Purification and Characterization of Heparan Sulfate 2-Sulfotransferase from Cultured Chinese Hamster Ovary Cells*

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Heparan sulfate 2-sulfotransferase, which catalyzes the transfer of sulfate from adenosine 3′-phosphate 5′-phosphosulfate to position 2 of L-iduronic acid residue in heparan sulfate, was purified 51,700-fold to apparent homogeneity with a 6% yield from cultured Chinese hamster ovary cells. The isolation procedure included a combination of affinity chromatography on heparin-Sepharose CL-6B and 3′,5′-ADP-agarose, which was repeated twice for each, and finally gel chromatography on Superose 12. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed two protein bands with molecular masses of 47 and 44 kDa. Both proteins appeared to be glycoproteins, because their molecular masses decreased after N-glycanase digestion. When completely desulfated and N-resulfated heparin and mouse Engelbreth-Holm-Swarm tumor heparan sulfate were used as acceptors, the purified enzyme transferred sulfate to position 2 of L-iduronic acid residue but did not transfer sulfate to the amino group of glucosamine residue or to position 6 of N-sulfoglucosamine residue. Heparan sulfates from pig aorta and bovine liver, however, were poor acceptors. The enzyme showed no activities toward chondroitin, chondroitin sulfate, dermatan sulfate, and keratan sulfate. The optimal pH for the enzyme activity was around 5.5. The enzyme activity was minimally affected by dithiothreitol and was stimulated strongly by protamine. The $K_m$ value for adenosine 3′-phosphate 5′-phosphosulfate was 0.20 μM.

Heparan sulfate and heparin bind to a variety of proteins, such as growth factors and protease inhibitors, suggesting their involvement not only in a fundamental cellular behaviors such as cell growth, differentiation, and cell adhesion, but also in the anticoagulation process and some pathological processes such as growth factors and protease inhibitors, suggesting that the biosynthesis of heparan sulfate proteoglycans on cell surfaces as well as in extracellular matrix may have such specific structures and regulate the biological activity of basic fibroblast growth factor (9). In fact, the response of neural cells to either acidic or basic fibroblast growth factor (FGF-1 or FGF-2, respectively) seems to be regulated by developmentally modulated forms of heparan sulfate proteoglycans (10). Thus, microheterogeneity in the heparan sulfate structures, particularly in the sulfation positions may be an important factor to control the biological activity of basic fibroblast growth factor. In this regard, it is important to study how the microheterogeneity is caused and regulated.

Various types of sulfotransferases have been shown to be responsible for the sulfation of heparan and heparin sulfate: sulfation of 2-N (11–14), 6-O (13, 15, 16), and 3-O (17) of glucosamine residue, sulfation of 2-O (16) of L-iduronic acid residue, and sulfation of 2-O (18) of α-glucuronic acid residue. The sulfate donor in these reactions is adenosine 3′-phosphate 5′-phosphosulfate (PAPS) that is synthesized in the cytosol and transported into the lumen of the Golgi to serve as substrate (19). N-Sulfotransferases have been purified to homogeneity from rat liver and mouse mastocytoma (11, 12). Recently, molecular cloning studies have suggested that these N-sulfotransferases were closely related to but were clearly distinct from each other (20–23), suggesting that the biosynthesis of heparan sulfate and heparin may be catalyzed by different enzymes and independently regulated.

It has been suggested that O-sulfation is the final step in the modification of the structure during the biosynthesis of heparan and heparin sulfate in the lumen of the Golgi apparatus (24, 25). We recently purified heparan sulfate 6-sulfotransferase (HS6ST) that catalyzes the transfer of sulfate to position 6 of N-sulfoglucosamine residue in heparan sulfate, with a high yield from the serum-free culture medium of Chinese hamster ovary cells (CHO). HS6ST, heparan sulfate 2-sulfotransferase; CHO, Chinese hamster ovary; CDSNS-heparin, completely desulfated and N-resulfated heparin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ΔDi-6S, 2-acetamido-2-deoxy-4-O-(4-deoxy-α-L-threo-4-enepypyranosylyl)uronic acid-6-O-sulfo-α-D-glucose; ΔDi-NS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-threo-4-enepyranosyl)uronic acid-α-D-glucose; ΔDi-(6, DiS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-threo-4-enepyranosyl)uronic acid)-6-O-sulfo-α-D-glucose; ΔDi-(U)DiS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-threo-4-enepyranosyl)uronic acid)-6-O-sulfo-α-D-glucose; AMan, 2,5-anhydro-o-mannose when a sub-
Purification of Heparan Sulfate 2-Sulfotransferase from the Crude Extract of CHO Cells

All operations were performed at 4°C.

