Biochem. J. (1987) 247, 267–276 (Printed in Great Britain)

The detection of substructures within proteoglycan molecules

Electron-microscopic immuno-localization with the use of Protein A–gold

John K. SHEEHAN,*‡ Anthony RATCLIFFE,† Ken OATES* and Tim E. HARDINGHAM†

*Department of Biological Sciences, University of Lancaster, Bailrigg, Lancaster LA1 4YQ, U.K., and †Kennedy Institute, 6 Bute Gardens, Hammersmith, London W6 7DW, U.K.

Proteoglycan monomers from pig laryngeal cartilage were examined by electron microscopy with benzyldimethylalkylammonium chloride as the spreading agent. The proteoglycans appeared as extended molecules with a beaded structure, representing the chondroitin sulphate chains collapsed around the protein core. Often a fine filamentous tail was present at one end. Substructures within proteoglycan molecules were localized by incubation with specific antibodies followed by Protein A–gold (diameter 4 nm). After the use of an anti-(binding region) serum the Protein A–gold (typically one to three particles) bound at the extreme end of the filamentous region. A small proportion of the labelled molecules (10–15%) showed the presence of gold particles at both ends. A monoclonal antibody specific for a keratan sulphate epitope (MZ15) localized a keratan sulphate-rich region at one end of the proteoglycan, but gold particles were not observed along the extended part of the protein core. This distribution was not changed by prior chondroitin AC lyase digestion of the proteoglycan. Localization with a different monoclonal antibody to keratan sulphate (5-D-4) caused a change in the spreading behaviour of a proportion (approx. 20%) of the proteoglycan monomers that lost their beaded structure and appeared with the chondroitin sulphate chains projecting from the protein core. In these molecules the Protein A–gold localized antibody (5-D-4) along the length of the protein core whereas in those molecules with a beaded appearance it labelled only at one end. Labelling with either of the monoclonal antibodies was specific, as it was inhibited by exogenously added keratan sulphate. The differential localization achieved may reflect structural differences within the proteoglycan population involving keratan sulphate and the protein core to which it is attached. The results showed that by this technique substructures within proteoglycan molecules can be identified by Protein A–gold labelling after the use of specific monoclonal or polyclonal antibodies.

INTRODUCTION

Proteoglycans are complex glycoproteins and are important components of the extracellular matrix of connective tissue. In cartilage the major proteoglycans are of high M_r (1×10^6–4×10^6) (Hardingham, 1981) and consist of an extended protein core that has three main regions: an *N*-terminal region, which has two globular domains (Wiedemann *et al.*, 1984), one of which binds to hyaluronate (binding region) and is involved in aggregation (Paulsson *et al.*, 1987), a keratan sulphate-rich domain, and a longer extended domain, which bears all the chondroitin sulphate and also contains some interspersed keratan sulphate and neutral oligosaccharide chains. A schematic diagram of the proposed structure is shown in Fig. 1. Aggregates are formed by many proteoglycans binding to each chain of hyaluronate (Hardingham & Muir, 1972), and each proteoglycan–hyaluronate bond is stabilized by a separate link protein (M_r 40000) (Hardingham, 1979; Bonnet *et al.*, 1985).

Electron microscopy of proteoglycan monomers and aggregates has provided supporting evidence for the proposed models of these structures. Under conditions that hold the glycosaminoglycan chains extended, the proteoglycan monomer appears with a fine extended filamentous protein core, and attached to this are many closely spaced side chains of chondroitin sulphate (Rosenberg *et al.*, 1970; Wellauer *et al.*, 1972; Thyberg *et al.*, 1975; Buckwalter & Rosenberg, 1982). Proteoglycans can also be observed with the glycosaminoglycan chains collapsed on to the protein core, and monomers attached to hyaluronate then appear to have two distinct regions, a thick segment that represents the chondroitin sulphate region, and a thin section that attaches directly to the hyaluronate (Rosenberg *et al.*, 1975; Buckwalter & Rosenberg, 1982). Immunoferritin labelling of proteoglycan monomers indicated this latter segment to contain the binding region (Buckwalter *et al.*, 1982).

Colloidal gold has been used in conjunction with specific antibodies as an electron-microscopic immunolabel providing detection of epitopes with high specificity and high resolution (Roth *et al.*, 1978; Roth, 1982). Together with the availability of specific antibodies to proteoglycan substructures (Ratcliffe & Hardingham, 1983; Caterson *et al.*, 1983; Couchman *et al.*, 1984; Zanetti *et al.*, 1985), this has permitted quantitative studies of the extracellular and intracellular localization of proteoglycans (Ratcliffe *et al.*, 1984, 1985). The

‡ Present address: Department of Biochemistry and Molecular Biology, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, U.K.

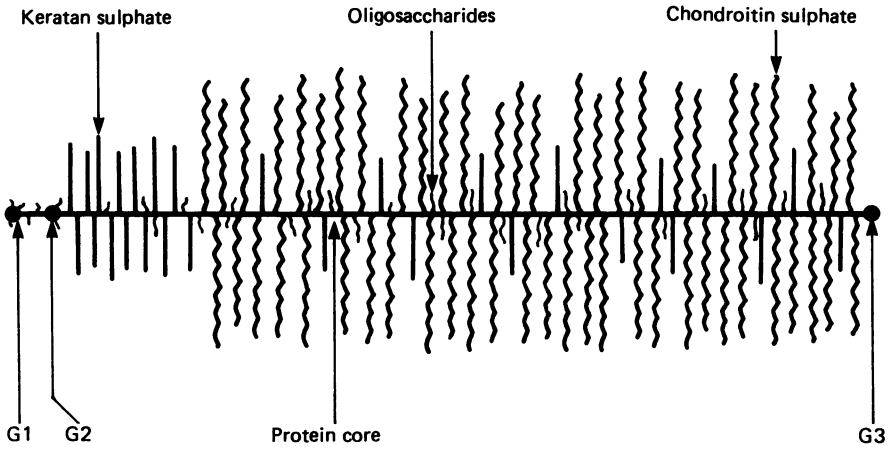

Fig. 1. Schematic diagram of the structure of aggregating proteoglycan from cartilage (Hardingham *et al.*, 1986)

The protein core contains three globular domains: the binding region (G1), which contains a specific hyaluronate-binding site involved in aggregation, a second globular domain (G2), and a *C*-terminal domain (G3). The extended part of the protein core contains a keratan sulphate-rich region and a long chondroitin sulphate-attachment region.

present paper describes the intramolecular localization of substructures of proteoglycan monomers labelled with antibodies and Protein A-coated gold particles and examined by electron microscopy. We have employed molecular spreading techniques as used in the examination of DNA with benzyldimethylalkylammonium chloride as the spreading agent (Koller *et al.*, 1969; Lang & Mitani, 1970). This technique has recently been used in a study of proteoglycans from bovine femoral-head cartilage (Thornton *et al.*, 1986) and of cervical, gastric and bronchial mucus glycoproteins (Sheehan *et al.*, 1986).

