PGII with HF in pyridine by the method of Mort & Lamport (1977). Similar experiments were not performed on PGI because

of shortage of material. Reduction and alkylation of PGI and PGII was carried out as described by Cöster *et al.* (1981).

Dermatan sulphate and hyaluronan

These were prepared from pig skin and from mesothelioma fluid respectively by the method of Scott (1960).

Electron microscopy

Proteoglycan samples were dissolved in phosphate-buffered saline (18.4 mM-Na_2HPO_4/11.6 mM-KH_2PO_4/142 mM-NaCl/4 mM NaOH) at a temperature of 22 °C and at a concentration of 50 μg/ml. This concentration was decreased to 25 μg/ml by adding an equal volume of 10 mM-acetic acid, giving a final pH of 7.4. Samples were mixed with glycerol to give a final glycerol concentration of 70% (w/w). A 10 μl drop of sample was then spread between two freshly-cleaved mica sheets (ruby mica from Mica and Micanite Supplies, London N.1, U.K.) each of area 6.5 cm^2 (Mould *et al.*, 1985; Ward *et al.*, 1986). The sheets were separated after 10 min, dried under vacuum for 1 h and rotary-shadowed with platinum/tungsten (Agar Aids, Stansted, Essex, U.K.) at a glancing angle of 5° before coating with carbon at 90° from a carbon-fibre evaporation source. The mica pieces were left in a desiccator over aq. 10% (v/v) acetic acid for 1.5 h (a step which helped subsequent removal of the replicas). The replicas were then floated on to double-distilled water, picked up on 400-mesh grids, blotted, and left to dry. Electron microscopy was performed on a JEOL JEM 100B instrument. Micrographs were taken at magnifications in the range 15000–40000× and calibrated by using a replica line grating with 2160 lines/mm.

RESULTS

PGI, the 'large' proteoglycan

Fig. 1 shows a series of images in which the proteoglycan is seen in increasingly large aggregates. The simplest forms, presumably the monomers, show a central filament with side branches and a clearly discernible globular 'head'. Dermatan sulphate chains isolated from skin after papain digestion appeared as rods of similar length to those of the side chains. Assuming that the side chains are glycosaminoglycuronans, their length (~ 40 nm) corresponds to an M_r of about 20000. By analogy to work on the cartilage proteoglycans (Wiedemann *et al.*, 1984) and that on the small PGII (see below), the globular regions are assumed to be protein. Dimers and higher aggregates are commonly seen. It is possible that some of the 'monomeric' forms which appear to contain two globular regions are dimers. There are indications that this can arise from both head-to-head and head-to-tail interaction (Fig. 1, ix). Fig. 2 shows a very large aggregate with over 20 monomers participating, based on the number of globular regions present. The number of such aggregates did not increase on further addition of hyaluronan to this preparation. After reduction and