Step 1: First Heparin-Sepharose CL-6B Chromatography—One-third volume (600 ml) of the crude extract prepared as above was applied to a column of heparin-Sepharose CL-6B (30 × 70 mm, 50 ml) equilibrated with 0.15 M NaCl in buffer A containing a mixture of the protease inhibitors at a flow rate of 76 ml/h. The column was washed with 500 ml of the same buffer, and then 35 ml of a column volume of buffer A containing a linear gradient from 0.15 to 1.2 M NaCl in the above buffer was then eluted with a linear gradient from 0.15 to 1.2 M NaCl in the above buffer (total volume, 1,000 ml). Fractions of 13 ml were collected. The fractions containing the sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 1), concentrated to about 100 ml with polyethylene glycol 20,000 (molecular weight 15,000–25,000), and dialyzed against 0.05 M NaCl in buffer A for the next purification step. Triton X-100 was added to 1% (v/v) and dialyzed against 0.05 M NaCl in buffer A for the next purification step.

Step 2: First Chromatography on 3'5'-ADP-agarose—The first 1 fraction was applied to a column of 3'5'-ADP-agarose (14 × 100 mm, 15 ml, 1.9 μmol of 3'5'-ADP/ml of gel) equilibrated with buffer A containing 0.05 M NaCl at a flow rate of 13 ml/h. The column was washed with 120 ml (8 volumes of a column) of buffer A containing 0.05 M NaCl and then eluted with 75 ml (5 volumes of a column) of 0.2 M 3'5'-ADP in buffer A containing 0.05 M NaCl. This fraction, containing sulfotransferase activity, was brought to 0.15 M NaCl by adding buffer A containing 1 M NaCl. The above processes (steps 1 and 2) were repeated three times, and the fractions thus obtained were combined.

Step 3: Second Heparin-Sepharose CL-6B Chromatography—The first 1 fraction combined was applied to a heparin-Sepharose CL-6B column (16 × 50 mm, 10 ml) equilibrated with buffer A containing 0.15 M NaCl at a flow rate of 26 ml/h. The column was washed with 30 ml (3 volumes of a column) of buffer A containing 0.15 M NaCl. The sulfotransferase activity was eluted with a linear gradient from 0.15 to 1.0 M NaCl in buffer A (total volume, 300 ml). The fractions containing sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 2), brought to 1% (w/v) Triton X-100, and dialyzed against 1 M NaCl in buffer A and then against 0.05 M NaCl in buffer A for the next purification step.

Step 4: Second Chromatography on 3'5'-ADP-agarose—The first 1 fraction was subjected to the second 3'5'-ADP-agarose chromatography (the first 3'5'-ADP-agarose chromatography was performed). Sulfotransferase activity was eluted with 0.2 M 3'5'-ADP in buffer A containing 0.05 M NaCl. This fraction was brought to 0.15 M NaCl in buffer A, and concentrated to about 8 ml with polyethylene glycol. After dialysis against 0.1 M NaCl in buffer A, the fraction was applied onto a small heparin-Sepharose CL-6B column (bed volume, 0.5 ml) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 5 ml of 0.1 M NaCl in buffer B (buffer A containing not 0.13 but 0.02% Triton X-100) and then eluted with 2.5 ml of 1 M NaCl in buffer B in order to remove 3'5'-ADP. The eluate was then concentrated to about 0.5 ml, and dialyzed against 1 M NaCl in buffer A for the next step.

Step 5: Chromatography on Superose 12—Superose 12 HR 10/30 was equilibrated with buffer A containing 2 M NaCl. The step 4 fraction was applied to the column. The sulfotransferase activity was pooled and dialyzed against 2 M NaCl at a flow rate of 0.25 ml/min. The fractions (0.25 ml each) containing sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 3A) and dialyzed against 0.15 M NaCl in buffer A. The fraction was stored at –20°C.

