PHYSICOCHEMICAL PROPERTIES AND PROTEOGLYCANS OF HAGFISH (MYXINE GLUTINOSA) NOTOCHORD

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Cyclostomes retain the notochord as the sole, axial, skeletal support throughout their adult life. In lampreys, the notochord consists of a central core of large, vesiculated epithelial cells surrounded by a dense collagenous sheath (Potter & Welsch, J. Zool. 226, 1-30, 1992). Histochemically, sulfated glycosaminoglycans were found associated predominantly with the collagenous sheath, although occasional deposits were localized within epithelial cells (Welsch et al. Acta Histochem. 91, 59-65, 1991). We have observed a similar organization in the notochord of the hagfish, Myxine glutinosa (Trotter & Koob, unpublished). Although these histochemical studies indicated the likely presence of proteoglycans in the notochord sheath of lampreys and hagfish, the nature of the proteoglycans remains unresolved. The present report describes our biochemical characterization of the hagfish notochord proteoglycans and some initial experiments examining the physicochemical properties of the notochord.

Notochords were excised from propylene phenoxetol anesthetized hagfish (Myxine glutinosa). Whole notochord or sheath and central core separated by dissection were minced and then extracted with 6M guanidine-HCl, 50 mM Na acetate, pH 6.5 for 24 hours at 4°C. The extracts were centrifuged at 27,000 x g for 30 min. The supernatant was collected and immediately exchanged by dialysis into 7M urea, 50 mM Tris-HCl, pH 8.0. The extract was applied to a 50 ml column of DEAE-cellulose equilibrated in the urea buffer. After washing the column with 150 ml of the urea buffer, the column was eluted with a linear gradient of 0 - 1.0 M NaCl in the urea buffer. Orcinol positive fractions (i.e. containing uronic acid) eluting between 0.3 and 0.6 M NaCl were subsequently chromatographed on a dissociative 90 x 1.5 cm Sepharose CL-2B column eluted with 4M guanidine-HCl, 50 mM Na acetate, pH 6.5. Fractions were analyzed by 4-20% linear gradient SDS/PAGE followed by Coomassie Brilliant blue and Alcian blue staining. Glycosaminoglycan composition of proteoglycans was characterized by digestion of isolated proteoglycans with chondroitinase AC II, chondroitinase ABC and keratanase II followed by SDS/PAGE analysis.

For fixed charge density measurements, the following theory (Maroudas & Thomas, Biochim. Biophys. Acta 215, 214-216, 1970) was applied: when tissue is equilibrated in a dilute electrolyte solution, free electrolyte is virtually excluded from the tissue because of the Donnan equilibrium; the ions remaining in the tissue are those counterbalancing the negatively charged fixed groups; measurement of these ions gives a total concentration of these negative charge groups. Excised notochords were cut into 2 cm long sections, each end was ligated with 00-silk suture, and the specimens were then incubated in neutral phosphate buffered salt solutions of varying NaCl concentrations at 4°C for 24 hr. Following incubation, the central portion of each specimen was excised to remove ligatures, blotted and weighed, then dried at 65°C and weighed again. Sodium

was eluted from the dried specimens in 1 N HCl and finally measured in diluted aliquots by flame photometry. Sodium concentration in the incubation fluids was also determined by flame photometry.

Bulk free swelling tests were performed on notochord segments prepared with ligatures as described above. Segments were blotted and weighed, incubated at 4°C for 24 hr in neutral phosphate buffered salt solutions with NaCl concentrations varying between 0.15M and 2.0M, then blotted and weighed again. Swelling is expressed as percentage change in wet weight.

Figure 1 shows SDS/PAGE analysis of the anion exchange purified proteoglycans eluted from Sepharose CL-2B. Three distinct populations of proteoglycans were found: 1) a large proteoglycan eluting just after the void volume (fr 2-4) which barely entered the resolving gel; treatment of this proteoglycan with chondroitinase ACII resulted in a reduction in alcian staining and apparent molecular weight, however, a significant amount of alcian staining remained; further digestion with keratanase II resulted in elimination of alcian staining and the appearance of a core protein with an apparent molecular weight of 240 kDa; these results indicate that this large proteoglycan contains chondroitin sulfate and keratan sulfate; 2) a disperse proteoglycan population (fr 5-7) which eluted from the column with a K_{av} of approximately 0.5 yet it barely entered the resolving gel, suggesting aggregation of smaller species; diluted aliquots of this proteoglycan population resolved two proteoglycans with apparent molecular weights of 350 kDa and 100 kDa (data not shown); and 3) a small proteoglycan (fr 7-10) with an apparent molecular weight of approximately 46 kDa. When extracts of the sheath were analyzed separately, the predominant proteoglycan found was the small, 46 kDa species. Chondroitinase ABC digestion of this proteoglycan eliminated the glycosaminoglycan chain and produced a core protein doublet with an apparent molecular weight of 36 kDa. In contrast, chondroitinase ACII was only partially