METHODS

Preparation of proteoglycan monomers

Proteoglycans were extracted from pig laryngeal cartilage in 4 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8, in the presence of proteinase inhibitors as described by Hardingham (1979). The proteoglycan components were separated by equilibrium-density-gradient centrifugation (Hardingham & Muir, 1974) as described previously (Ratcliffe & Hardingham, 1983). Associative equilibrium-density-gradient centrifugation produced a fraction (A1) containing proteoglycan aggregate, and this was fractionated by dissociative equilibrium-density-gradient centrifugation to produce a fraction (A1D1) containing proteoglycan monomers.

Proteoglycan monomers were digested with chondroitin AC lyase (0.1 unit/mg of proteoglycan) in 0.1 M-sodium acetate in 0.1 M-Tris/HCl buffer, pH 7.4, for 90 min at 37 °C (Yamagata *et al.*, 1968).

Preparation of the antiserum and monoclonal antibodies

The antiserum to the binding region of proteoglycan from pig laryngeal cartilage was raised in rabbits as described previously. It was shown to be specific and contains antibodies to both native and denatured binding region, but does not cross-react with any other cartilage components (Ratcliffe & Hardingham, 1983; Ratcliffe *et al.*, 1984).

The monoclonal antibodies MZ15 and 5-D-4 are specific for keratan sulphate (Zanetti *et al.*, 1985; Caterson *et al.*, 1983). It has been shown (Mehmet *et al.*, 1986) that they both bind to pentasulphated hexasaccharides and larger related oligosaccharides of keratan sulphate, and this suggests that they selectively bind to highly sulphated sequences. Antibody MZ15 optimally bound to nonasulphated decasaccharides, but the optimal antigenic determinant for antibody 5-D-4 may involve a longer sequence, or one containing different linkages or patterns of sulphation (Mehmet *et al.*, 1986). Antibody 5-D-4 was generously given by Dr. B. Caterson, West Virginia University, Morgantown, WV, U.S.A.

Preparation of Protein A–gold

Colloidal gold of 4 nm diameter was prepared by reduction of tetrachloroauric acid (BDH Chemicals, Poole, Dorset, U.K.) with $NaBH_4$ (Tschopp *et al.*, 1982). A 100 ml portion of 0.01% tetrachloroauric acid was cooled to 4 °C and a solution of 0.1 M-$NaBH_4$ was added dropwise with constant stirring until no further colour was observed. The gold solution was then stirred for a further 10 min. Complexes of protein A (Pharmacia, Uppsala, Sweden) and gold were prepared as described by Slot & Geuze (1981). The gold particles had a diameter of 4 ± 1 nm.

Preparation of proteoglycan–antibody–gold complexes

Proteoglycan–antibody complexes were formed under conditions where there was an excess of antibody, and this was typically achieved by mixing 50 μl of solution (50 mM-magnesium acetate, pH 7.0) containing approx. 5 μg of proteoglycan with 50 μl of antiserum. After 30 min the excess antibody was removed by chromatography of the mixture on a small column (150 mm × 10 mm) of Sepharose CL2B (Fig. 2*a*). When the column was run at approx. 400 μl/min the proteoglycan–antibody complex was eluted in the void volume of the column over an 8–12 min period after application and the excess antibody and serum proteins were eluted after 20 min. The column effluent was monitored at 280 nm with a Uvichord spectrophotometer, and to a sample (100 μl) of the void-volume peak 100 μl of the

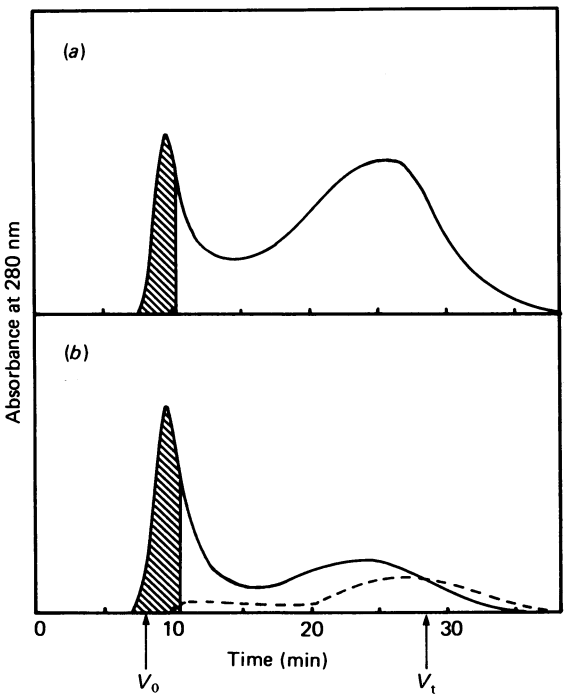

Fig. 2. Preparation of the proteoglycan–antibody–gold complex

(*a*) Proteoglycan monomer was incubated with monoclonal antibody or antiserum, and the mixture was then applied to a Sepharose CL2B column (150 mm × 10 mm) to separate the proteoglycan–antibody complex (hatched area), which was eluted in the void volume of the column from the unbound material, which was eluted away from the void volume. (*b*) The proteoglycan–antibody complexes were then incubated with excess Protein A–gold, and applied to a Sepharose CL2B column (150 mm × 10 mm) to separate the proteoglycan–antibody–gold complexes (hatched area), which were eluted in the void volume of the column, from the unbound Protein A–gold, which was eluted in the included volume of the column (----).

Protein A–gold plus bovine serum albumin (100 μg) was added. The bovine serum albumin was included to block non-specific interactions of gold with protein-rich regions of the proteoglycan. After 30 min this mixture was chromatographed on the same column (Fig. 2*b*) to remove the excess gold. The whole of the void-volume peak containing the proteoglycan–antibody–gold complex was collected (1.5 ml) and diluted to 10 ml with 50 mM-magnesium acetate yielding a final concentration of proteoglycan of 0.1–0.5 μg/ml.

