Purification and Characterization of N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase from the Squid Cartilage*

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N-Acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST), which transfers sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to position 6 of N-acetylgalactosamine 4-sulfate in chondroitin sulfate and dermatan sulfate, was purified 19,600-fold to apparent homogeneity from the squid cartilage. SDS-polyacrylamide gel electrophoresis of the purified enzyme showed a broad protein band with a molecular mass of 63 kDa. The protein band coeluted with GalNAc4S-6ST activity from Toyopearl HW-55 around the position of 66 kDa, indicating that the active form of GalNAc4S-6ST may be a monomer. The purified enzyme transferred sulfate from PAPS to chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate. The transfer of sulfate to chondroitin sulfate A and dermatan sulfate occurred mainly at position 6 of the internal N-acetylgalactosamine 4-sulfate residues. Chondroitin sulfate E, kerratan sulfate, heparan sulfate, and completely desulfated N-resulfated heparin were not efficient acceptors of the sulfotransferase. When a trisaccharide or a tetrasaccharide having sulfate groups at position 4 of N-acetylgalactosamine was used as acceptor, efficient sulfation of position 6 at the nonreducing terminal N-acetylgalactosamine 4-sulfate residue was observed.

Chondroitin sulfate E (CS-E) contains a repeating disaccharide, Glcβ1–3GalNAc(4,6-bis-SO4), and was originally found in squid cartilage (1, 2). CS-E was subsequently found in various cells such as mast cells (3–6), polymorphonuclear granulocytes (7, 8), monocytes (9–12), and macrophages (13) and was thought to be involved in histamine release from mast cells (14), binding of platelet factor 4 to neutrophils (8), regulation of procoagulant activity (12), and binding of lipoprotein lipase to macrophages (13). CS-E was reported to promote neurite outgrowth from rat embryonic hippocampal neurons (15). Rat glomeruli (16) and rat mesangial cells (17) were reported to synthesize CS-E as well as glycosaminoglycans containing IdoA1–3GalNAc(4,6-bis-SO4) units, which had been found in hag fish notochord (18). The GalNAc4,6-bis-SO4 residue was also found in the nonreducing terminal of chondroitin sulfate (19–21). The proportion of the nonreducing terminal GalNAc(4,6-bis-SO4) residue of aggrecan was found to be decreased in human osteoarthritis (22). The anticoagulant activity of thrombomodulin was reported to depend on the presence of chondroitin sulfate having GalNAc(4,6-bis-SO4) residues at the nonreducing end (23). These observations suggest that CS-E or GalNAc(4,6-bis-SO4) residues at the nonreducing terminal of chondroitin sulfate should play important roles in various cellular interactions.

Sulfotransferases capable of producing GalNAc(4,6-bis-SO4) residues have been examined in squid cartilage (24), quail ovoduct (25), and human serum (26, 27). The sulfotransferase from the quail ovoduct and human serum mainly catalyzed sulfation of position 6 of nonreducing terminal GalNAc(4-SO4) residues (25–27), whereas sulfotransferase partially purified from the squid cartilage mainly catalyzed transfer of sulfate to position 6 of internal GalNAc(4-SO4) residues (24, 27). However, strict substrate specificities of these sulfotransferases are still obscure because no homogeneous preparations of these sulfotransferases have been obtained. In the present study, we purified to apparent homogeneity a sulfotransferase from squid cartilage that transfers sulfate to position 6 of the GalNAc(4-SO4) residue of chondroitin sulfate. This sulfotransferase was found to transfer sulfate to position 6 of both internal and nonreducing terminal GalNAc(4-SO4) residue. Thus, this enzyme may be described as an N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST).