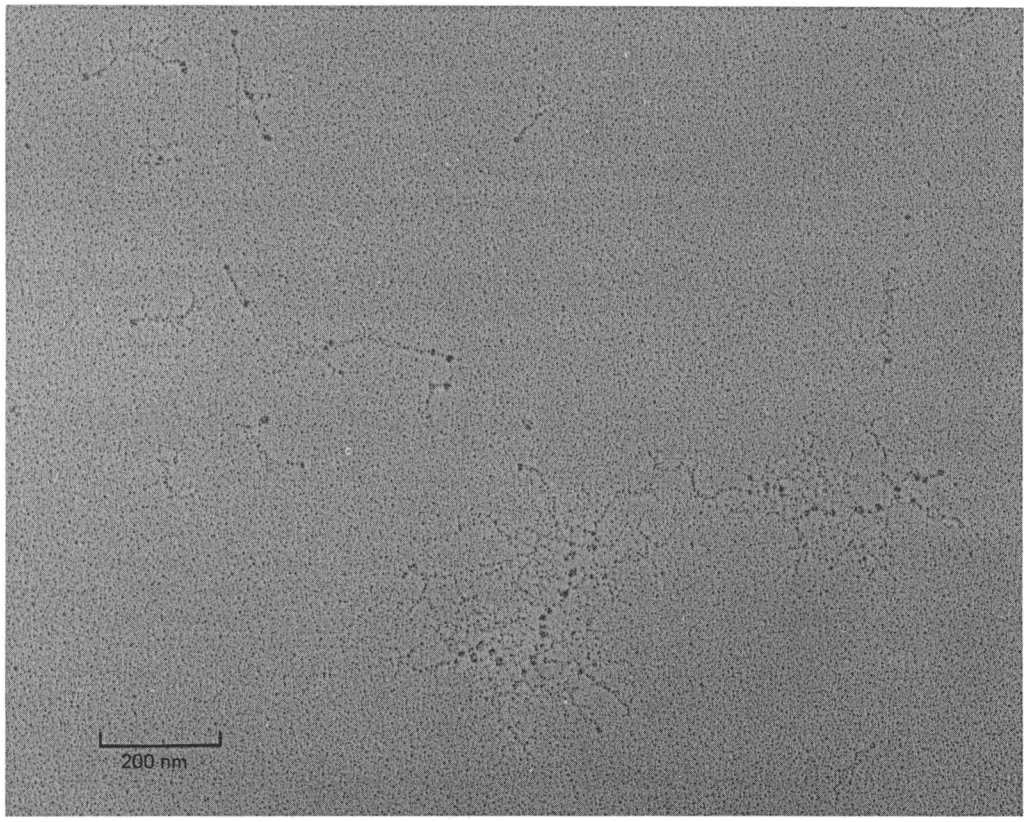

Fig. 2. Large aggregate of PGI, with globular regions close together, but not always in contact

Side chains clearly interlock. Some monomers and dimers are visible (top left-hand corner). This aggregate is very similar in appearance to that of the large cartilage chondroitin sulphate proteoglycan–hyaluronan complex (Weidemann *et al.*, 1984).