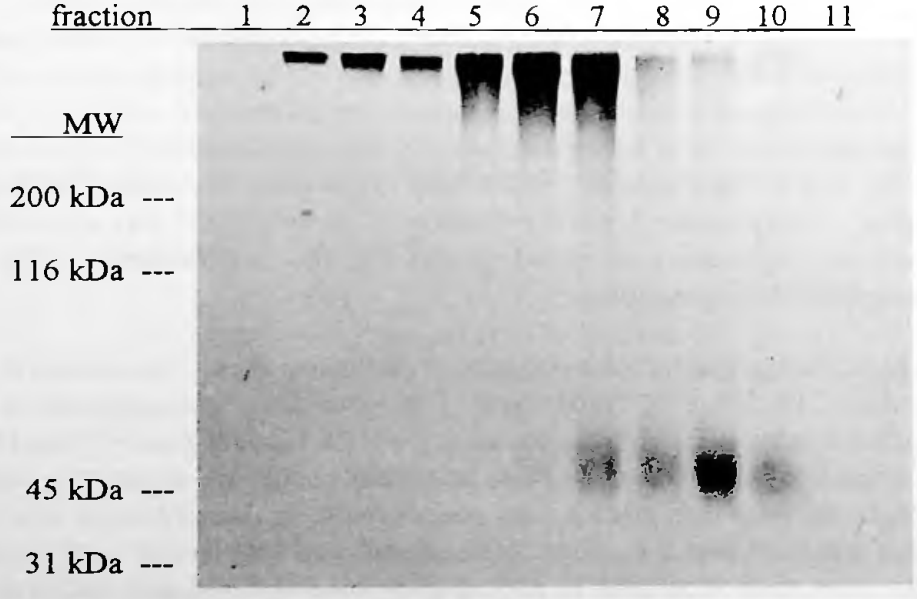

Figure 1. 4 - 20% linear gradient SDS/PAGE analysis of anion exchange purified notochord proteoglycans eluted from a 90 x 1.5 cm Sepharose CL-2B column.

effective at digesting the glycosaminoglycan chain and did not produce a distinct core protein. These results indicate that this proteoglycan belongs to the class of small, dermatan sulfate proteoglycans normally associated with collagen fibrils.

Notochord sheath and core were extracted separately to determine the distribution of the four proteoglycans described above. Extracts were analyzed by anion exchange chromatography followed by SDS/PAGE of bound material. Core extracts contained little detectable proteoglycan. The predominant material extracted from core was a single protein with an apparent molecular weight of 47 kDa which eluted from DEAE-cellulose between 0 and 0.2 M NaCl. This protein formed large aggregates (>500 kDa) which could only be dissociated by reducing agents. The identity of this protein is not known. Extracts of the sheath contained all four proteoglycans described above. When the inner aspect of the sheath was scraped with a razor blade to remove tissue associated with both the core and sheath, the large proteoglycan was no longer detected in sheath extracts. These results suggest that the large proteoglycan is located in the basement membrane separating the core and sheath. This observation is consistent with the histochemical localization of glycosaminoglycans in the basement membrane of the lamprey notochord (Welsch et al., op. cit.). The 350 kDa, 100 kDa and 46 kDa proteoglycans appear to be integral components of the collagenous sheath.

The relationship between excess sodium measured in notochord segments after incubation in phosphate buffered NaCl solutions of varying ionic strength versus the solution sodium concentration is shown in Fig. 2. The best curve fit for this relationship was an exponential curve ($r^2 = 0.95$). By extrapolating to zero Na in the incubation fluid, the negative fixed charge density of the notochord is calculated to be 274 mM.