Electron microscopy

Carbon films were made by evaporation of carbon on to freshly cleaved mica. The films were floated off on water and deposited on 600-mesh grids (Gilder Grids, Grantham, Lincs., U.K.). Specimens for electron microscopy were prepared by a modification of the methods used in spreading DNA (Kleinschmidt & Zahn, 1959; Koller *et al.*, 1969; Lang & Mitani, 1970). The proteoglycan–antibody–gold complex (approx. 0.1 μg/ml) was put into a Teflon or plastic Petri dish. A small amount of fine graphite powder was sprinkled on the surface, which was then touched with a plastic pipette tip containing a solution (10 mg/ml) of benzyldimethylalkylammonium chloride so that approx. 1 μl was spread on the surface, displacing the graphite to the edge of the dish. After 5–10 min a grid was touched to the surface, stained in a solution of uranyl acetate (1 mM in 95% ethanol) and finally washed in ethanol (95%). After drying in air, the grids were typically subjected to either rotary or undirectional shadowing with platinum carbon at 10–15°. Electron microscopy was performed with a Jeol 100 CX scanning transmission instrument at 80 keV. Contour-length measurements of proteoglycan monomers were performed by tracing over photographic reproductions on a graphics tablet (Grafpad; British Micro, Watford, Herts., U.K.) connected to a BBC micro-computer.

RESULTS AND DISCUSSION

Proteoglycans spread with benzyldimethylalkylammonium chloride

Proteoglycans were observed with the chondroitin sulphate chains collapsed around the protein core when spread in 50 mM-magnesium acetate, pH 7.0, and benzyldimethylalkylammonium chloride (Fig. 3*a*). The molecules showed a pronounced beaded appearance, and were polydisperse in length with one to ten beads per molecule. On many particles a fine filamentous tail about 50–70 nm in length can just be discerned at one end, which previous studies have suggested to be the binding region (Buckwalter & Rosenberg, 1982). The distribution of particle lengths expressed as a number and a weight frequency is shown in Fig. 3(*b*). All molecules observed were included in the counting. The number-average length of the population ($L_n = \Sigma N_i{*}L_i/\Sigma N_i$) is 192 nm and the weight-average length ($L_w = \Sigma N_i{*}L_i{*}L_i/\Sigma N_i{*}L_i$) is 311 nm, with a length range of 10–550 nm. These lengths are comparable with those previously obtained by this and other techniques (Rosenberg *et al.*, 1970, 1975; Buckwalter & Rosenberg, 1982; Buckwalter, 1983; Thornton *et al.*, 1986) and appear to reflect two proteoglycan populations, as reported by Thornton *et al.* (1986). In the latter study of bovine femoral-head proteoglycan monomers with the use of the same electron-microscopy spreading technique, one population of proteoglycans appeared as extended molecules with a pronounced beaded appearance, having a number-average length of 214 nm and a weight-average length of 255 nm, and a significant proportion of proteoglycan monomers also appeared in a collapsed form, with diameters 10–80 nm. Fractionation by using gel filtration, agarose electrophoresis or rate-zonal centrifugation showed that the images appearing condensed by electron microscopy were of proteoglycans that in solution were of only slightly smaller average size than those that appeared extended. Comparison of the lengths/diameters of the two populations did not therefore accurately reflect their difference in size.

The beaded appearance noted here has been seen previously (Thornton *et al.*, 1986), and was not due to a staining artifact, as it was seen by shadowing alone. Whether it reflects some true clustering of the chondroitin sulphate chains on the protein core is not established. The average bead periodicity is 44 nm (range $\pm$10 nm). Assuming the chondroitin sulphate region of the proteoglycan to constitute 80% of the total weight of the

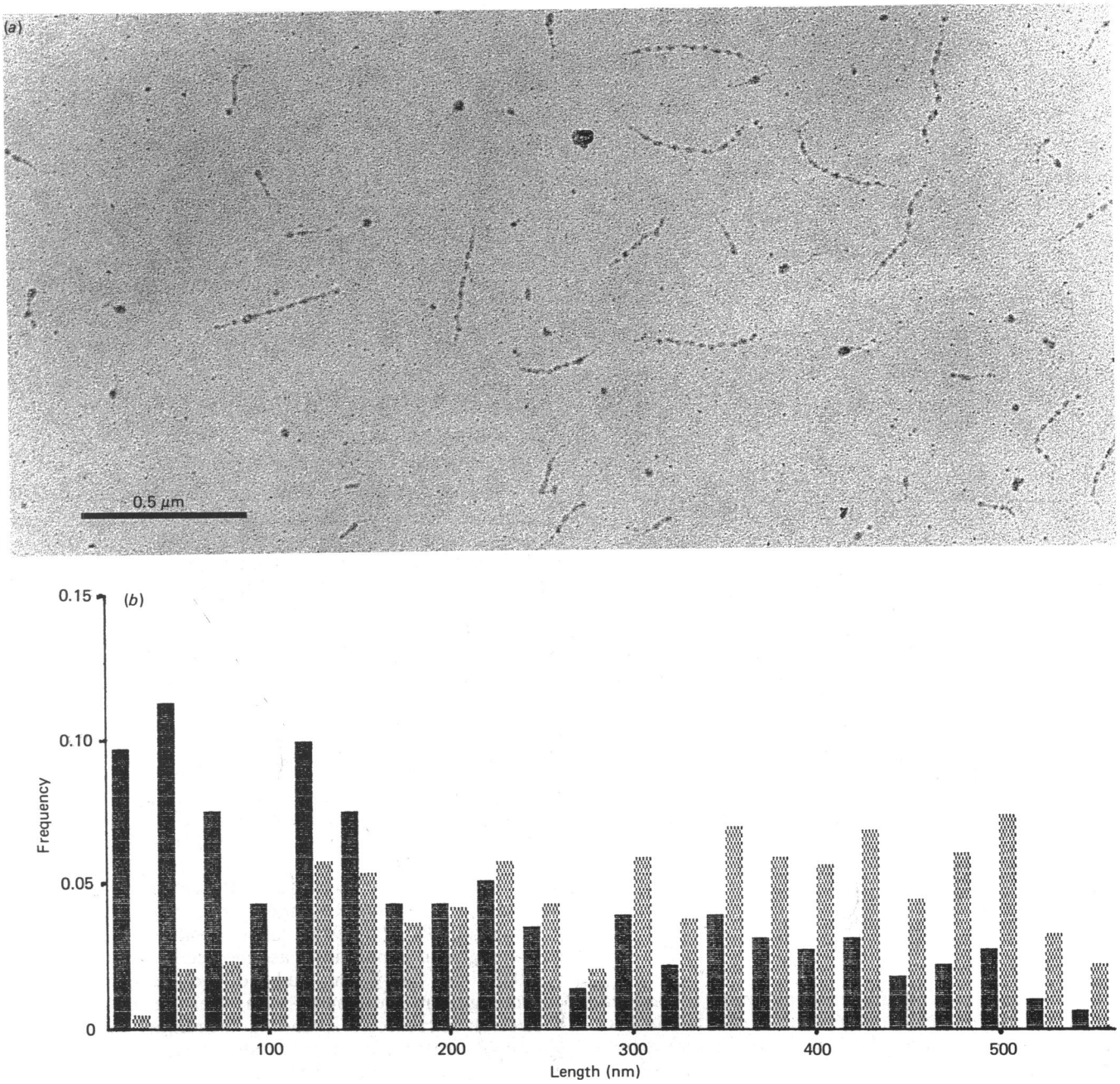

Fig. 3. Electron microscopy of proteoglycan spread in benzyldimethylalkylammonium chloride monolayers

