X-Ray Fibre Diffraction of Cartilage Proteoglycan Aggregates containing Hyaluronic Acid

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Ordered conformations of proteoglycan–hyaluronic acid aggregates in the intercellular matrix in cartilage were observed by X-ray diffraction. The sodium salt form of three samples, (a) aggregated proteoglycan, (b) disaggregated proteoglycan and (c) reconstituted disaggregated proteoglycan, give essentially similar X-ray fibre-type diffraction photographs. The patterns correlate with the chondroitin 4-sulphate component and can be interpreted as twofold helical conformations, similar to that observed previously for the free acid form of chondroitin 4-sulphate (Isaac & Atkins, 1973). The information takes us one step nearer the situation found in cartilage *in vivo*.

Recent biochemical studies by Hardingham & Muir (1972, 1973*a,b*, 1974) have established a specific interaction between hyaluronic acid and the proteoglycans in the intercellular matrix of cartilage. The essential features of their proposed model (Fig. 1) are that proteoglycans are able to bind along the entire length of a hyaluronic acid chain and that at saturation there is one proteoglycan bound to each region of hyaluronic acid of about 20 disaccharide units long (approx. 20 nm). Thus for a hyaluronic acid chain of 500 000 mol.wt. there are about 40 proteoglycans regularly distributed. Each proteoglycan can bind to only one hyaluronic acid chain, so the system does not readily form a network or gel by an interaction of the form hyaluronic acid–proteoglycan–hyaluronic acid.

Tsiganos *et al.* (1971) have also shown that the proteoglycans from pig laryngeal cartilage have several core proteins differing in length, type, distribution and number of carbohydrate chains attached. However, the average molecular weight of the bound chondroitin sulphate chains does not vary from one core to another. Part of the core protein does not have chondroitin sulphate chains attached, but functions in binding the proteoglycan to the hyaluronic acid chain (Hascall & Heinegård, 1974). It has been shown that the smallest site of attachment for strong binding is a decasaccharide of hyaluronic acid (4–5 nm) (Hardingham & Muir, 1973*b*; Hascall & Heinegård, 1974), and this is less than the average separation of the proteoglycans bound along the hyaluronic acid chain (approx. 20 nm), which is to be expected since there is probably a steric restriction on the proximity with which adjacent proteoglycan molecules can approach each other. With the optimum interaction proportions of hyaluronic acid and proteoglycan, the mixture consists of 99% proteoglycan, and hence the complex contains approx. 84% chondroitin 4-sulphate. In the

naturally occurring aggregate the binding is stabilized by two protein-link components (Gregory, 1973).

Techniques have been developed recently for crystallization of the pure glycosaminoglycans which have yielded much valuable information (Atkins *et al.*, 1972). Fibre-diffraction patterns of these crystalline samples have shown that there are a number of different molecular conformations for hyaluronic acid, the chondroitin sulphates, dermatan sulphate, heparin and heparan sulphate (Atkins *et al.*, 1974*a*; Nieduszynski & Atkins, 1973, 1974; Atkins & Nieduszynski, 1974). In particular, chondroitin 4-sulphate (i.e. polysaccharide chains which have been cleaved from the protein backbone to which they normally are attached) has so far shown two

Fig. 1. Two-dimensional representation of the proteoglycan–hyaluronic acid (HA) complex

This also forms the basis of the proteoglycan aggregate which contains additional protein-link components.

distinct conformations, namely a threefold helical structure crystallizing in a hexagonal unit cell, and a twofold helical structure indexing on an orthorhombic unit cell (Isaac & Atkins, 1973).

On applying these crystallizing techniques to the proteoglycan-hyaluronic acid complex, we expected that the chondroitin 4-sulphate chains might crystallize, since they form 84% of the complex, but we were interested in seeing what effect the presence of the protein and hyaluronic acid might have. Moreover, if the complex were to crystallize in a form similar to that observed with pure chondroitin 4-sulphate it would lend weight to the biological relevance of the technique, since in the complex the chondroitin sulphate is in a state closer to the form in which it occurs in cartilage.

Experimental

The proteoglycans used were prepared from pig laryngeal cartilage.

(a) Aggregated proteoglycans were prepared by extraction of cartilage slices in 4M-guanidinium chloride and purified by equilibrium density-gradient centrifugation under 'associative' conditions (Hascall & Sajdera, 1969) as described by Hardingham & Muir (1974). The composition of the preparation was approx. 80% chondroitin 4-sulphate, 9% keratan sulphate, 10% protein and 1% hyaluronic acid.

(b) Disaggregated proteoglycans were prepared from the aggregated proteoglycans by equilibrium density-gradient centrifugation under 'dissociative' conditions in the presence of 4M-guanidinium chloride (Hascall & Sajdera, 1969) as described by Hardingham & Muir (1974). The composition of the preparation was approx. 85% chondroitin 4-sulphate, 8% keratan sulphate and 7% protein. It contained no hyaluronic acid.

(c) Disaggregated proteoglycans were mixed with sufficient hyaluronic acid (ex umbilical cord) to bind all the proteoglycans. The composition of the mixture was 99% disaggregated proteoglycans and 1% hyaluronic acid.