EXPERIMENTAL PROCEDURES

Materials—The following commercial materials were used: [35S]sulfuric acid was from PerkinElmer Life Sciences, chondroitin ACII, chondroitin ABC, chondro-6-sulfatase, CS-A (whale cartilage), CS-C (shark cartilage), DS (pig skin), heparan sulfate (bovine kidney), completely desulfated N-resulfated heparin (CDNSS-heparin), ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-diS and ΔDi-diS were from Seikagaku Corp., recombinant N-glycosidase F was from Roche Molecular Biochemicals; Partisil-10 SAX was from Whatman; Toyopearl HW-55 was from Tosoh, Tokyo; 3′,5′-ADP, unlabeled PAPS, 3′,5′-ADP-agarose, N-acetylgalactosamine 4-sulfate, N-acetylgalactosamine 6-sulfate, N-acetylgalactosamine 4,6-bis-sulfate, hyaluronidase (bovine testis), β-glucuronidase (bovine liver, Type B-3), 2-acetamido-2-deoxy-2-galactonic acid-1,4-lactone and molecular weight standards for SDS-PAGE and for gel chromatography were from Sigma; Hicload Superdex 30 HR 16/60, Fast Desalting Column HR 10/10, heparin-Sepharose CL-6B were from Amersham Pharmacia Biotech; Matrex Gel Red A (cross-

Received for publication, December 6, 1999, and in revised form, June 22, 2000. Published, JBC Papers in Press, June 27, 2000, DOI 10.1074/jbc.M909633199

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linked 6% agarose with covalently coupled dye) was from Millipore. Fresh squids, _Ommastrephes sloani pacificus_, were obtained locally. Human meniscus CS-C was a gift from Dr. H. Habuchi, Institute for Molecular Science of Medicine, Aichi Medical University.

**Purification of GalNAc(4SO4) 6-O-Sulfotransferase**—All operations were performed at 4 °C.  

**Step 1. Preparation of Crude Extracts**—Squid cranial cartilage was disintegrated with a blender, added with cotton wool, and cut into slices with a razor. The slices were placed in 3 volumes of ice-cold 0.5 M NaCl in buffer A (10 mM Tris-HCl, pH 7.2, 20 mM MgCl2, 2 mM CaCl2, 10 mM 2-mercaptoethanol, 0.5% Triton X-100, 20% glycerol) containing protease inhibitors (5 μM Nα-p-tosyl-L-lysine chloromethyl ketone, 3 μM Nα-tosyl-L-phenylalanyl chloromethyl ketone, 30 μM phenylmethylsulfonyl fluoride, and 3 μM pepstatin A), and homogenized with a Polytron homogenizer 5 times at speed 7 for 30 s. After gentle stirring for 24 h, the homogenate was centrifuged at 100,000 × g for 60 min. The clear supernatant fraction was used as the crude extract.

**Step 2. Protopine Precipitation**—Proteoglycans contained in the crude extract could be removed as insoluble complexes with protamine sulfate. Protopine sulfate was added to the crude extract with continuous stirring to a final concentration of 0.80 mg/ml protamine. The precipitates formed were removed by centrifugation at 100,000 × g for 30 min. The apparent enzyme activity was markedly increased after protamine precipitation. The degree of the purification in each step was measured as the relative value of the specific activity of each step 30 min. The apparent enzyme activity was markedly increased after dialyzed solution from Step 6 was applied to a column of heparin-agarose fraction was dialyzed against 0.05 M NaCl in buffer A. The adsorbed sulfotransferase was eluted with 15 ml of buffer A containing 1.0 M NaCl. The collected fraction was dialyzed against buffer A containing 0.05 M NaCl. This step was adopted for the concentration of the enzyme solution. The purified enzyme was stored at 20 °C.

**Assay of Sulfotransferase Activity**—GalNAc45S-6ST activity was assayed by the method described previously (24, 32). The standard reaction mixture contained, in a final volume of 50 μl, 2.5 μM of imidazole-HCl, pH 6.8, 1 μM of CaCl2, 1 μM of reduced glutathione, 25 nmol (as galactosamine) of CS-A, 50 μmol of [35S]PAPS (about 5.0 × 106 cpm), and enzyme. The reaction mixtures were incubated at 25 °C for 20 min, and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, [35S]-labeled glycosaminoglycans were isolated by precipitation with ethanol followed by gel chromatography with a Fast Desalting Column as described previously (32), and radioactivity was determined. One unit of enzyme activity is defined as the amount required to catalyze the transfer of 1 μmol of sulfate per min. For determining the position of sulfate transferred to CS-A and DS, [35S]-labeled glycosaminoglycans were digested with chondroitinase ACII, chondroitinase ABC, chondroitinase ACII plus chondro-6-sulfatase, or chondroitinase ABC plus chondro-6-sulfatase. The radioactive products formed after the enzymatic digestion were separated with HPLC using a Whatman Partisil-10 SAX column as described below, and 32P radioactivity was determined. When oligosaccharides were used as acceptors, the reaction mixtures were applied directly to the Superdex 30 column as described below, and the [35S]-labeled oligosaccharides were separated from [35S]sulfate and [35S]PAPS.