(*a*) Proteoglycans were spread in 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon. The molecules appear as flexible 'beaded' chains of various lengths and as round blobs, both of which have, in many cases, a fine filamentous tail (see Thornton *et al.*, 1986). (*b*) Histograms of proteoglycan length distributions. The black bars show the number frequency of the length distribution [$f(N_i) = N_i/\Sigma N_i$], and the number-average length ($L_n = \Sigma N_i{}^* L_i/\Sigma N_i$) is 192 nm. The stippled bars show the weight frequency of the length distribution [$f(N_i{}^* L_i) = N_i L_i/\Sigma N_i{}^* L_i$], and the weight-average length ($L_w = \Sigma N_i{}^* L_i{}^* L_i/\Sigma N_i{}^* L_i$) is 311 nm.

proteoglycan (Hardingham, 1981; Hardingham *et al.*, 1986), then the weight-average M_r of this region for pig laryngeal proteoglycans is about 2×10^6 (Nieduszynski *et al.*, 1980) and its average length is approx. 240 nm. There are about 100 chondroitin sulphate chains (M_r approx. 20000) on an average proteoglycan, which has about five or six beads, and calculation suggests that a single 'bead' therefore accounts for as many as 15–20 chondroitin sulphate chains. Analysis of the trypsin/chymotrypsin-cleavage products of bovine nasal cartilage proteoglycan showed the presence of fragments containing two to ten chondroitin sulphate chains (Heinegård & Hascall, 1974), which were suggested to represent 'clusters' of chondroitin sulphate chains on the protein core. These 'clusters' appear considerably smaller than the estimates of the number of chondroitin sulphate

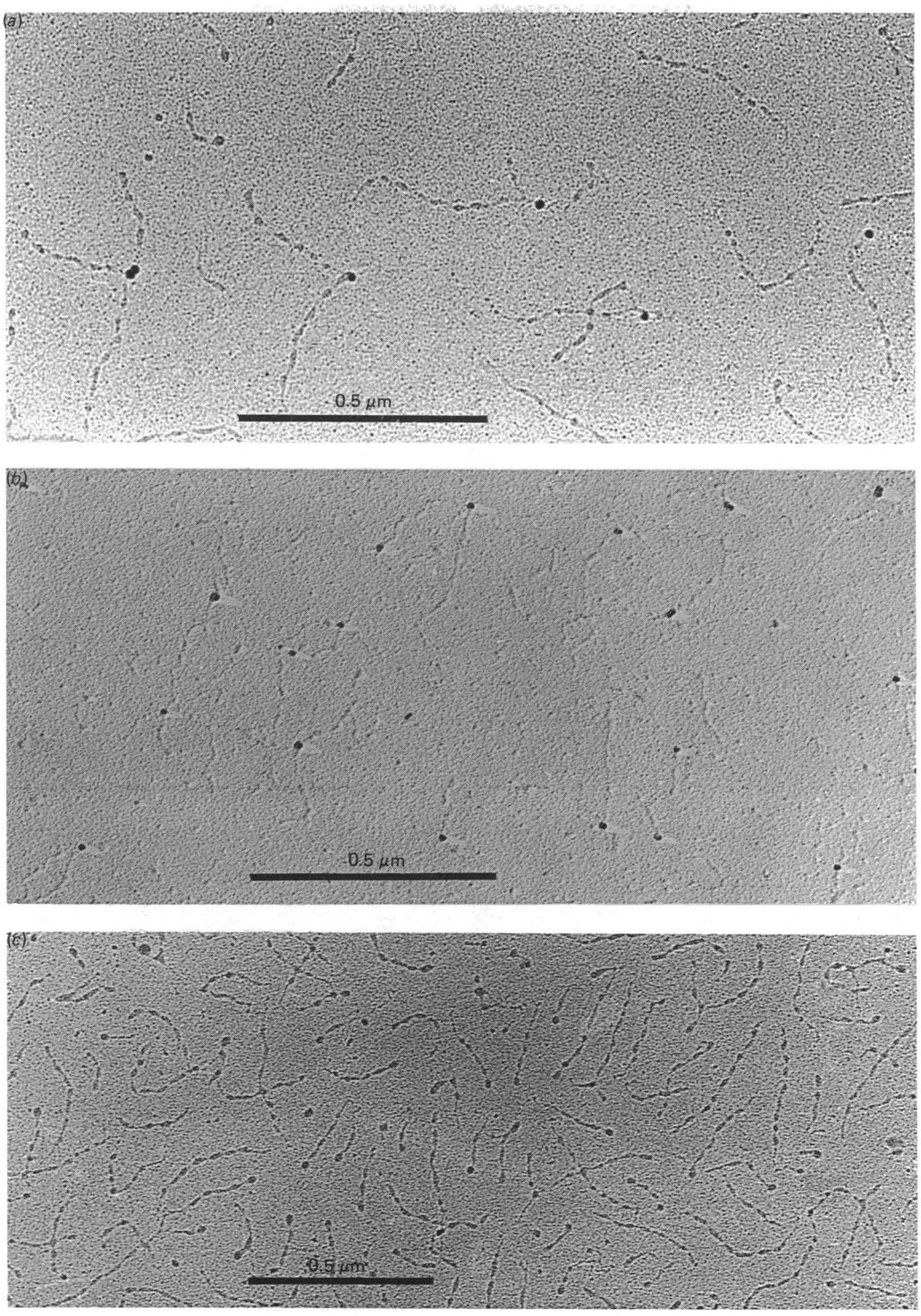

Fig. 4. Interaction of colloidal gold with proteoglycans in the absence of Protein A and antibodies

(*a*) An excess of colloidal gold was mixed with proteoglycans and after 1–2 h was chromatographed as described in the Methods section. The void fraction was collected for electron microscopy. The gold binds only at one end of the molecule (probably the binding region), one gold particle sometimes linking two or three proteoglycan molecules. (*b*) Proteoglycans treated with chondroitin AC lyase for 2 h were mixed with colloidal gold and the experiment as in (*a*) above was performed. The molecules were detected by unidirectional shadowing with platinum/carbon (no molecules were seen with the use of positive staining alone). Gold particles were only observed at the ends of the molecules. (*c*) The interaction between colloidal gold and the proteoglycans can be inhibited by the presence of an excess of a small protein such as cytochrome *c*, bovine serum albumin or Protein A with the colloidal gold. By this means non-specific binding can be decreased to less than 5% of the total number of gold particles bound.

