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# The core protein of fibroblast proteoheparan sulphate consists of disulphide-bonded subunits

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Fibroblast proteoheparan sulphate has a disulphide-bonded subunit structure. The core protein appears to consist of two polypeptides each of $M_r$ 80000–100000. As shown elsewhere [Carlstedt, Cöster, Malmström & Fransson (1983) *J. Biol. Chem.* in the press], both polypeptide molecules carry four to six heparan sulphate side chains (approx. $M_r$ 20000) and an unknown number of oligosaccharide units, giving the whole macromolecule an $M_r$ in the range 300000–400000.

Most animal cells synthesize proteoheparan sulphate, which is mainly deposited in the pericellular matrix. Structural characterization has been performed on cell-associated proteoheparan sulphates from hepatocytes (Oldberg *et al.*, 1979), hepatomas (Mutoh *et al.*, 1980; Oldberg *et al.*, 1982), endothelial cells (Oohira *et al.*, 1983), lung and skin fibroblasts (Vogel & Peterson, 1981; Carlstedt *et al.*, 1983), rat ovarian granulosa cells (Yanagishita & Hascall, 1983) and mouse mammary epithelial cells (Rapraeger & Bernfield, 1983) and from synaptic vesicles of cholinergic neurons (Stadler & Dowe, 1982), as well as from basement-membrane-producing cell lines (PYS-2) or sarcomas (Hassel *et al.*, 1980; Oohira *et al.*, 1982). Proteoglycans from the different sources appear to fall into three size classes: the hepatic ones with $M_r$ 70000–150000 and three or four side chains/protein core, the latter with $M_r$ less than 40000, a medium-sized one with $M_r$ 300000–400000 of widespread occurrence (epithelial, neuronal, mesenchymal and endothelial sources) and with as many as ten side chains/protein core, the latter with $M_r$ approx. 200000, and a large-sized one with $M_r$ 400000–750000 containing more than ten side chains/protein core. The last-mentioned proteoglycans are usually associated with basement membranes. Despite considerable variation in size of the core protein, common antigenic determinants have been detected even for the extreme variants. In the present study the core protein of proteoheparan sulphate from human embryonic skin fibroblasts has been examined.

## Experimental

### *Materials*

Human embryonic skin fibroblasts in culture were incubated with [$^3$H]leucine and [$^{35}$S]sulphate (Carlstedt *et al.*, 1981) for 72h, and proteoglycans were extracted from the cell monolayer with 4M-guanidinium chloride in the presence of $Na_2EDTA$, di-isopropyl phosphorofluoridate and *N*-ethylmaleimide as proteinase inhibitors. The presence of *N*-ethylmaleimide should also minimize the possibility of disulphide–thiol exchange reactions. Proteoheparan sulphate was purified by isopycnic density-gradient centrifugation in CsCl/4M-guanidinium chloride followed by gel-permeation and ion-exchange chromatography after degradation of contaminating proteodermatan sulphates with chondroitin ABC lyase in the presence of proteinase inhibitors (Carlstedt *et al.*, 1983). The final preparation represented 80% of the cell-associated heparan sulphate.

Heparan sulphate lyase (EC 4.2.2.8) from *Flavobacterium heparinum* was a produce of Seikagaku Kogyo Co., Tokyo, Japan. Proteins for calibration of gel-chromatography columns were purchased from Pharmacia, and other chemicals were obtained from sources listed previously (Carlstedt *et al.*, 1981, 1983).

### *Methods*

The heparan sulphate side chains were removed from the core protein of the proteoglycan by digestion with heparan sulphate lyase (6 munits/ml) in 3mM-calcium acetate buffer, pH 7.0, containing 0.5mg of ovomucoid (Sigma)/ml at 37°C for 4h in tubes that were pretreated with ovalbumin (0.5mg/ml). (One unit of the enzyme releases 1$\mu$mol of unsaturated hexuronate/min.)

Reduction of disulphide bonds was conducted in 4M-guanidinium chloride/10mM-dithiothreitol/50mM-sodium acetate buffer, pH 5.8, at 37°C for 5h. Alkylation was subsequently achieved by the addition of a molar excess of iodoacetamide over

dithiothreitol and by keeping the solution in the dark overnight.