**Digestion with β-Glucuronidase, Chondroitinase ACII, Chondroitinase ABC, and Chondroitinase ACII plus Chondro-6-Sulfatase**—Digestion with β-glucuronidase was carried out for 5.5 h at 37 °C in a reaction mixture containing, in a final volume of 40 μl, [35S]-labeled or -unlabeled tetrasaccharide (40 nmol as galactosamine), 2 μmol of sodium acetate buffer, pH 4.5, 20 nmol of 2-acetamido-2-deoxy-D-galactonic acid-1,4-lactone, 0.8 μmol of sodium fluoride, and 18 units of β-glucuronidase. Under these conditions, removal of the nonreducing terminal GlcA was complete, and no release of inorganic sulfate was observed. Digestion with chondroitinase ACII on various glycosaminoglycan was carried out for 4 h at 37 °C in the reaction mixture containing, in a final volume of 25 μl, [35S]-labeled glycosaminoglycans, 1.25 μmol of Tris acetate buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 50 milliunits of chondroitinase ACII or chondroitinase ABC. After the reaction with chondroitinase ACII or chondroitinase ABC was over, the reaction mixtures were immersed in a boiling water bath for 1 min. Chondro-6-sulfatase (75 milliunits) was added to the reaction mixtures, and the reaction was continued for a further 30 min for the complete degradation of ADP-Sa or for 5 h for the complete degradation of GalNAc (4,6-bis-SO4).

**SDS-Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis of proteins in SDS was carried out on 10% polyacrylamide gels under reducing or nonreducing conditions as described (33). Protein bands were detected by silver stain or Coomassie Brilliant Blue.

**Gel Chromatography of the Sulfotransferase on Toyopearl HW-55**—A Toyopearl HW-55 column (1.4 × 96 cm) was equilibrated with a buffer containing 2 mM NaCl, 10 mM Tris-HCl, pH 7.2, 20 mM MgCl2, 2 mM CaCl2, 0.1% Triton X-100, and 20% glycerol. Fractions (1.2 ml) were collected at a flow rate of 2.0 ml/h.

**Superdex 30 Chromatography and HPLC**—A Superdex 30 16/60 column was equilibrated with 0.2 mM NH4HCO3 and run at a flow rate of 1 ml/min. Fractions were collected. Separation of the products formed from [35S]-labeled glycosaminoglycans was carried out by HPLC using a Whatman Partisil-10 SAX column (4.6 mm x 25 cm) equilibrated with 10 mM KH2PO4. The column was developed with 10 mM KH2PO4 for 10 min followed by a linear gradient from 10 to 450 mM KH2PO4 as indicated in Fig. 8. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min and a column temperature of 40 °C.

**Other Methods**—The galactosamine and glucosamine contents of glycosaminoglycans and oligosaccharides were determined by the Morgan-Elson method as modified by Strominger et al. (35) after hydrolysis with
**RESULTS**

**Preparation of Crude Extract and Removal of Proteoglycans from the Crude Extract**—When sliced squid cartilage was homogenized with a Polytron homogenizer, addition of sodium chloride to buffer A at a final concentration of 0.5 mM augmented the yield of the activity by 1.35-fold. A clear supernatant solution (crude extract) obtained after centrifugation of the homogenate contained a significant amount of proteoglycan as judged from the content of uronic acid (about 7 μmol/ml as glucuronic acid). Most of the proteoglycans contained in the crude extract were removed as insoluble proteoglycan-proteoglycan complexes by centrifugation after addition of protamine sulfate. In contrast, the sulfotransferase activity was markedly increased after the removal of proteoglycans (Fig. 1, Table I).