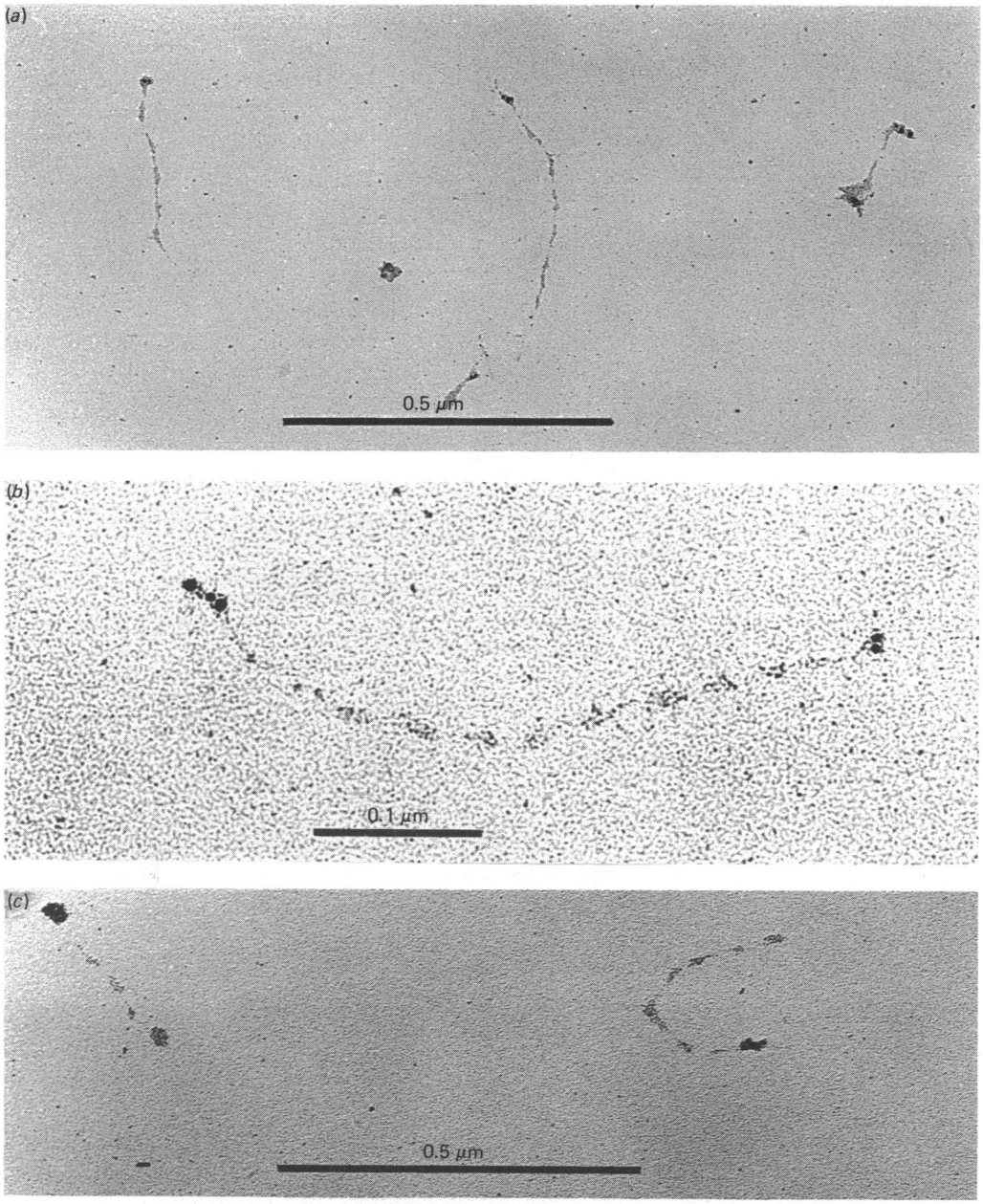

Fig. 5. Immuno-localization of binding region within proteoglycans

(*a*) Proteoglycan–antibody–gold complexes were prepared and spread as described in the Methods section. The gold particles (typically one to four) bound only at the ends of the molecules. Some 70% of the proteoglycans bound the gold, and about 10–15% of this number bound the gold at both ends of the molecule. (*b*) A micrograph of a proteoglycan binding gold at both ends (positively stained only). The long filamentous region at the left is probably the binding-region end. (*c*) Proteoglycans after reduction with dithiothreitol show a tendency to bind more gold particles (two to eight) to the binding region.

chains in each 'bead' of the extended structures shown in this study. However, analysis of the size of clusters isolated after enzyme digestion of pig laryngeal cartilage proteoglycan has not been reported.

Interaction of colloidal gold with proteoglycans in the absence of antibodies

Colloidal gold consists of negatively charged hydrophobic particles and will form stable complexes with hydrophobic domains on many globular proteins (Geoghegan & Ackerman, 1977), but not with other hydrophilic structures such as glycosaminoglycans. Colloidal gold in the absence of Protein A bound only to the extreme end of the proteoglycan, i.e. that associated with the binding region (Fig. 4*a*), one gold particle often linking groups of two to four molecules. No gold particles found sites on the main body of the protein core even after chondroitin AC lyase digestion of the proteoglycans (Fig. 4*b*). In a count of 200 proteoglycans 70% of the clearly identifiable molecules were found to be associated with gold particles. A proportion of the Protein A–gold complex used in these studies was also