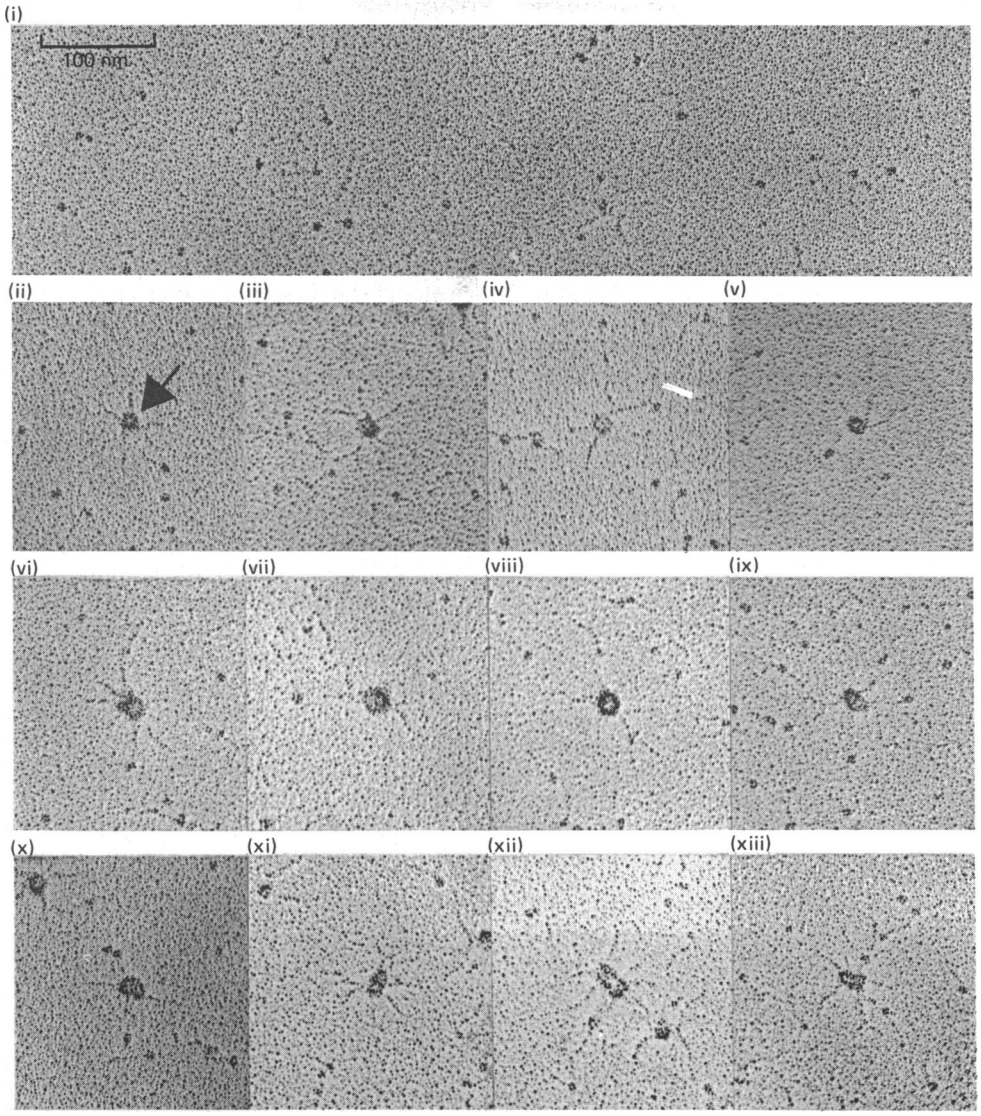

Fig. 3. Rotary-shadowed images of the small sceral proteoglycan (PGII), showing aggregates of increasing size from (i) (top row) to (xiii)

The monomers in (i) are tadpole-like, with a globular head and a single tail. The globule is proteinaceous, and the tail is GAG (see the text). The aggregates in (ii)–(xiii) are all centred around globule–globule interactions (arrowed), with the GAG side chains radiating outwards.

alkylation there was evidence of aggregation between the globular regions, but with no large aggregates of the type seen in Fig. 1. It is noteworthy that the globular head survives after reduction and alkylation.

PGII, the 'small' proteoglycan

Fig. 3 shows a series of images of increasingly large aggregates. The simplest form, presumably the monomer, consists of a single tail with a globular head. HF treatment leaves only the globular head (Fig. 4), and so the tail is assumed to be GAG. The length of the tail (maximum about 40 nm) corresponds to an M_r of about 20000. There is no central polypeptide chain as there is in PGI and also in the large cartilage chondroitin sulphate proteoglycan (Wiedemann *et al.*, 1984).

The electron micrographs show that aggregates form by protein–protein interaction, resulting in large conglomerates of globular regions with their GAG tails radiating outwards. The larger the central aggregate, the more numerous the GAG side chains. This suggests that most of the GAG side chains are not involved in the aggregation mechanism. The largest aggregates have a central region of very low electron density, indicative of low levels of metal deposition, and therefore probably of the absence of proteoglycan mass in this region. After reduction and alkylation there appeared to be many fewer head-to-head aggregates, and a large number of dumb-bell-like structures were seen, presumably dimers, in which the tails were aligned side by side, extending from head to head (Fig. 5). The globular head is still present after reduction and alkylation

DISCUSSION

Our results confirm and extend the conclusions drawn from biochemical studies on scleral dermatan sulphate proteoglycans PGI and PGII (Cöster *et al.*, 1981). M_r determination by light-scattering and ultracentrifugation