Assay for Sulfotransferase Activity

Sulfotransferase activity was determined as described previously (15). Briefly, the standard reaction mixture (50 μl) contained 2.5 μmol of D-glucose, 10 mM HCl, pH 6.8, 3.7 μg of potassium chloride, 25 nmol (as hexosamine) of CDNSNS-heparin, 50 pmol of [35S]PAPS (about 5 × 107 cpm), and enzyme. When the activity of HS2ST was monitored at each purification step, 10 mM dithiothreitol (DTT) was added to the standard reaction mixture. After incubation at 37°C for 20 min, the reaction was terminated by heating at 100°C for 1 min. Carrier chondroitin sulfate A and B was added to the reaction mixture for calibration. The reaction mixtures were then subjected to precipitation with ethanol containing 1.3% potassium acetate and 0.5 mM EDTA and by subsequent gel chromatography on a Fast desalting column to remove [35S]PAPS and its degradation products. The incorporation of [35S]Sulfate was linear with the amounts of enzyme proteins and with the incubation time under the above conditions (up to 5.8 ng of protein and 50 min when the purified enzyme was used) (data not shown). One unit of enzyme activity was defined as the amount required to transfer 1 pmol of sulfate at 20°C.
Purification of Heparan Sulfate 2-Sulfotransferase

We successfully purified HS2ST from the extract of CHO cells to a apparent homogeneity with about 51,700-fold purification. Table II shows a summary of the purification of HS2ST from 1,800 ml of the crude extract (3.3 x 10^10 cells). In this experiment, HS2ST activity was specifically determined using the standard assay mixtures containing 10 mM DTT as described under "Experimental Procedures." The concentration had little effect on HS2ST activity as shown later, while HS6ST activity was substantially decreased (15).

The details for each purification step are as follows.

**Step 1: First Heparin-Sepharose Chromatography—The**

**Distribution of heparan sulfate 2-sulfotransferase in CHO cell culture**

In our previous study using CHO cells cultured on plates (15), HS2ST activity was hardly detected in the culture medium, although the CHO cell heparan sulfate contained hexuronic acid residue in heparan sulfate, (6)diS. As is evident from the summarized results (Table I), the major sulfotransferase activity retained in the cell layer catalyzed the transfer of sulfate to position 2 of hexuronic acid residue in heparan sulfate, i.e. HS2ST. The similar results were also obtained when CHO cells were cultured in suspension in a spinner flask (data not shown), which enabled us to culture cells on a large scale. Therefore, spinner culture was chosen in place of adhesion culture to obtain CHO cells enough to purify HS2ST.

**Table I.**

**Distribution of heparan sulfate 6-sulfotransferase and 2-sulfotransferase in CHO cell culture.**

| Step | Volume | Total activitya | Total protein | Specific activity | Purification | Recovery |
|------|--------|-----------------|---------------|------------------|--------------|----------|
|      | ml     | 10^3 x unitsb   | mg            | 10^4 x units/mg  | -fold        | %        |
| Crude extract | 1,800 | 20.0 | 4,560 | 0.0000439 | 1 | 100 |
| First heparin-Sepharose | 300 | 37.5 | 390 | 0.00962 | 22 | 188 |
| First 3.5'-ADP-agarose | 225 | 23.6 | 5.60 | 0.421 | 959 | 118 |
| Second heparin-Sepharose | 96 | 12.9 | 1.22 | 1.06 | 2,420 | 65 |
| Second 3.5'-ADP-agarose | 75 | 4.75 | 0.0405 | 11.7 | 26,700 | 24 |
| Superoxide | 12 | 1.75 | 0.00546 | 22.7 | 51,700 | 6 |

a One unit of activity is defined as 1 pmol/min sulfate transferred to CDSNS-heparin used as substrate as described under "Experimental Procedures."
b One unit of activity is defined as 1 pmol/min sulfate transferred to CDSNS-heparin used as substrate as described under "Experimental Procedures."

**RESULTS**

**Distribution of Heparan Sulfate 6-Sulfotransferase and 2-Sulfotransferase Activities in CHO Cell Culture**

The galactosamine and glucosamine contents of glycosaminoglycans were determined by the Elson-Morgan method as modified by Strominger et al. (30) after hydrolysis of the glycosaminoglycans with 6 M HCl at 100°C for 4 h. Uronic acid was determined by the method of Strominger et al. (30) after hydrolysis of the glycosaminoglycans with 6 M HCl at 100°C for 4 h. Uronic acid was determined by the method of Bitter and Muir (31). Protein concentration was determined by a micro-BCA protein assay reagent kit (Pierce) using bovine serum albumin as a standard. 6.6 ng of the enzyme protein was digested with 0.5 unit of BCA protein assay reagent kit (Pierce) using bovine serum albumin at 37°C for 2 h. The digested products were injected together with standard unsaturated disaccharides into a column of PAMN (4.6 mm x 25 cm). The fractions corresponding to 35S-labeled disaccharides were collected and analyzed by HPLC on a Partisil-10 SAX column.