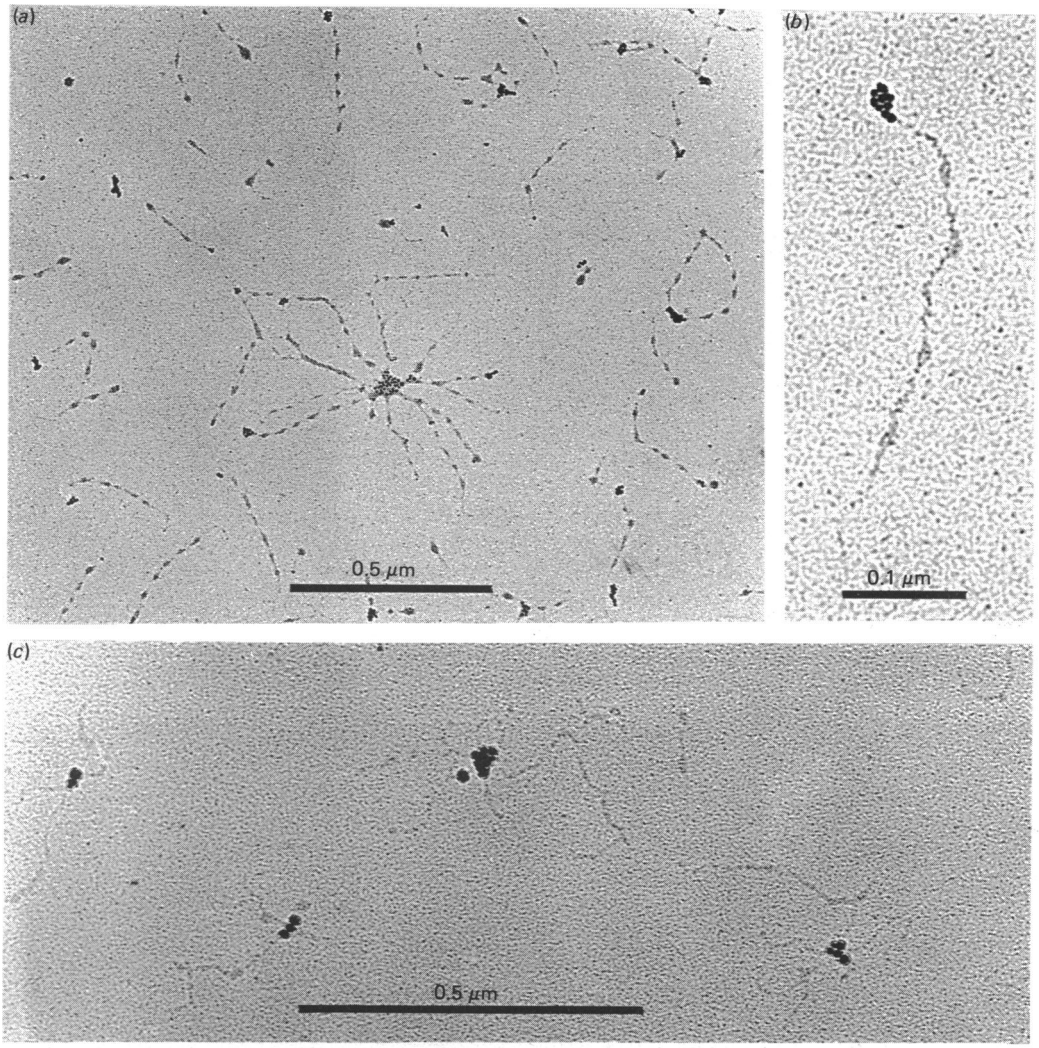

Fig. 6. Immuno-localization of keratan sulphate chains by the use of the MZ15 monoclonal antibody

(*a*) Proteoglycan antibody MZ15–gold complexes were made and spread as described in the Methods section. The proteoglycan molecules occur singly and also occasionally as aggregates with gold particles (two to 15) clustered around one end. (*b*) A detail of a single proteoglycan (positively stained only) showing a cluster of nine gold particles at the end. (*c*) Proteoglycans digested with chondroitin AC lyase and labelled with antibody MZ15 and Protein A–gold only bound the gold particles at one end; no labelling of the Protein A–gold was observed along the extended protein core.

found to bind to the proteoglycans in the same region in the absence of antibodies. This may mean that not all colloidal gold particles carried Protein A. This non-specific binding activity was substantially decreased to less than 5% by including an excess of bovine serum albumin together with the proteoglycan before adding the gold (Fig. 4*c*). An excess of Protein A itself was also a very effective inhibitor of virtually all gold binding to the proteoglycans.

Localization of substructures with the use of specific antibodies

Localization of the binding region. After the use of anti-(binding region) serum the Protein A–gold particles (typically one to three) were found to localize at the extreme end of the filamentous region of the spread proteoglycan (Fig. 5*a*). A high proportion (70%) of the proteoglycan monomers were labelled, and 10–15% of these showed the presence of gold particles at both ends (Fig. 5*b*). Reduction with dithiothreitol of the disulphide bonds within the proteoglycans gave increased clustering of gold particles in the filamentous region (Fig. 5*c*). This may reflect the exposure of more epitopes for the binding of antibodies; however the possible multivalent nature of the gold particles themselves means that the number of gold particles bound in a cluster may not have quantitative significance.

Localization of keratan sulphate. Keratan sulphate was localized within the proteoglycan monomer by use of the monoclonal antibody MZ15 (Zanetti *et al.*, 1985), and the distribution of gold particles on proteoglycans after exposure to antibody MZ15 is shown in Fig. 6(*a*). Distribution clusters of two to 15 gold particles were seen at the extreme end of over 70% of the molecules (Fig. 6*b*). The appearance of the proteoglycans was very