**Purification of GalNAc(4SO₄)₆-O-Sulfotransferase**—GalNAc4S-6ST was purified to apparent homogeneity about 20,000-fold over the specific activity of the supernatant fraction after protamine precipitation. Table I summarizes the purification of the sulfotransferase from 1750 g of squid cartilage.

As observed in most of the glycosaminoglycan sulfotransferases, GalNAc4S-6ST was adsorbed to heparin-Sepharose CL-6B. GalNAc4S-6ST was eluted at about 0.7 mM NaCl from the column. The NaCl concentration was much higher than the concentration at which C6ST and C4ST were eluted from the same column. On 3',5'-ADP-agarose chromatography, GalNAc4S-6ST was eluted at about 5 mM NaCl (Fig. 2). The concentration of NaCl required for the elution of GalNAc4S-6ST from 3',5'-ADP-agarose was much higher than that required for the elution of glucosaminyl 3-O-sulfotransferase (37). Purification of GalNAc4S-6ST was achieved after rechromatography on 3',5'-ADP-agarose. The second heparin-Sepharose CL-6B fraction was used for the later experiments on the purified GalNAc4S-6ST.

**Purity of GalNAc4S-6ST**—The different fractions in the purification of GalNAc4S-6ST were separated by SDS-PAGE under nonreducing conditions and stained with silver nitrate (Fig. 3, lanes 1–6). A broad protein band of 63 kDa was the predominant band in the second heparin-Sepharose CL-6B fraction (lane 6). When the second heparin-Sepharose CL-6B fraction was treated with sample buffer containing 2-mercaptoethanol and was stained with Coomassie Brilliant Blue, the mobility of the protein was not altered (lane 9). The second heparin-Sepharose CL-6B fraction also showed a broad band around the origin of the gel (lane 6). This band appeared to be protein aggregates formed under the nonreducing conditions because this band became weaker under the reducing conditions when stained with Coomassie Brilliant Blue (lane 9) or silver nitrate (data not shown). The protein bands of 63 kDa disappeared after N-glycosidase F digestion, and new protein bands of 38 and 40 kDa appeared (lane 7), indicating that the purified protein contained N-linked oligosaccharides. To confirm that the protein bands observed in SDS-PAGE corresponded to GalNAc4S-6ST, the purified GalNAc4S-6ST was applied to a Toyopearl HW-55 column, and elution profiles of the GalNAc4S-6ST activity and protein were determined (Fig. 4). The protein band of 63 kDa appeared almost exclusively when the peak fraction (tube number 40 in Fig. 4A) was subjected to SDS-PAGE before N-glycosidase F digestion (Fig. 4B). When the peak fraction was subjected to SDS-PAGE after N-glycosidase F digestion, protein bands of 38 and 40 kDa appeared almost exclusively (Fig. 4C). A protein band with slightly higher molecular weight was observed when the fraction 39 was subjected to SDS-PAGE before N-glycosidase F digestion (Fig. 4B); however, the heterogeneity in the molecular weight observed before N-glycosidase F digestion seemed to be caused by microheterogeneity of N-linked oligosaccharides attached to the enzyme protein because only protein bands of 38 and 40 kDa were also detected in fraction 39 after N-glycosidase F digestion (Fig. 4C). A weak band of 54 kDa was observed in Fig. 4, B and C, but this band was thought to be an impurity that was artificially brought into the sample during the Toyopearl HW-55 chromatography, because this band was not observed in the purified fraction (Fig. 3, lanes 6, 7, 9). Molecular mass of the peak fraction determined from the elution position in the Toyopearl HW-55 chromatography (Fig. 4A) was 66 kDa, which agreed well with molecular mass determined by SDS-PAGE. These results suggest that GalNAc4S-6ST may behave as a monomer. The sulfotransferase activity toward DS and the sulfotransferase activity toward CSA were eluted from this column at exactly the same position, suggesting that sulfation of CSA and DS may be catalyzed by the same protein.