**Other Methods**

The sulfotransferase activities were determined under standard assay conditions containing 10 mM DTT as described under "Experimental Procedures." In the case of the products by the spent medium, major radioactivity was recovered in a peak of ΔDi-(U)diS (ΔDi-(6)diS:ΔDi-(U)diS = 96:4) as described previously (15). On the other hand, in the case of the products by the cell extract, major radioactivity was recovered in a peak of ΔDi-(U)diS, and only a small amount of radioactivity was in a peak of ΔDi-(6)diS (ΔDi-(U)diS:ΔDi-(6)diS = 92:8). As is evident from the summarized results (Table I), the major sulfotransferase activity retained in the cell layer catalyzed the transfer of sulfate to position 2 of hexuronic acid residue in heparan sulfate, i.e. HS2ST.

**Analysis of Enzymatic Reaction Products**

Analysis of enzymatic reaction products was performed by HPLC as described previously (15) with some modification. Briefly, [35S]labeled products were digested with a mixture of 10 units of heparitinase I, 1 milliunit of heparitinase II, and 10 milliunits of heparitinase III in 40 μl of 50 mM Tris-HCl, pH 7.2, 1 mM CaCl2, 4 μg of bovine serum albumin at 37°C for 2 h. The digested products were injected together with standard unsaturated disaccharides into a column of PAMN (4.6 mm x 25 cm). Fractions of 0.6 ml were collected and mixed with 3 ml of Ready Safe Scintillator (DuPont NEN), and the radioactivity was determined. Degradation of the [35S]labeled glycosaminoglycans with nitric acid at pH 1.5 and reduction with NaBH4 were carried out as described by Shively and Conrad (29). Anomalous deamination products were cleaved by the additional treatment of mild acid hydrolysis (25 mM H2SO4 at 80°C for 30 min) (16, 17). The final products were subjected to gel filtration on a Superdex 30 equilibrated with 0.5 M NH4HCO3. The fractions corresponding to [35S]labeled disaccharides were collected and analyzed by HPLC on a Partisil-10 SAX column.

**TABLE I.**

| Step | Volume | Total activitya | Total protein | Specific activity | Purification | Recovery |
|------|--------|-----------------|---------------|------------------|--------------|----------|
|      | ml     | 10^3 x unitsb   | mg            | 10^4 x units/mg  | -fold        | %        |
| Crude extract | 1,800 | 20.0 | 4,560 | 0.0000439 | 1 | 100 |
| First heparin-Sepharose | 300 | 37.5 | 390 | 0.00962 | 22 | 188 |
| First 3.5'-ADP-agarose | 225 | 23.6 | 5.60 | 0.421 | 959 | 118 |
| Second heparin-Sepharose | 96 | 12.9 | 1.22 | 1.06 | 2,420 | 65 |
| Second 3.5'-ADP-agarose | 75 | 4.75 | 0.0405 | 11.7 | 26,700 | 24 |
| Superoxide | 12 | 1.75 | 0.00546 | 22.7 | 51,700 | 6 |

a The sulfotransferase activities were determined under standard assay conditions containing 10 mM DTT as described under "Experimental Procedures."
b One unit of activity is defined as 1 pmol/min sulfate transferred to CDSNS-heparin used as substrate as described under "Experimental Procedures."
of CHO cells was applied to a heparin-Sepharose column as described under “Experimental Procedures.” After wash with buffer containing 0.15 M NaCl, the column was eluted with a linear gradient of NaCl. Fractions of 13 ml were collected. Sulfotransferase activity in the absence (○) or presence (□) of 10 mM DTT, and protein concentration (- - -) of each fraction were assayed. The broken line (- - -) indicates the concentration of NaCl.

The crude extract from CHO cells was applied to a heparin-Sepharose column equilibrated with buffer A containing 0.15 M NaCl (Fig. 1). More than 75% of total proteins passed through the column, and most of the proteins adsorbed to the column were eluted at the low NaCl concentration earlier than HS2ST activity. The sulfotransferase activity inhibited by DTT was noted in the early fractions (fraction number approximately 6). This chromatography brought a 1.9-fold increase of the total activity of HS2ST, suggesting that some inhibitors for HS2ST or degrading enzymes for substrate PAPS might have been removed at this step.