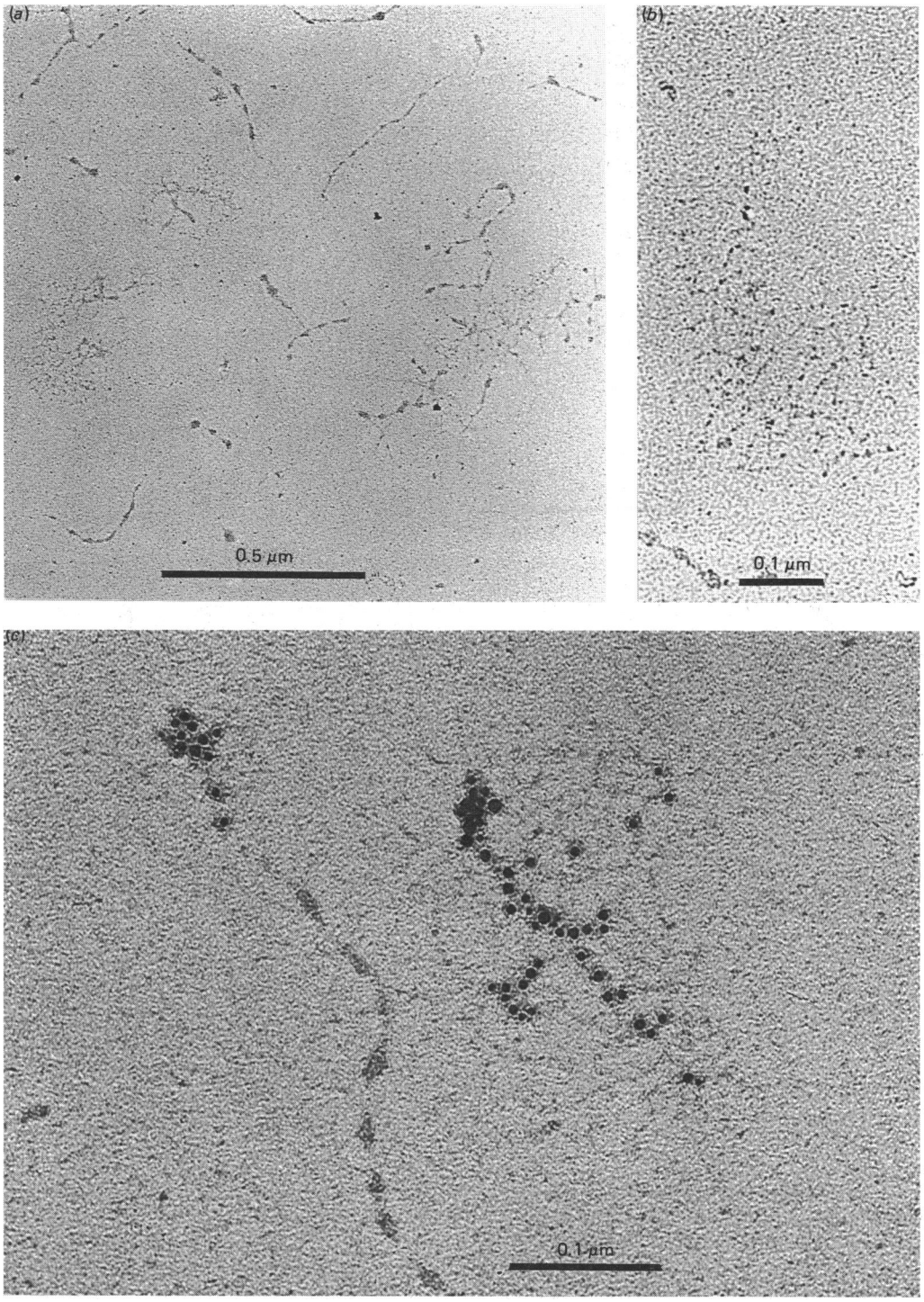

Fig. 7. Immuno-localization of keratan sulphate chains by the use of the 5-D-4 monoclonal antibody

(*a*) Proteoglycan–antibody complex was prepared, the Protein A–gold labelling was omitted and the preparation was spread as described in the Methods section. Approx. 80% of the particles have the usual beaded appearance, but the remainder have their chondroitin sulphate chains spread on the carbon substrate radiating away from their protein core. (*b*) A detail of a proteoglycan with chondroitin sulphate chains spread. (*c*) Proteoglycan–antibody–gold complexes show the same differential spreading as in (*a*). Approx. 80% of the particles have a beaded appearance, and in these molecules the gold particles labelled antibody only at one end. The remaining 20% showed the chondroitin sulphate chains radiating away from the protein core and had Protein A–gold particles labelling keratan sulphate along the length of the protein core. This micrograph shows the two types of labelled proteoglycan side by side.

similar to those spread in the absence of antibody and Protein A–gold (see Fig. 5*a*) except for the occasional presence of cross-linked aggregates. Very few molecules appeared to bind any gold within the main body of the protein core itself. In order to test whether this was due to an absence of epitopes or their concealment by steric exclusion caused by the chondroitin sulphate chains, experiments were performed on proteoglycans digested with chondroitin AC lyase. No increase of gold binding to the core region was noted after this procedure. The binding of all gold particles to the proteoglycans could be inhibited by including an excess of keratan sulphate chains together with the proteoglycans before the antibodies were added.

Keratan sulphate was also localized by use of the monoclonal antibody 5-D-4 (Caterson *et al.*, 1983). Unlike antibody MZ15, antibody 5-D-4 caused a change in the spreading behaviour of a proportion (approx. 20%) of the proteoglycans even in the absence of the colloidal gold. Fig. 7(*a*) shows the presence of two molecular forms, one with the typical beaded appearance and the other with chains spread, and although the individual chains cannot be discerned the lightly contrasted groups of entangled chains radiating from the protein core can be seen (Fig. 7*b*). These observations were supported by experiments in which the Protein A–gold was used to localize the antibodies. Two distinct populations were observed, i.e. those that bound gold only at the extreme ends of the molecules and those that bound gold throughout the core region. These two types occurred in the same ratio as previously noted, and the molecules with gold distributed along the core also had chondroitin sulphate chains spread. An example of both types of labelling is shown in Fig. 7(*c*). As with the MZ15 antibody, the gold binding was inhibited by an excess of exogenous keratan sulphate chains.

GENERAL DISCUSSION

Colloidal gold has been extensively used in various techniques for staining proteins, and in light and electron microscopy for localization after the use of specific antibodies. In the present study we have extended the use of colloidal gold in conjunction with specific antibodies to form an intramolecular structural probe. In histochemical applications of immuno-gold staining tissue sections are labelled with Protein A–gold complex after incubation with the antibody under study. This strategy could not be adopted in molecular spreading experiments owing to a high non-specific attachment of Protein A–gold to the carbon substrate as well as the possible shielding of the epitopes when the molecules are attached to the carbon. However, these problems were avoided by carrying out both the formation of the proteoglycan–antibody complex and its interaction with Protein A–gold in free solution. Cross-linking effects due to the multivalent nature of the antibodies and the colloidal gold were decreased to a low level by working with an excess of these reagents.

The position of the binding region on cartilage proteoglycans has been identified previously by chemical and electron-microscopic studies. In the presence of antibodies to the binding region there was specific labelling of one end of the molecule, and the proportion of molecules labelled (approx. 70%) corresponded with the proportion of proteoglycans typically able to aggregate in these preparations (Hardingham *et al.*, 1976). A small proportion (approx. 10%) were also labelled at both ends, which may suggest that these molecules contain a *C*-terminal domain, and that the anti-(binding region) antibodies may show cross-reaction with features on the *C*-terminal domain of the protein core.

Differences in the binding of MZ15 and 5-D-4 antibodies have provided an unexpected insight into the potential value of this technique for probing molecular structure. It appears that the epitopes most favoured by antibody MZ15 were available only at one end of the molecule (a recognized region of high keratan sulphate chain density). However, radioimmunoassay and biochemical analysis have shown that the chondroitin sulphate-rich region also contains keratan sulphate epitopes recognized by the MZ15 antibody (A. Ratcliffe & T. E. Hardingham, unpublished work). The pattern of labelling observed may thus reflect co-operative binding of the antibody in the region containing a high concentration of keratan sulphate epitope. In contrast, the 5-D-4 antibody distinguished between two groups of proteoglycans, namely those that had epitopes available for antibody binding at one end of the proteoglycan only (as for antibody MZ15) and those that also had epitopes available to the antibodies throughout the core protein. Although both antibodies recognize similar epitopes (Mehmet *et al.*, 1986), some differences in their detailed specificity or affinity may have resulted in the differential labelling. The ability of antibody 5-D-4 to label two different proteoglycan forms suggests that they are heterogeneous with respect to keratan sulphate, in the number and distribution of keratan sulphate chains, their content of epitope or its accessibility to antibody. Previous studies have shown that populations of proteoglycans, differing with respect to their size, keratan sulphate composition and distribution, could be identified in many cartilaginous tissues (Heinegård *et al.*, 1985; Stanescu *et al.*, 1980).