**Properties of the Purified GalNAc4S-6ST**—The pH optimum for the GalNAc4S-6ST was around 6.2. This value is similar to the pH optimum of C6ST (38) but slightly lower than that of C4ST (39). The apparent $K_m$ for PAPS was $5.0 \times 10^{-7}$ M; this value is the same order of magnitude as the $K_m$ values for C6ST (38) and C4ST (39). The $K_m$ values for CS-A and DS (expressed as the concentration of galactosamine) were $1.1 \times 10^{-6}$ M and $1.3 \times 10^{-7}$ M, respectively. GalNAc4S-6ST was activated 2.5 to 3.5-fold with various divalent ions such as Mn²⁺, Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, and Co²⁺ at 20 mM. About 2-fold activation was observed in the presence of 0.1 mM NaCl, 0.1 mM KCl, or 0.15 mg/ml proteamine. Reduced glutathione caused 33% stimulation at 20 mM. Other sulfhydryl compounds such as dithiothreitol and 2-mercaptoethanol showed only minimal effects on GalNAc4S-6ST activity.

**Sulfation of Glycosaminoglycans with GalNAc4S-6ST**—For determining acceptor specificity, the purified GalNAc4S-6ST was incubated with different glycosaminoglycans in the presence of varying amount of CaCl₂. The disaccharide composition of these glycosaminoglycans is shown in Table II. Fig. 5 shows that the purified GalNAc4S-6ST was able to transfer sulfate to CSA, CS-C, and DS. A low activity was observed toward squid skin chondroitin. CS-E, keratan sulfate, and heparan sulfate, and CDSINS-heparin did not serve as acceptor. The optimum concentration of CaCl₂ was 20 mM for CSA and CS-C, but 100
mM for DS. When two CS-C preparations were compared, CS-C from shark cartilage showed higher activity than CS-C from human meniscus. The ratios of GlcAβ1–3GalNAc(4-SO4)6-ST or GlcAβ1–3GalNAc6-SO4 of CS-C from shark cartilage and CS-C from human meniscus were 0.49 and 0.17, respectively (Table II). The lower ratio may result in the lower acceptor activity. The incorporation into CS-A was markedly inhibited by CS-E. In the presence of an equal amount of CS-E, incorporation into CS-A was decreased to 35% of control (indicated by an open rhombus in Fig. 5). Sulfation of CS-A with the purified GalNAc4S-6ST was inhibited by dermatan sulfate in a dose-dependent manner (Fig. 6B) and sulfation of DS was also inhibited by CS-A (Fig. 6B) suggesting that the sulfation of both CS-A and DS is catalyzed by the same enzyme or at least the same catalytic site.

To determine the position of the sulfate groups transferred to CS-A and DS, we digested 35S-labeled glycosaminoglycans with chondroitinase ACII or chondroitinase ABC and analyzed the digestion products by Superdex 30 gel chromatography (Fig. 7) and Partisil-10 SAX HPLC (Fig. 8). When the 35S-labeled CS-A was digested with chondroitinase ACII, the major radioactive peak was detected at the position of ΔDi-diS6 (Figs. 7B and 8A). On the other hand, the 35S-labeled DS was hardly depolymer-
Digestion with chondro-6-sulfatase for 30 min (Fig. 8B) disappeared after digestion for 5 h (data not shown). Squid skin chondroitin was mainly composed of GlcA and GalNAc(4S,6ST). Because squid skin chondroitin was mainly composed of GlcA and GalNAc(4S,6ST), the concentration of CaCl$_2$ was varied. The sulfotransferase reaction was carried out as described under “Experimental Procedures” using various concentrations of CS-A and DS, which are indicated under the graph. The concentrations of these glycosaminoglycans were expressed as the concentration of galactosamine. After the sulfotransferase reaction, 35S-labeled glycosaminoglycans were isolated and digested with chondroitinase ACII. To the digests, 3 volumes of ethanol containing 1.3% potassium acetate was added, and the mixtures were centrifuged. 35S radioactivity, which became soluble in ethanol after chondroitinase ACII or chondroitinase ABC digestion was determined. Additionally, the sulfated disaccharide units were about 0.2% of total repeating disaccharide units (Table II), the sulfate group transferred to chondroitin ACII was further digested with chondro-6-sulfatase and subjected to SAX-HPLC (Fig. 8B). After digestion with chondro-6-sulfatase, 35S radioactivity was shifted to the position of inorganic sulfate. Essentially the same results were obtained from 35S-labeled DS after chondroitinase ABC and chondro-6-sulfatase digestion (data not shown). These results clearly indicate that 35S-labeled GalNAc(4S,6ST) was transferred to position 6 of GalNAc(4,6-bis-SO$_4$)$_2$ residues in GlcA$\beta$1–3GalNAc(4,6-bis-SO$_4$)$_2$ units of CS-A or IdoA$\alpha$1–3GalNAc(4,6-bis-SO$_4$)$_2$ units of DS. When the 35S-labeled CS-A was digested with chondroitin ACII, small amounts of radioactivity were detected at the position of GalNAc(4,6-bis-SO$_4$)$_2$ (Fig. 8A). The radioactivity observed at the position of GalNAc(4,6-bis-SO$_4$)$_2$ did not disappear after digestion with chondro-6-sulfatase for 30 min (Fig. 8B) but disappeared after digestion for 5 h (data not shown).