Step 2: First 3',5'-ADP-agarose Chromatography—This column chromatography resulted in a 44-fold purification of HS2ST. Since the sulfotransferase activity appeared to be stable in the higher concentration of NaCl, each fraction was collected in a tube containing 1 ml NaCl in buffer A to make the 0.15 M NaCl solution. HS2ST was also strongly inhibited by 3',5'-ADP, as observed with other glycosaminoglycan sulfotransferases (12, 15, 32). The concentration of 3',5'-ADP giving a 50% inhibition of this sulfotransferase activity was found to be less than 2.5 μM (data not shown). HS2ST activity at this step, therefore, was determined after the removal of 3',5'-ADP by adsorbing HS2ST to a small heparin-Sepharose column (bed volume, 0.5 ml) and eluting it with 1 M NaCl in buffer A.

Step 3: Second Heparin-Sepharose Chromatography—The elution was performed with a linear gradient of NaCl concentration (Fig. 2). HS2ST activity was eluted with most of the proteins adsorbed to the column. The sulfotransferase activity inhibited by DTT was noted in the early fractions (fraction number approximately 6). This chromatography resulted in an 11-fold purification of HS2ST (Fig. 3). 35S-labeled CDSNS-heparin produced by incubation with [35S]PAPS and this DTT-inhibited fraction gave a major disaccharide of ΔDi-(6)diS (data not shown). The fractions indicated by a horizontal bar in Fig. 3A were pooled, dialyzed as described under “Experimental Procedures,” and used for the subsequent experiments as purified HS2ST.

Purity of the Heparan Sulfate 2-Sulfotransferase

SDS-PAGE for the fractions (nos. 35–47) obtained by the Superose 12 chromatography showed that the fractions around a peak of HS2ST activity contained two protein bands that were migrated closely to each other with apparent molecular weights of 47,000 and 44,000, respectively (Fig. 3B). Considering the HS2ST activity in those fractions, these protein bands appeared to correspond to HS2ST. When an aliquot of the step 4 fraction was applied to chromatography on heparin-Sepharose CL-6B and every four fractions around the peak of the HS2ST activity were subjected to SDS-PAGE (Fig. 4), two protein bands of M, 47,000 and 44,000 appeared exclusively in the fractions containing the HS2ST activity. Their staining intensity with silver nitrate fairly corresponded to the activity, and their relative intensities were almost constant, which further suggested the correspondence of these two protein bands to HS2ST.

Samples at each purification step (0.47 μg of protein for each) were also analyzed by SDS-PAGE (Fig. 5A). Both protein bands of M, 47,000 and 44,000 were stained more intensely with silver nitrate in the samples at the higher fold purification, and were the most predominantly stained in the Superose 12 fraction ("purified HS2ST") (Fig. 5A, lane 7). In addition, the fraction that passed through the second 3',5'-ADP-agarose column and had the little activity exhibited little if any of the two protein bands on SDS-PAGE (Fig. 5A, lane 6). Such a concomitancy between the two protein bands and the HS2ST activity
again suggested that the two protein bands correspond to HS2ST.

When the N-glycanase digest of the purified HS2ST was subjected to SDS-PAGE, the two protein bands of 47 and 44 kDa disappeared, but two sharp protein bands of 38 and 34 kDa appeared newly (Fig. 5B), indicating that both proteins are glycoproteins containing almost equal contents of carbohydrates (approximately 20%). The result further suggested that both proteins are closely related to each other.

However, it was worthy of note that the molecular weights of the two protein bands were half or one-third as much as the molecular weight of the HS2ST activity determined by gel chromatography on Superose 12 using standard M₉ proteins (Fig. 3A). When the purified HS2ST was again applied to the Superose 12 gel column, which was this time equilibrated with buffer A containing 4 M guanidine HCl (known to be a “dissociative” solvent), the HS2ST activity mostly disappeared in the fractions around M₉. 130,000 but, instead of it, a little but significant activity (<0.3% of the starting activity) was recovered in the fractions where ovalbumin (43 kDa) was eluted. Subsequent SDS-PAGE of the fractions revealed that the two proteins of M₉, 47,000 and 44,000 newly appeared in the fractions around 43 kDa (data not shown). The observed discrepancy between their elution position on gel chromatography and their mobilities on SDS-PAGE could be explained by the possibility that HS2ST may exist as a dimer or trimer, although other possibilities could also be considered.