A sample of the sodium salt of each preparation was prepared in water solution. Each solution was placed on a glass slide, the water allowed to evaporate and the resulting thin film stretched in a similar way to that described by Atkins *et al.* (1972). Similar X-ray fibre-diffraction patterns were obtained for each sample and the photographs, shown in Plate 1, indicate different degrees of orientation in individual films. In addition variations in the degree of crystallinity with humidity were observed. The longer arcs of the aggregate (Plate 1a) show that this sample was not as well orientated as the disaggregated proteoglycan or proteoglycan-hyaluronic acid complex (Plates 1b and 1c). This difference may have resulted from the presence of additional protein-link components and a

higher proportion of keratan sulphate relative to chondroitin 4-sulphate in the aggregate preparation, but it is not thought to be of great significance, since the patterns index on the same unit cell. The features of the pattern, namely a twofold helical conformation, are similar to a crystalline form of chondroitin 4-sulphate obtained by Isaac & Atkins (1973) by dipping a crystalline specimen of sodium chondroitin 4-sulphate in a methanol-HCl bath. It is not known at this stage how far this method converts the sample into the free acid form. The X-ray data, however, record a conformational change from a threefold helix to a twofold helix. Only when this conversion is repeated under controlled pH conditions can the significance of this conformational change be considered. The proteoglycan-hyaluronic acid complex samples used in this investigation were not treated in this manner.

The photographs indicate a twofold helix, and index on an orthorhombic unit cell with dimensions: $a = 1.76 \text{ nm}$, $b = 0.77 \text{ nm}$, c (fibre axis) $= 1.95 \text{ nm}$. The density of the samples was measured by using the flotation method, which showed that there were two chains running through the unit cell. The evidence so far suggests that these chains are parallel.

The chain sense of the crystalline sample is important when we consider the overall structure of the matrix. If, as suggested above, the chains are parallel, then it is likely that the chondroitin sulphate chains have crystallized with others from the same protein backbone, and hence would not form a network. However, if they are anti-parallel, they would be expected to have crystallized by interdigitation with chains from other molecules.

It is remarkable that the chondroitin 4-sulphate should crystallize in these proteoglycan preparations in which all the chains are attached to a protein core and moreover to do so in a form that has already been observed with free chondroitin sulphate chains. This implies that the presence of protein, keratan sulphate and hyaluronic acid in the aggregate preparations does not significantly alter the ability of the chondroitin 4-sulphate to crystallize. This may be because the proteoglycan and proteoglycan-hyaluronic acid complex are sufficiently flexible molecules under the conditions used in this study to permit the ordered packing of chondroitin sulphate chains. This is not the only example of crystallization of complexes of this kind, since Atkins *et al.* (1974b) have obtained X-ray fibre patterns for macromolecular heparin.

For other biopolymers, such as nucleic acids and proteins, structure has provided much insight to function, but this is unlikely to be so with proteoglycans, where tertiary structure may be much more influenced by changes in the molecular environment. In the cartilage from which these proteoglycans were prepared the concentration of proteoglycan aggregates is about 60–80 mg/g wet wt. of tissue. The con-

EXPLANATION OF PLATE I

X-ray fibre-diffraction patterns of the proteoglycan-hyaluronic acid complex

(a) Aggregated proteoglycan; (b) disaggregated proteoglycan; (c) disaggregated proteoglycan reattached to hyaluronic acid. The longer arcs of (a) indicate that this specimen is not as well oriented as in (b) and (c).

centration of proteoglycan is thus at least 60–80 mg/ml of tissue fluid. The concentration of proteoglycan in the orientated fibres at approx. 90% humidity is approx. 2×10^3 – 3×10^3 mg/ml and is thus considerably higher than the situation *in vivo*, but in regions of locally high concentration there may be some packing of chondroitin sulphate chains in these ‘crystalline’ forms in cartilage. It remains to be determined how far this would contribute to the stability of the matrix or affect other interactions of proteoglycans in the matrix, as for example the interaction with collagen.

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- Atkins, E. D. T. & Nieduszynski, I. A. (1974) in *Heparin: Basic Research and Clinical Implications*, Plenum Press, New York, in the press
- Atkins, E. D. T. & Sheehan, J. K. (1972) *Nature (London) New Biol.* **235**, 253–254
- Atkins, E. D. T., Phelps, C. F. & Sheehan, J. K. (1972) *Biochem. J.* **128**, 1255–1263
- Atkins, E. D. T., Isaac, D. H., Nieduszynski, I. A., Phelps, C. F. & Sheehan, J. K. (1974a) *Polymer* **15**, 263–271
- Atkins, E. D. T., Nieduszynski, I. A. & Horner, A. (1974b) *Biochem. J.* **143**, in the press
- Gregory, J. D. (1973) *Biochem. J.* **133**, 383–386
- Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
- Hardingham, T. E. & Muir, H. (1973a) *Biochem. Soc. Trans.* **1**, 282–284
- Hardingham, T. E. & Muir, H. (1973b) *Biochem. J.* **135**, 905–908
- Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565–581
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* in the press
- Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396
- Isaac, D. H. & Atkins, E. D. T. (1973) *Nature (London) New Biol.* **244**, 252–253
- Nieduszynski, I. A. & Atkins, E. D. T. (1973) *Biochem. J.* **135**, 729–733
- Nieduszynski, I. A. & Atkins, E. D. T. (1974) *Proc. Symp. Colston Res. Soc.* **26**, in the press
- Tsiganos, C. P., Hardingham, T. E. & Muir, H. (1971) *Biochim. Biophys. Acta* **229**, 529–534