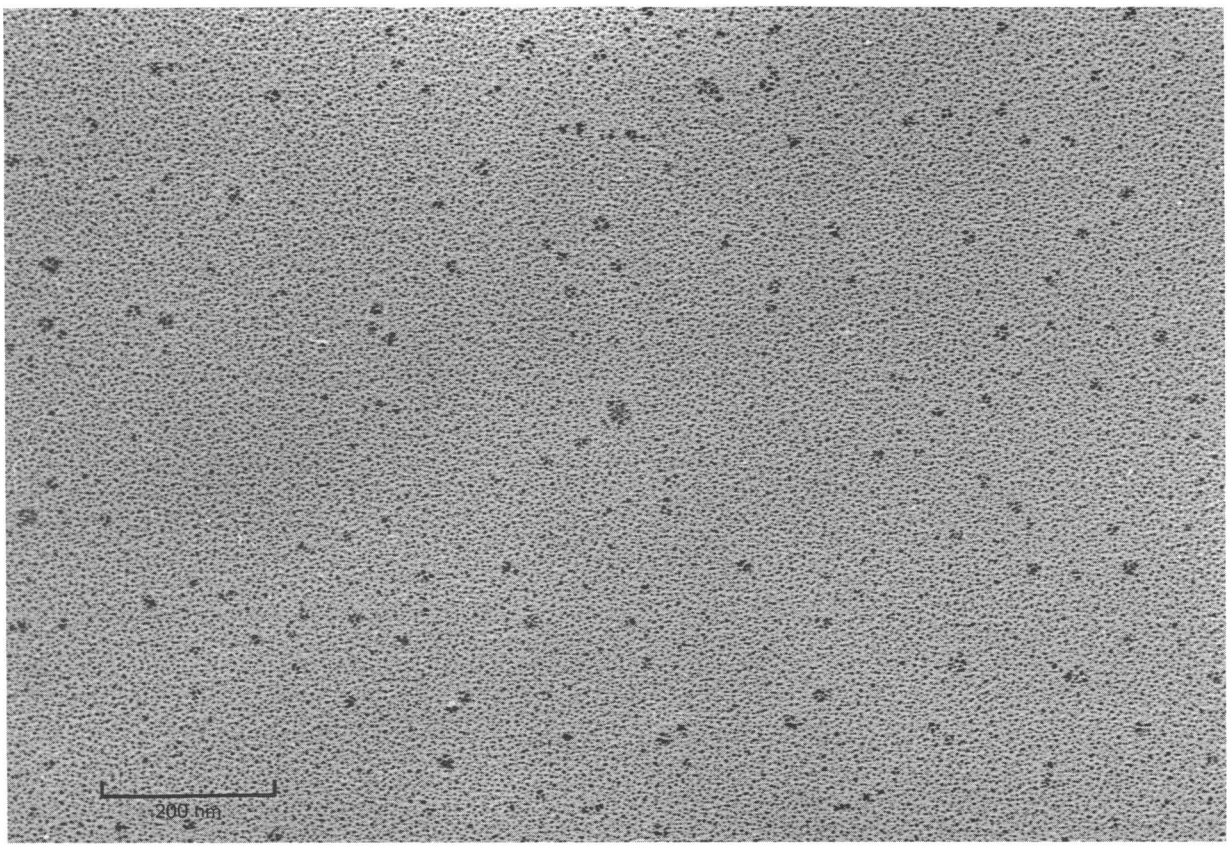

Fig. 4. Rotary-shadowed images of HF-treated PGII

The globules vary in size, suggesting that some may be aggregates of several proteinaceous globular regions. There are no tails, or tadpole-like forms, suggesting that the tails were GAG and were cleaved by HF treatment.

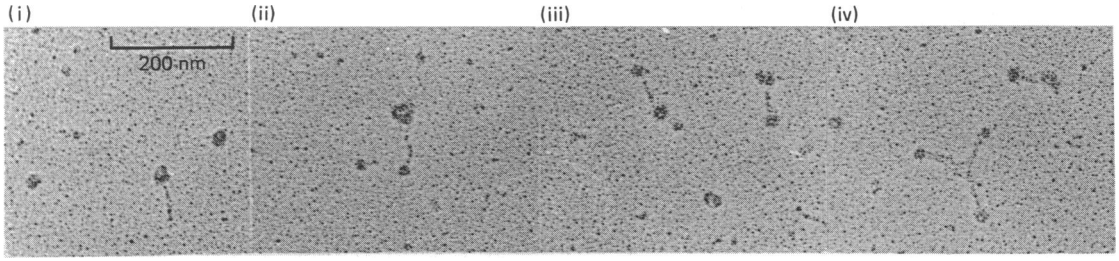

Fig. 5. Rotary-shadowed images of PGII after reduction and alkylation

(i) Shows a typical monomer with globular head and GAG tail. (ii)–(iv) show dumb-bell-shaped aggregates, with at least two, or possibly three, monomers involved, apparently via side-chain interactions. (iv) Shows a Y-shaped aggregate with at least three monomers interacting via side chains. The size of some of the globular regions suggests that more than one monomer protein core is involved at that point.

of intact proteoglycans, and those digested with chondroitinase ABC or papain, suggested that PGI and PGII had protein cores to which five to seven or one or two glycosaminoglycuronan side chains respectively were attached (Cöster & Fransson, 1981). Our electron micrographs confirm these ideas and give new information on the protein cores.