Characterization of Purified Heparan Sulfate 2-Sulfotransferase
Specificity for Acceptor Substrate—The purified fraction of HS2ST was incubated with different acceptors. The purified HS2ST was able to transfer sulfate to CDSNS-heparin and mouse EHS tumor heparan sulfate (Table III). Interestingly,

![Fig. 3. Superose 12 gel chromatography of the second 3',5'-ADP-agarose fraction.](image)

**TABLE III**

Incorporation of [35S]sulfate from [35S]PAPS into various glycosaminoglycan acceptors by the purified heparan sulfate 2-sulfotransferase

| Substrate | Activity (unit)/ml
|-----------|------------------|
| CDSNS-heparin | 7.19 (100)² |
| Heparin | 1.44 (20) |
| Heparan sulfate (mouse EHS tumor) | 6.59 (92) |
| Heparan sulfate (pig aorta) | 0.81 (11) |
| Heparan sulfate (bovine liver) | 1.12 (16) |
| Chondroitin² | 0 |
| Chondroitin sulfate A | 0.05 (0.7) |
| Chondroitin sulfate C | 0 |
| Dermatan sulfate | 0 |
| Keratan sulfate | 0 |
| CDSNS-heparin + heparin³ | 2.66 (37) |
| CDSNS-heparin + chondroitin sulfate C⁴ | 6.61 (92) |

²One unit of activity is defined as 1 pmol/min sulfate transferred to each glycosaminoglycan used as substrate as described under “Experimental Procedures.”

³The values in parentheses indicate the percentage of the enzyme activity compared with that of CDSNS-heparin.

⁴The reaction mixture contained 1.25 μg of protamine chloride instead of 3.75 μg.

The unsaturated disaccharide compositions of heparan sulfate from EHS tumor, pig aorta, and bovine liver in Table III are expressed as percent of total disaccharides, respectively.

**TABLE IV**

Unsaturated disaccharide compositions of heparan sulfates from EHS tumor, pig aorta, and bovine liver

| Heparan sulfate | Unsaturated disaccharides | % of total | 
|-----------------|---------------------------|-----------|
| Mouse EHS tumor ² | ΔDi-(5)S ΔDi-(5)NS ΔDi-(5)6S ΔDi-(6)S | 2.6 2.3 4.4 3.8 |
| Pig aorta² | 63.9 18.5 6.0 | 2.6 2.3 4.4 3.8 |
| Bovine liver (highly sulfated fraction) | 21.9 12.7 18.2 | 2.6 2.3 4.4 3.8 |

²Data were cited from the previous report (4).
EHS tumor heparan sulfate, 35S-labeled product derived from the position of the sulfate group transferred to CDSNS-heparin or EHS tumor heparan sulfate. The results indicated that the purified enzyme showed inhibition of the transfer of sulfate to CDSNS-heparin, while chondroitin, chondroitin sulfate A, chondroitin sulfate B, dermatan sulfate, and keratan sulfate. Moreover, heparin, heparan sulfate from pig aorta, and heparan sulfate from bovine liver (highly sulfated fraction) were poor acceptors. The disaccharide compositions of these heparan sulfates were determined as shown in Table IV. The enzyme showed no activity toward chondroitin, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, and keratan sulfate. Moreover, heparin, which is rich in the unit of the reaction product by HS2ST, C, dermatan sulfate, and keratan sulfate. Furthermore, heparin, heparan sulfate from pig aorta, and heparan sulfate from bovine liver (highly sulfated fraction) were poor acceptors.

Characterization of the Reaction Products—To determine the position of the sulfate group transferred to CDSNS-heparin or EHS tumor heparan sulfate, 35S-labeled product derived from each glycosaminoglycan acceptor was digested with a mixture of heparitinase I, II and III, and the digest was then subjected to HPLC on a PAMN column as described under “Experimental Procedures” (Fig. 6). In either case, most of radioactivity was eluted at the position of A-Di-(U)-diS. Furthermore, HPLC on a Partisil-10 SAX column of 35S-labeled disaccharides produced by nitrous acid degradation at pH 1.5 showed that most of radioactivity was eluted at the position of IdoA-GlcNSO3 unit in CDSNS-heparin or EHS tumor heparan sulfate (Fig. 6).

Properties of the Heparan Sulfate 2-Sulfotransferase—The pH dependence of HS2ST activity was shown in Fig. 9A. The maximal activity was observed at pH 5.4–5.8. The enzyme activity increased with the increase in PAPS concentration up to 1 μM and then reached the maximal level (approximately 310 unit/μg enzyme protein) (Fig. 9E). The apparent Km value of HS2ST for PAPS was 2.0 × 10⁻⁷ M.