Polyacrylamide-gel electrophoresis was performed on 6% gels (T = 6%; C = 2.5%) as described previously (Carlstedt *et al.*, 1981).

## Results

The proteoheparan sulphate from the cell surface of cultured fibroblasts chromatographed with a $K_{av.}$ of 0.30 on Sepharose CL-4B in 4M-guanidine/1% Triton X-100 (Fig. 1*a*), corresponding to an $M_r$ of 350000 (see Carlstedt *et al.*, 1983). After reduction and alkylation the entire population was displaced to a $K_{av.}$ of 0.42 (Fig. 1*b*), suggesting that the proteoglycan was cleaved into subunits. The core protein was obtained by treating the proteoglycan with heparan sulphate lyase followed by gel chromatography in the presence of sodium dodecyl sulphate (Figs. 2*a* and 2*b*). The $K_{av.}$ for the core protein was 0.6, which corresponds to an apparent $M_r$ of 150000. Reduction and alkylation of this material afforded one peak (Fig. 2*c*), with a $K_{av.}$ of

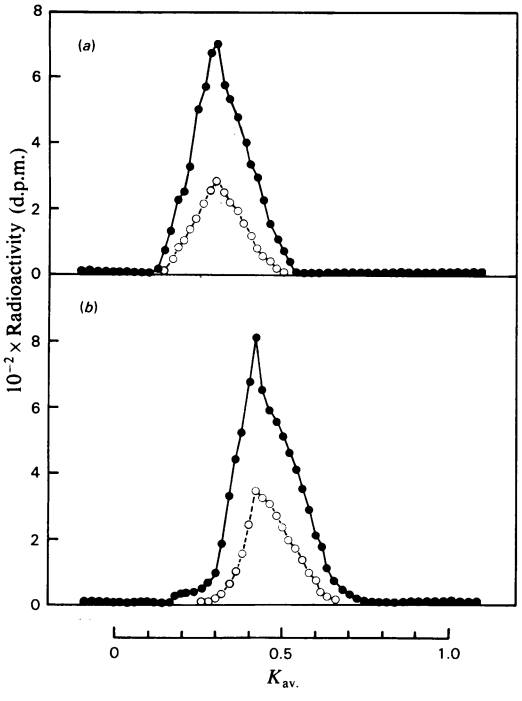


Fig. 1. *Gel chromatography of proteoheparan sulphate (a) before and (b) after reduction and alkylation*

The [$^3$H]leucine- and [$^{35}$S]sulphate-labelled proteoglycan was purified and treated as described in the Experimental section. Chromatography was performed on a column (1.8cm × 145cm) of Sepharose CL-4B eluted with 4M-guanidinium chloride/1% Triton X-100/50mM-sodium acetate buffer, pH 5.8, at a flow rate of 6ml/h. Fractions were assayed for radioactivity: ●, $^3$H; ○, $^{35}$S. As the $K_{av.}$ for a particular substance can vary between different batches of Sepharose, all experiments were performed with the same batch.

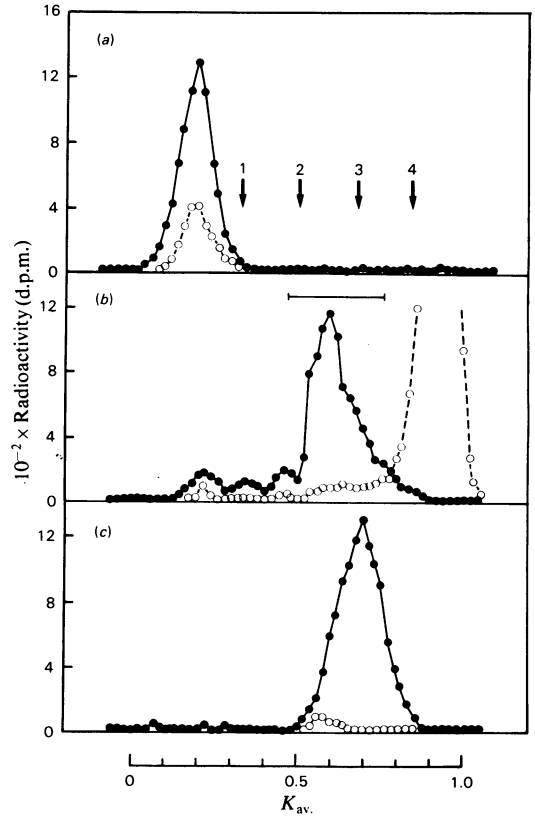


Fig. 2. *Gel chromatography of (a) intact proteoheparan sulphate, (b) the protein core obtained after treatment with heparan sulphate lyase and (c) the protein core after reduction and alkylation*