Squid skin chondroitin served as acceptor for the purified GalNAc4S-6ST. Because squid skin chondroitin was mainly composed of GlcA$\beta$1–3GalNAc units and the contents of 4-sulfated disaccharide units were about 0.2% of total repeating disaccharide units (Table II), the sulfate group transferred to chondroitin ACII was expected to be located at position 6 of GalNAc residues. But, when the sulfated products were digested with chondroitinase ACII or chondroitinase ABC, the amounts of sulfate transferred to dermatan sulfate were calculated from the difference between the radioactivity that became soluble in ethanol after chondroitinase ACII and the radioactivity that became soluble in ethanol after chondroitinase ACII.

### Table II

| Glycosaminoglycans | 0S | 4S | 6S | diS$_b$ | diS$_d$ | diS$_e$ | 4S/6S$^b$ |
|-------------------|----|----|----|--------|--------|--------|-----------|
| Chondroitin       | 99.6| 0.2| 0.2| ND     | ND     | ND     | ND        |
| CS-A              | 1.6 | 74.2| 22.9| ND     | 0.8    | 0.5    | 3.24      |
| CS-C (shark cartilage) | 3.3 | 26.8| 55.1| ND     | 12.4   | 2.4    | 0.49      |
| CS-C (human meniscus) | 4.0 | 13.5| 81.0| ND     | 4.4    | 0.4    | 0.17      |
| DS                | 2.8 | 89.9| ND  | ND     | 7.3    | ND     | ND        |
| Chondroitinase ABC and subjected to SAX-HPLC (Fig. 8B). | | | | | | | |

$^a$ OS, 4S, 6S, diS$_b$, diS$_d$, and diS$_e$ represent ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS$_b$, ΔDi-diS$_d$, and ΔDi-diS$_e$, respectively, formed after chondroitinase ABC digestion.

$^b$ Molar ratio of ΔDi-4S/ΔDi-6S.