Both proteoglycans possess a globular structure with, in the case of PGII, a single unbranched extension or tail and, in the case of PGI, a branched extension. The tail of PGII was removed by treatment with HF, suggesting that the tail was of GAG and that the globule was proteinaceous. Dermatan sulphate chains isolated after papain digestion appeared as rods of similar length to those of PGII tails. PGII thus resembles the small cartilage proteoglycan (Wiedemann *et al.*, 1984). Small proteoglycans from various tissues have similar amino acid compositions and core-protein sizes and share immunological cross-reactivity (Heinegård *et al.*, 1985). By analogy to PGII and the large cartilage proteoglycan (Wiedemann *et al.*, 1984) the globule of PGI is assumed to be proteinaceous in nature, with an extension of polypeptide to which are affixed the GAG side chains. The M_r values for the GAG side chains measured by

light-scattering and ultracentrifugation (~ 25000) are similar to those estimated from the lengths of the GAG side chains in our electron micrographs.

Although PGI has an overall structure similar to that of the large cartilage proteoglycan monomer (Wiedemann *et al.*, 1984), there are far fewer GAG side chains in the former, and only one globular region, as opposed to two in the latter.

Aggregation of the proteoglycans

The rotary-shadowed proteoglycans showed clear evidence of aggregates (Fig. 1), some of which were large. The globular regions of PGII interacted with each other and the GAG side chains radiate away from the central globules. This suggests that most of the GAG chains did not participate in aggregation and that globular-protein interactions were responsible. Reduced and alkylated PGII formed dimers and some meshworks via side-chain–side-chain interactions (Fig. 5). Thus both the protein and the side chains are able to self-aggregate, but protein–protein interactions seem to be preferred in the 'native' state. Occasional images suggested that aggregation proceeds by the formation of a cylinder of monomer heads somewhat like tobacco-mosaic virus or perhaps as a stack of discs, each disc containing at least four monomers.

In spite of the presence of a hyaluronan-binding region (Heinegård *et al.*, 1985), biochemical studies tended to exclude the possibility that either PGI or PGII could aggregate with hyaluronan (Cöster *et al.*, 1981), in contrast with the behaviour of large cartilage chondroitin sulphate-rich proteoglycan. It is therefore interesting that some PGI aggregates were quite different from those of PGII and reminiscent in appearance of rotary-shadowed hyaluronan–cartilage proteoglycan complexes (Fig. 2) (Wiedemann *et al.*, 1984). The biochemical evidence is against the presence of hyaluronan in these preparations (Cöster *et al.*, 1981). When hyaluronan was added in an amount at least 1000 times greater than that which could have been in PGI before rotary shadowing, there was no observable increase in the number or size of the aggregates. We conclude that most PGI does not aggregate with hyaluronan, a finding in agreement with that from biochemical studies. However a small percentage of PGI molecules may possess the ability to combine with hyaluronan.

GAG chains from PGI self-associate, and hence PGI might itself aggregate through GAG–GAG interactions (Cöster *et al.*, 1981). However, in 1.0 M-KCl/0.02 M-EDTA solutions, self-association of GAG chains is abolished, whereas that of PGI is not (Cöster *et al.*, 1981). Reduction and alkylation prevented aggregation of PGI, suggesting that the protein core was involved (Cöster *et al.*, 1981). Rotary-shadowed PGI aggregates are equivocal on the question of globular-region–globular-region aggregation, in contrast with PGII, but the electron micrographs are compatible with protein-core–protein-core interaction, perhaps helped by additional 'lateral' GAG–GAG complexing (Fig. 2). It is clear that there is a strong tendency for the native PGI aggregate to grow lengthwise, with the globular regions linearly distributed, and somewhat separated, rather than radially, as in the case of PGII, with the globules in very close proximity or overlapped.

Light-scattering showed both the proteoglycans to have very high M_r values (PGI, 12×10^6; PGII, 39×10^6) in non-dissociating conditions (low or zero concentrations of guanidinium chloride). Some rotary-shadowed aggregates of PGI and II were indeed large, being equivalent to 20 or more monomers. However, most of both PGs were not aggregated much beyond the dimer stage under the conditions in which they were spread on to mica (iso-osmotic buffered saline/glycerol). It seems probable that the light-scattering results (Cöster *et al.*, 1981) reflect the tendency of this technique to detect larger aggregates with greater sensitivity than it detects small ones.

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