Binding of gold particles alone to the proteoglycans in the absence of bovine serum albumin also showed interesting results. No binding occurred to the heavily glycosylated regions of proteoglycan containing chondroitin sulphate, keratan sulphate or oligosaccharide structures, and the gold alone appeared to bind selectively at one end of the molecule in the region of known globular protein structure. It may thus form a useful probe for the localization of protein domains within heavily glycosylated structures, and preliminary studies on mucus glycoproteins confirm this (J. K. Sheehan, unpublished work).

Further binding sites for the gold were not revealed by chondroitin AC lyase digestion, which suggests that appropriate hydrophobic sites were not present within the chondroitin sulphate-rich region or that they continued to be protected by the residual sugar-bearing structures such as oligosaccharides.

This method complements other techniques for the identification of specific substructures within large macromolecules. The specificity and resolution are limited only by the selection of appropriate antibodies and by the size of the gold particles. Furthermore the binding of antibodies when carried out in free solution can influence the subsequent conformation and this may help to elucidate further structural details.

This investigation was supported by grants from the Cystic Fibrosis Research Trust (to J.K.S.) and the Arthritis and

Rheumatism Research Council (to A.R. and T.E.H.). Mrs. Ruth Berry is thanked for valuable photographic assistance.

REFERENCES

Bonnet, F., Dunham, D. R. & Hardingham, T. E. (1985) Biochem. J. **228**, 77–85
Buckwalter, J. A. (1983) J. Bone Jt. Surg. Am. Vol. **65**, 958–974
Buckwalter, J. A. & Rosenberg, L. C. (1982) J. Biol. Chem. **257**, 9830–9839
Buckwalter, J. A., Poole, A. R., Reiner, A. & Rosenberg, L. C. (1982) J. Biol. Chem. **257**, 10529–10532
Caterson, B., Christner, J. E. & Baker, J. R. (1983) J. Biol. Chem. **258**, 8848–8854
Couchman, J. R., Caterson, B., Christner, J. E. & Baker, J. R. (1984) Nature (London) **307**, 650–652
Geoghegan, W. O. & Ackerman, G. A. (1977) J. Histochem. Cytochem. **25**, 1187–1200
Hardingham, T. E. (1979) Biochem. J. **177**, 237–247
Hardingham, T. E. (1981) Biochem. Soc. Trans. **9**, 489–497
Hardingham, T. E. & Muir, H. (1972) Biochim. Biophys. Acta **279**, 401–405
Hardingham, T. E. & Muir, H. (1974) Biochem. J. **139**, 565–581
Hardingham, T. E., Ewins, R. J. F. & Muir, H. (1976) Biochem. J. **157**, 127–143
Hardingham, T. E., Beardmore-Gray, M., Dunham, D. G. & Ratcliffe, A. (1986) Ciba Found. Symp. **124**, 30–46
Heinegård, D. & Hascall, V. C. (1974) Arch. Biochem. Biophys. **165**, 427–441
Heinegård, D., Wieslander, J., Sheehan, J., Paulsson, M. & Sommerin, Y. (1985) Biochem. J. **225**, 95–106
Kleinschmidt, A. K. & Zahn, R. K. (1959) Z. Naturforsch. B **146**, 770–779
Koller, T., Harford, A. G., Lee, Y. K. & Beer, M. (1969) Micron **1**, 110–118
Lang, D. & Mitani, M. (1970) Biopolymers **9**, 373–379
Mehmet, H., Scudder, P., Tang, P. W., Hounsell, E. F., Caterson, B. & Feizi, T. (1986) Eur. J. Biochem. **157**, 385–391
Nieduszynski, I., Sheehan, J. K., Phelps, C. F., Hardingham, T. E. & Muir, H. (1980) Biochem. J. **185**, 107–114
Paulsson, M., Mörgelin, M., Wiedemann, H., Beardmore-Gray, M., Dunham, D., Hardingham, T., Heinegård, D., Timpl, R. & Engel, J. (1987) Biochem. J. **245**, 763–772
Ratcliffe, A. & Hardingham, T. (1983) Biochem. J. **213**, 371–378
Ratcliffe, A., Fryer, P. R. & Hardingham, T. (1984) J. Histochem. Cytochem. **32**, 193–201
Ratcliffe, A., Fryer, P. R. & Hardingham, T. (1985) J. Cell Biol. **101**, 2355–2365
Rosenberg, L. C., Hellman, W. & Kleinschmidt, A. K. (1970) J. Biol. Chem. **245**, 4123–4130
Rosenberg, L. C., Hellman, W. & Kleinschmidt, A. K. (1975) J. Biol. Chem. **250**, 1877–1883
Roth, J. (1982) in Techniques in Immunocytochemistry, vol. 1, (Bullock, G. & Patrusy, P., eds.), pp. 108–131, Academic Press, London
Roth, J., Bendayan, M. & Orci, L. (1978) J. Histochem. Cytochem. **26**, 1074–1081
Sheehan, J. K., Oates, K. & Carlstedt, I. (1986) Biochem. J. **239**, 147–153
Slot, J. & Geuze, H. J. (1981) J. Cell Biol. **90**, 533–536
Stanescu, V., Maroteaux, P. & Sobczak, E. (1980) Biochim. Biophys. Acta **629**, 371–381
Thornton, D. J., Nieduszynski, I. A., Oates, K. & Sheehan, J. K. (1986) Biochem. J. **240**, 41–48
Thyberg, J., Lohmander, S. & Heinegård, D. (1975) Biochem. J. **151**, 157–166
Tschopp, J., Podack, E. & Müller-Eberhard, H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7474–7478
Wellauer, P., Wyler, T. & Buddecke, E. (1972) Hoppe-Seyler's Z. Physiol. Chem. **353**, 1043–1052
Wiedemann, H., Paulsson, M., Timpl, R., Engel, J. & Heinegård, D. (1984) Biochem. J. **224**, 331–333
Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) J. Biol. Chem. **243**, 1523–1535
Zanetti, M., Ratcliffe, A. & Watt, F. (1985) J. Cell Biol. **101**, 53–59

Received 15 December 1986/23 March 1987; accepted 23 June 1987