* Not detected.
nase ACII (Fig. 9). The elution profiles detected by the absorption at 210 nm showed that after chondroitinase ACII digestion the trisaccharide (Fig. 9B), the tetrasaccharide treated with β-glucuronidase (Fig. 9C) and the pentasaccharide (Fig. 9D) gave GalNAc(4SO₄) and Di-4S as main products, although a small peak of Di-6S (Fig. 9, B and D) and GalNAc6-SO₄ (Fig. 9D) were detected. The molar ratio of monosaccharides/disaccharides was calculated from the elution profiles (Table III). These data appear to confirm that the length of these oligosaccharides. GalNAc4S-6ST was found to catalyze the efficient sulfation of 4-sulfated oligosaccharides. The rates of sulfation of trisaccharide, tetrasaccharide, and pentasaccharide were 90, 94, and 87%, respectively, of the rate of sulfation of CS-A under the standard assay conditions. ³⁵S-Labeled trisaccharide, tetrasaccharide, and pentasaccharide were eluted from the Superdex 30 column at 80, 77, and 72 min, respectively, in the symmetrical peaks. These elution times were 2–3 min earlier than the elution time of the respective acceptor oligosaccharides (data not shown). We showed previously that monosulfated sialyl N-acetyllactosamine trisaccharide was eluted from this column 3 min earlier than sialyl N-acetyllactosamine trisaccharide (40). From the comparison of the elution time between the ³⁵S-labeled products and the respective acceptor oligosaccharides, only one sulfate group appeared to be transferred to each oligosaccharide acceptor. When the ³⁵S-labeled trisaccharide and ³⁵S-labeled pentasaccharide were digested with chondroitinase ACII and applied to SAX-HPLC, the radioactivity appeared at the position of GalNAc(4,6-bis-SO₄) and ΔDi-diS₄. The proportion of GalNAc(4,6-bis-SO₄) was 27 and 40% of the total radioactivity for ³⁵S-labeled trisaccharide and ³⁵S-labeled pentasaccharide, respectively (Fig. 10, A and C). When ³⁵S-labeled tetrasaccharide was digested with chondroitinase ACII after β-glucuronidase digestion, the radioactivity appeared mainly at the position of ΔDi-diS₄ with a small peak at the position of GalNAc(4,6-bis-SO₄). The proportion of GalNAc(4,6-bis-SO₄) was 11% of the total radioactivity. These observations suggest that the rate of sulfation of the penultimate GalNAc(4-SO₄) residue of the tetrasaccharide is lower than the rate of sulfation of nonreducing terminal GalNAc(4-SO₄) residue of the trisaccharide. To establish the position to which ³⁵S-labeled SO₄ was transferred, we digested ³⁵S-labeled trisaccharide with chondro-6-sulfatase after digestion with chondroitinase ACII. Although GalNAc(4,6-bis-SO₄) was refractory to chondro-6-sulfatase digestion when ΔDi-diS₄ was completely converted to ΔDi-4S (Fig. 11B), prolonged digestion with chondro-6-sulfatase allowed nearly quantitative conversion of GalNAc(4,6-bis-SO₄) to GalNAc4S-6ST (Fig. 11C). When ³⁵S-labeled trisaccharide was subjected to the prolonged digestion with chondro-6-sulfatase after chondroitinase ACII digestion, the radioactivity of not only ΔDi-diS₄ but also GalNAc(4,6-bis-SO₄) disappeared and was shifted to the position of inorganic sulfate (Fig. 10B). These results clearly indicate that GalNAc4S-6ST is able to transfer sulfate to position 6 of both reducing and nonreducing terminal GalNAc(4-SO₄) residues of the 4-sulfated trisaccharide. The Kₐ value expressed as the concentration of galactosamine for the trisaccharide was 1.6 × 10⁻⁵ M. When affinities for reducing-end GalNAc(4-SO₄) and nonreducing-end GalNAc(4-SO₄) were determined separately from the radioactivity of GalNAc(4,6-bis-SO₄) and ΔDi-diS₄, respectively, formed after chondroitinase ACII digestion,
there was no significant difference in the affinity between the reducing and nonreducing terminal GalNAc(4-SO₄) residue of the trisaccharide (data not shown). The $K_m$ value for the tetrasaccharide could not be determined because the Lineweaver-Burk plot did not show a straight line.

DISCUSSION

In the present study, we have purified GalNAc(4SO₄) 6-O-sulfotransferase to apparent homogeneity. The purified sulfotransferase showed two protein bands on SDS-PAGE after N-glycosidase F digestion as observed in C6ST (32), C4ST (39), heparan sulfate 6-sulfotransferase (41), and heparan sulfate 2-sulfotransferase (42). Although the identity of the two protein bands is not clear at present, it is possible that both protein bands correspond to GalNAc4S-6ST because the protein bands were both coeluted with GalNAc4S-6ST activity from Toyopearl HW-55. GalNAc4S-6ST activity was increased after removal of endogenous proteoglycans. The observed increase in the sulfotransferase activity after the proteolytic precipitation may be due mainly to the removal of proteoglycans with inhibitory activity to the sulfotransferase because the purified GalNAc4S-6ST was strongly inhibited with CS-E from squid cartilage (Fig. 5). Affinity chromatography on 3',5'-ADP-agarose was critical for the purification. The concentration of NaCl required for the elution of the enzyme from 3',5'-ADP-agarose was very high, suggesting that the affinity of GalNAc4S-6ST for 3',5'-ADP-agarose is higher than for any other glycosaminoglycan sulfotransferases so far purified.