The [$^3$H]leucine- and [$^{35}$S]sulphate-labelled proteoglycan was treated with heparan sulphate lyase as described in the Experimental section. The core protein was recovered (horizontal bar in *b*) and subjected to reduction and alkylation (*c*). The column (0.6cm × 150cm) contained Sepharose CL-4B and was eluted with 1% sodium dodecyl sulphate/50mM-sodium carbonate buffer, pH 7.0. Fractions were assayed for radioactivity: ●, $^3$H; ○, $^{35}$S. The standards were: 1, thyroglobulin ($M_r$ 330000); 2, ferritin subunit ($M_r$ 220000); 3, phosphorylase *b* ($M_r$ 94000); 4, catalase subunit ($M_r$ 60000). The $K_{av.}$ values of the standards were plotted against log $M_r$ (weight-average) and a straight line was obtained for proteins 2–4. The $K_{av.}$ for the non-reduced core protein corresponded to an $M_r$ of approx. 150000, whereas the reduced and alkylated core protein gave an $M_r$ of approx. 80000.

0.7, corresponding to an apparent $M_r$ of 80000. Although highly glycosylated proteins are notoriously difficult to analyse by disc-gel electrophoresis, attempts were made to determine the $M_r$ for the core protein by electrophoresis in the presence of sodium dodecyl sulphate. Whereas the non-reduced material did not enter the gel, the reduced core protein usually gave a major component with an apparent $M_r$ of approx. 80000–100000.

As shown elsewhere (Carlstedt *et al.*, 1983), the proteoheparan sulphate isolated from the culture medium is smaller ($M_r$ 140000) and unaffected by reduction.

## Discussion

Heparan sulphate lyase treatment of proteoheparan sulphate yields a core protein molecule with eight to twelve saccharide remnants of heparan sulphate and an unknown number of glycoprotein-type oligosaccharides (Carlstedt *et al.*, 1983). This preparation had an apparent $M_r$ of 150000, as determined by gel chromatography in the presence of sodium dodecyl sulphate. Estimations of $M_r$ for the subunit obtained after reduction of disulphide bonds gave values of 80000 (gel chromatography) and 80000–100000 (disc-gel electrophoresis). We conclude that the core protein of fibroblast proteoheparan sulphate appears to consist of two subunits linked by disulphide bonds. Both subunits are substituted with heparan sulphate chains.

The macromolecular design is similar to that of the transferrin receptor, a ubiquitous membrane glycoprotein of proliferating cells (Newman *et al.*, 1982). The latter also consists of two subunits with $M_r$ 90000 joined by disulphide bonds. The transferrin receptor is considered to be the only membrane glycoprotein with a dimeric disulphide-bonded subunit composition (Bleil & Bretscher, 1982). However, proteoglycans are frequently left unnoticed, because they do not readily penetrate polyacrylamide gels during electrophoresis.

Proteoheparan sulphates from hepatocytes (Kjellén *et al.*, 1981), glial cells and fibroblasts (Norling *et al.*, 1981) as well as epithelial cells (Rapraeger & Bernfield, 1983) have been found to be membrane-associated. The proteoglycan studied in the present work is presumably also a membrane-associated form. It remains to be seen whether one or both of the core polypeptides carry hydrophobic peptide portions.

The structure of proteoheparan sulphates could conceivably be built up from a common protein subunit of $M_r$ 90000 or lower. A single subunit with three to six side chains (each of $M_r$ approx. 20000) would have an $M_r$ of 150000–210000, one with two subunits an $M_r$ of 300000–420000, and so on. The hepatic form of proteoheparan sulphate would fit into the first category, the fibroblast ones into the second and the basement-membrane forms into a higher-order structure. In addition, the larger variants of proteoheparan sulphate may carry a greater number of longer side chains.

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