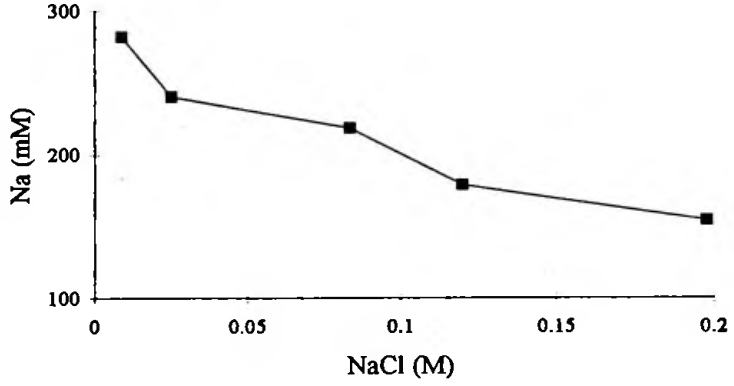

Figure 2. Concentration of excess sodium in notochord specimens incubated in neutral phosphate buffered saline containing NaCl at the indicated concentrations. Excess Na plotted on the ordinate was the sodium concentration in the specimen above that in the incubation fluid. n = 5 replicate specimens/NaCl concentration.

When placed in solutions of varying ionic strength, notochord segments changed volume as measured by a change in wet weight (Fig. 3). Notochord swelled in neutral phosphate buffered solutions with NaCl concentrations of 0.15 and 0.3 M. Little change occurred in 0.5M NaCl. The samples shrank when incubated in solutions of NaCl higher than 0.5 M, and the amount of shrinkage was directly related to NaCl concentrations between 0.7 and 1.5 M. These results

establish that the hagfish notochord is osmotically active and further suggest that fixed charge density contributes to the generation of osmotic pressure.

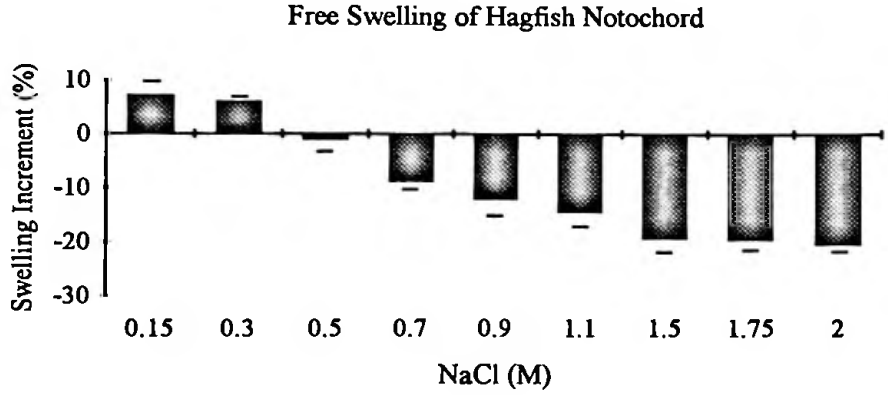

Figure 3. Bulk free swelling of notochord segments incubated for 24 hr at 4°C in 1 mM NaH_2PO_4, pH 7.0 containing NaCl at the indicated concentrations. Swelling increment represents the relative change in wet weight over the starting wet weight. n = 5/group.

The large notochord proteoglycan identified here resembles the large aggregating proteoglycan characteristic of vertebrate intervertebral discs and cartilage (aggrecan) in having both chondroitin sulfate and keratan sulfate as constituent glycosaminoglycan chains. In addition, the size of the core protein is similar to that of aggrecan. However, we were unable to detect hyaluronic acid in the notochord, suggesting that aggregation of this large proteoglycan does not occur in the notochord. The small 46 kDa proteoglycan is similar to decorin, the small proteoglycan of fibrous connective tissues periodically associated with collagen fibrils, except that the dermatan sulfate chain is much smaller than that on mammalian decorins (10 kDa versus 100 kDa). Since this is the predominant proteoglycan found in the notochord sheath, it may be the basis for the cuprolinic blue precipitates observed periodically associated with collagen fibrils in hagfish (Trotter & Koob, unpublished) and lamprey notochord sheaths (Welsch et al., op. cit.).

The fixed charge density of the notochord (0.27 mequivalents/g tissue) is similar to that found in both the nucleus pulposus of the intervertebral disc and articular cartilage (0.17 - 0.28 mequiv./g; Urban & Maroudas, Biochim. Biophys. Acta 586, 166-178, 1979). The swelling properties of the notochord are also similar to those of articular cartilage. Whether the notochord proteoglycans are the fundamental basis for these physicochemical properties, as they are in mammalian intervertebral disc and cartilage, is not yet known. However, since the core of the notochord does not contain proteoglycan yet it accounts for over 90% of the total volume, other charged and osmotically active molecules may well be the principal contributors to the physicochemical properties of the hagfish notochord.

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