GalNAc4S-6ST activity was activated by $Ca^{2+}$; the optimum concentration of $Ca^{2+}$ was 20 mM for CS-A and 100 mM for DS.

**Table III**

**Analysis of 4-sulfated oligosaccharides used for acceptors**

| Oligosaccharides | GalNAc(4-SO₄) | GalNAc(6-SO₄) | ΔDi-4S | ΔDi-6S |
|------------------|--------------|--------------|--------|--------|
| Trisaccharide    | 1.00         | 0.99         | 0.03   |        |
| Tetrasaccharide  | 1.00         | 1.15         |        |        |
| Pentasaccharide  | 1.00         | 0.17         | 2.04   | 0.09   |

a Not detected.
although the reason that DS requires a higher concentration of Ca\(^{2+}\) than CS-A is not clear, it is possible that the difference in the optimal concentration of Ca\(^{2+}\) between DS and CS-A may reflect the different conformation of these glycosaminoglycans induced by the addition of Ca\(^{2+}\). Reduced glutathione was found to activate GalNAc4S-6ST as observed in C4ST (39) but the degree of the activation was much lower than that found to activate GalNAc4S-6ST, indicating that molecular size of the acceptor may contribute to the affinity between the acceptors and the enzyme.

Squid skin chondroitin was sulfated, but sulfate was transferred to position 6 of nonreducing terminal GalNAc(4-SO\(_4\)) residues, which were minor components of chondroitin. These results clearly indicate that GalNAc4S-6ST absolutely requires the presence of GalNAc(4-SO\(_4\)) residues for the activity. Proportion of ΔDi-4S released after chondroitinase ACII digestion of squid skin chondroitin to the total unsaturated disaccharides was about 0.2%. The concentration of internal GalNAc(4-SO\(_4\)) residue in the reaction mixtures was thus calculated as 1 × 10\(^{-6}\) M. Because the \(K_m\) for CS-A was 1.1 × 10\(^{-6}\) M, sulfation of GalNAc(4-SO\(_4\)) residues contained in chondroitin may also occur.

We have previously purified and cloned C6ST (44). The purified C6ST transferred sulfate to position 6 of GalNAc residues of chondroitin and Gal residues of keratan sulfate (38) or sialyl N-acetyllactosamine oligosaccharides (40). However, C6ST could not catalyze the sulfation of GalNAc(4-SO\(_4\)) residues even when CS-A was used as acceptor. In contrast, the purified GalNAc4S-6ST from squid cartilage could not transfer sulfate to the GalNAc residues of chondroitin.

At present, it is not clear whether sulftotransferases with the substrate specificity similar to the specificity of squid GalNAc4S-6ST are present in the vertebral tissues. GalNAc4S-6ST activity was reported in the human serum (26, 27) and quail oviduct (25), but these sulfotransferases may be distinguished from the squid GalNAc4S-6ST in the substrate specificity because when chondroitin sulfate was used as acceptor, both human and quail enzymes transferred sulfate preferentially to position 6 of nonreducing terminal GalNAc(4-SO\(_4\)) residues, whereas the purified squid GalNAc4S-6ST transferred sulfate mainly to the internal GalNAc(4-SO\(_4\)) residues. Molecular cloning of the squid GalNAc4S-6ST would offer important clues about the molecular nature of the sulfotransferase involved in the biosynthesis of CS-E in the vertebral tissues.

Acknowledgment—We thank Dr. H. E. Conrad, Dept. of Biochemistry, University of Illinois, for critical reading and valuable suggestions on the manuscript.

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