DISCUSSION

We have purified HS2ST to an apparent homogeneous level from the extract of the cultured CHO cells by affinity chromatography with heparin-Sepharose and 3',5'-ADP-agarose. As was also the case with heparan sulfate/heparin N-sulfotransferase, HS6ST and chondroitin 6-sulfotransferase (11, 12, 15, 32), affinity chromatography on those columns yielded successful purification of this enzyme. Furthermore, gel chromatogra-
The purification of Heparan Sulfate 2-Sulfotransferase

The purified HS2ST fraction was found to transfer sulfate exclusively to position 2 of the L-iduronic acid residue of IdoA-GlcNSO$_3$ unit in CDSNS-heparin or EHS tumor heparan sulfate (Fig. 8), and none of the activity was observed to transfer sulfate to position 2 of O-glucuronic acid residue, position 6 of N-sulfoglucosamine residue or amino group of glucosamine residue. Wlad et al. (33) have recently purified ~60-kDa enzyme capable of catalyzing both the 2-O- and 6-O-sulfotransferase reactions from mouse mastocytoma tissue. A proteolytic fragment (~20 kDa) of this original enzyme remains capable of promoting 2-O-sulfation but has lost the 6-O-sulfotransferase activity, indicating that the two reactions are catalyzed by separate active sites derived from a single protein. We previously showed that HS6ST from the culture medium of CHO cells transfers sulfate exclusively to position 6 of N-sulfoglucosamine residue (15). The difference between the reports by Wlad et al. and us suggests that our HS6ST and HS2ST from CHO cells are distinct from their enzyme from mouse mastocytoma tissue capable of catalyzing both the 2-O- and 6-O-sulfotransferase reactions. Supposing that HS2ST and HS6ST be derived from a composite enzyme containing distinct domains, each committed to a specific O-sulfotransferase reaction, the composite enzyme would be expected to have a molecular mass of 90–100 kDa. This again suggests that our sulfotransferases may be genetically different from the sulfotransferase purified by Wlad et al. It is very likely that sulfotransferases prepared from CHO cells are engaged in the biosynthesis of heparan sulfate and the enzyme from mouse mastocytoma tissue is mainly involved in the biosynthesis of heparin. This is also the case with N-sulfotransferases (20–23). Considering these results, the molecular organization of the O-sulfation process may differ between heparin and heparan sulfate.

Modification reactions in the biosynthesis of heparin/heparan sulfate are thought to occur in the following sequences: N-deacetylation/N-sulfation of glucosamine, epimerization of O-glucuronic to L-iduronic acid, 2-O-sulfation of L-iduronic acid, and finally, 6-O- and 3-O-sulfation of glucosamine residue (1). In these sequential reactions, each product in the respective reaction becomes the substrate for the subsequent reaction and may be controlled by the substrate specificities of the enzymes involved. HS6ST has a capacity to catalyze 6-O-sulfation of N-sulfoglucosamine residue irrespective of the 2-O-sulfation of the neighboring L-iduronic acid residue (15), while HS2ST is unable to catalyze 2-O-sulfation of L-iduronic acid residue adjacent to GlcNSO$_3$(6SO$_3$) residue. This difference strongly supports the above modification reaction sequence. In relation to this control mechanism, it should be of note that HS2ST was much less active toward heparan sulfates from pig aorta and bovine liver (Table III). There seem to be some differences in the acceptor efficiency between EHS tumor heparan sulfate and the above two heparan sulfates as the good and poor acceptor substrates, respectively. The content of HexA-GlcNSO$_3$ unit in heparan sulfate may be one of factors affecting the substrate efficiency, because the content of this unit in EHS tumor heparan sulfate is the highest (Table IV). However, heparan sulfates from pig aorta and bovine liver contain significant amount of HexA-GlcNSO$_3$ unit, although they are very poor acceptors, suggesting that other factors such as the higher content of IdoA-GlcNSO$_3$ unit or a longer oligosaccharide sequence containing IdoA-GlcNSO$_3$ unit may be required for the recognition by HS2ST. Furthermore, HS2ST may be negatively controlled by its reaction products, IdoA(2SO$_4$)-GlcNSO$_3$ and IdoA(2SO$_4$)-GlcNSO$_3$ units, because heparin, a form of heparan sulfate containing the highest contents of these units, appeared to act as an inhibitor and, in addition, heparan sul-
Sulfations of position 2 of GlcA (a) and of position 2 of IdoA adjacent to GlcNSO₃(6SO₄) (b) were not observed.

It is interesting to compare the properties of HS2ST with those of other purified glycosaminoglycan sulfotransferase. DTT had little effect on HS2ST, while it substantially inhibited HS6ST (15). This finding was useful to monitor activities of both sulfotransferases separately in the present study. In contrast, DTT stimulated chondroitin 4-sulfotransferase (34). HS2ST appeared to exist as a dimer or trimer, because the enzyme behaved on gel chromatography 2–3 times larger than on SDS-PAGE (Fig. 3). However, considering the effect of Triton X-100 micelles on molecular mass determinations of membrane proteins, there is another possibility that HS2ST may exist as a monomer. Mass determinations for proteins embedded in the micelles might have caused the overestimation by the mass of the detergent micelle. The guanidine treatment would have dissociated the micelles, and, therefore, the enzyme might have run as a monomer independently of the detergent. Similarly to HS2ST, chick chondrocyte chondroitin 6-sulfotransferase apparently showed a single broad protein band of Mₗ 75,000 on SDS-PAGE although it was eluted at the position with an apparent molecular mass of 160,000 on gel chromatography (32, 35). A majority of proteins intrinsic to the Golgi apparatus membrane appear to be dimers in situ (36). However, this is not always the case. HS6ST from CHO cells may be a monomer (15). Rat liver heparan sulfate N-deacetylgalactosaminylsulfotransferase is also a monomer (11, 37). The apparent Kₘ value for PAPS of the purified HS2ST was 2.0 × 10⁻⁷ M, which was in the same order of that of HS6ST (4.4 × 10⁻⁷ M) (15). In contrast, that of heparan sulfate N-sulfotransferase of rat liver was 1.08 × 10⁻⁴ M (38). HS2ST as well as HS6ST appeared to have a higher affinity for PAPS than heparan sulfate N-sulfotransferase. The large difference in the Kₘ value for PAPS between our sulfotransferases and heparan sulfate N-sulfotransferase, however, might be due to the differences in the assay conditions used. For example, we added cationic activator, protamine, to the assay mixture in this study, while the assay mixture reported for heparan sulfate N-sulfotransferase (38) included Mg²⁺ and Mn²⁺, instead of protamine. We previously showed that cationic proteins such as protamine and histone stimulated chondroitin 6-sulfotransferase by decreasing the Kₘ value for PAPS (27). The low Kₘ values for PAPS of HS2ST as well as HS6ST may have been caused by the presence of protamine.

SDS-PAGE of the purified HS2ST gave only two protein bands of 47 and 44 kDa, which were both sensitive to N-glycanase digestion to the same extent (Fig. 5), as was also observed with HS6ST (15) and chondroitin 6-sulfotransferase (32, 35). Both proteins were always comigrated in the SDS gel whenever the fractions containing the HS2ST activity were subjected to SDS-PAGE (see Figs. 3 and 4 for the Superose 12 column chromatography and the heparin-Sepharose CL-6B column chromatography, respectively). These findings strongly support that both the 47- and 44-kDa proteins bear HS2ST activity and the size difference may be due to some protein modification such as limited proteolytic cleavage as we discussed previously in the case of HS6ST (15). Several glycosyltransferases that exhibit catalytically active multiple forms with different molecular weights have been shown to be de-
rived from the single genes (39–42). However, since our several trials to detect the HS2ST activity in the gel segments after SDS-PAGE of the purified HS2ST were unsuccessful, probably owing to a difficulty of renaturation of the enzyme activity (data not shown), one could still argue that either or both of the 47- and 44-kDa proteins represent contaminants. Relating to this possibility, one could also argue that the extent of purification was rather low (51,700-fold), compared with those of other Golgi enzymes. Because HS6ST was purified 10,700-fold when proteins originally present in the Golgi apparatus such as glycosyltransferases are secreted (42, 43). The molecular cloning of both sulfotransferases should provide us with a clue to answer these possibilities.

It should be noted that in cultured CHO cells more than 90% of the HS6ST activity was secreted into the medium, while 97% of the HS2ST activity was retained in the cells. Several reports have shown that the stem regions are cleaved off proteolytically when proteins originally present in the Golgi apparatus such as glycosyltransferases are secreted (42, 43). The molecular cloning of chondroitin 6-sulfotransferase suggests that the enzyme has shown that the stem regions are cleaved off proteolytically owing to a difficulty of renaturation of the enzyme activity (data not shown), one could still argue that either or both of the 47- and 44-kDa proteins represent contaminants. Relating to this possibility, one could also argue that the extent of purification was rather low (51,700-fold), compared with those of other Golgi enzymes. Because HS6ST was purified 10,700-fold when proteins originally present in the Golgi apparatus such as glycosyltransferases are secreted (42, 43). The molecular cloning of both sulfotransferases should provide us with a clue to answer these possibilities.

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