Purification and Characterization of *N*-Acetylglucosamine-6-sulfate Sulfatase from Bovine Kidney: Evidence for the Presence of a Novel Endosulfatase Activity†

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ABSTRACT: *N*-Acetylglucosamine-6-sulfate sulfatase (NG6SS) is an enzyme that catalyzes the hydrolysis of sulfate esters from the C-6 hydroxyl of *N*-acetylglucosamine. We report our purification and characterization of the enzyme and the discovery that it can remove sulfate from internally sulfated GlcNAc on glycopeptides and glycoproteins. The enzyme was purified from bovine kidney over 200000-fold using a combination of ion-exchange and size-exclusion chromatography. NG6SS is soluble and occurs as a single subunit with apparent solution molecular weight of 60.2 kDa on gel filtration chromatography and approximately 52.5 and 57.8 kDa on reducing and nonreducing SDS/PAGE, respectively. The enzyme is highly basic and exhibits a broad pH range with an optimum at pH 6.5 and a temperature optimum of 37 °C. Among the mono- and disaccharide sulfates tested, only GlcNAc-6-SO_4 is an effective substrate with a K_m of 4.7 mM, and either free sulfate or phosphate inhibits the activity. Unexpectedly, we found that the enzyme displays endosulfatase activity and quantitatively releases $^{35}SO_4$ from $^{35}SO_4$-labeled glycopeptides and intact glycoproteins isolated from human Molt-3 cells, which we have previously shown to synthesize glycoproteins containing GlcNAc-6-SO_4 residues within the sequence Galβ1-4[SO^-_3-6]-GlcNAcβ1-R of complex-type *N*-linked oligosaccharides. The *N*-terminal sequence of the bovine NG6SS was homologous to a human-liver-derived *N*-acetylglucosamine-6-sulfatase. The endosulfatase activity of bovine kidney NG6SS may be important in its potential role in the degradation of sulfated glycans and may make this enzyme a valuable reagent to study the biological functions of sulfated glycoproteins.

Sulfation is a common posttranslational modification of the carbohydrate moieties in many different glycoproteins and is a defining characteristic of many glycosaminoglycans. Sulfate esters occur at several positions on different monosaccharides, and a variety of *O*- and *N*-sulfated monosaccharides within glycoconjugates have been described (Sundblad et al., 1988a,b; Poduslo, 1990; Von Wurtemburg & Fries, 1989; Weaver et al., 1987; Braulke et al., 1987; Colburn & Bounassasi, 1987; Freeze & Varki, 1989; Griswold et al., 1986; Freeze & Wolgast, 1986; Stadler et al., 1983; Heifetz et al., 1980). Although the functions of sulfated carbohydrates in glycoproteins and glycosaminoglycans are not well understood, there are known systems in which sulfated glycans are recognized by specific receptors or have a definable activity. These include the recognition of GalNAc-4-sulfate within pituitary glycoproteins by a hepatic lectin (Fiete et al., 1991), the binding of heparin fragments containing GlcNAc-3-sulfate to antithrombin III (Lindahl et al., 1983; Atha et al., 1984), the effect of rhizobium-derived oligosaccharides containing GlcNAc-6-sulfate on plant nodulation (Lerouge et al., 1990), and the binding of the human endothelial glycoprotein GlyCAM-1 to L-selectin expressed by leukocytes (Imai et al., 1991).

In animals, GlcNAc-6-sulfate has been reported to occur in many glycoproteins including the complex oligosaccharides of gp120 from the HIV-1 infected human T-cell line Molt-3 (Shilatifard et al., 1993), chicken adipose lipoprotein lipase (Hoogewerf & Bensadoun, 1991), *Dictyostelium discoideum* lysosomal enzymes (Lacoste et al., 1989), bovine pulmonary artery endothelial cells (Roux et al., 1988), and human and bovine thyroglobulin (Spiro & Bhoyroo, 1988). One of the difficulties in defining the function(s) of GlcNAc-6-sulfate or other sulfated residues within an oligosaccharide has been the inability to enzymatically and efficiently remove sulfate from oligosaccharides. Thus far, only a small number of sulfatases have been characterized and, in some cases, purified and cloned (Freeman & Hopwood, 1987; Freeman et al., 1987; Neufeld & Muenzer, 1989; Basner et al., 1979).

Following our discovery of GlcNAc-6-sulfate within the lactosaminyl sequences of complex-type *N*-linked oligosaccharides in the envelope glycoprotein of HIV-1, we sought to define sulfatases, especially endosulfatases, that might help us study the biochemical significance of such a modification. An enzyme termed *N*-acetylglucosamine-6-sulfatase was previously purified from human liver and urine and was shown to have exosulfatase activity toward free GlcNAc-6-sulfate and terminally sulfated oligosaccharides (Freeman & Hopwood, 1987; Freeman et al., 1987; Basner et al., 1979). The difficulty in working with human tissues and the apparent restricted exosulfatase activity of the human enzyme prompted us to search for a GlcNAc-6-sulfate sulfatase from a more readily available source. Here, we report the purification and characterization of *N*-acetylglucosamine-6-sulfate sulfatase (NG6SS) from bovine kidney. In addition to its exosulfatase activity and release of sulfate from GlcNAc-6-sulfate, the enzyme also displayed an unexpected endosulfatase activity, as demonstrated by its ability to quantitatively release sulfate from $^{35}SO_4$-labeled Molt-3 glycopeptides and glycoproteins. The availability of this enzyme might enable a

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broader range of studies on the functions of GlcNAc-6-sulfate residues within glycoprotein oligosaccharides.

EXPERIMENTAL PROCEDURES

Materials. *N*-Acetylglucosamine-6-sulfate, glucose-6-sulfate, galactose-6-sulfate, *N*-acetylglucosamine-3-sulfate, *p*-nitrophenyl (PNP) derivatives of monosaccharides and sulfate, CNBr-activated Sepharose, QAE-Sephadex, phenylmethanesulfonyl fluoride (PMSF), aprotinin, pepstatin A, Leupeptin, Sephacryl S-200, CM-Sepharose, and RCA-I were obtained from Sigma Chemical Co. DE-52 was purchased from Whatman; XK-50, prepacked Superose-6, Mono-S, and prep grade Superose-6 and -12 resins were purchased from Pharmacia. Lactose and LNFP-III (Galβ1-4[Fucα1-3]-GlcNAcβ1-3Galβ1-4Glc) were obtained from V-Labs (Covington, LA). GluAβ1-4GalNAc-4-SO_4 and GluAβ1-4GalNAc-6-SO_4 were obtained from Seikagaku Fine Biochemicals. $Na_2{}^{35}SO_4$ (1050–1600 mCi/mmol) was purchased from DuPont NEN. Pronase (grade B) was purchased from CalBiochem. Fresh bovine kidneys were obtained from a local abattoir.

Buffers and Reagents. The following buffers were used in the purification of NG6SS: buffer A, 5 mM NaCl, 5 mM Tris base, 0.02% NaN_3, and 5 mM EDTA, pH 8.5; buffer B, 5 mM NaCl, 10 mM sodium acetate, 0.02% NaN_3, and 5 mM EDTA, pH 5.5; buffer C, 150 mM NaCl and 10 mM Tris-HCl, pH 7.5; buffer D, 2 mM pyridine acetate, pH 10.0; buffer E, 10 mM sodium acetate, 0.02% NaN_3, pH 5.5; and buffer F, 150 mM Na acetate, pH 6.5.

Assay for N-Acetylglucosamine-6-sulfate Sulfatase Activity. *N*-Acetylglucosamine-6-sulfate sulfatase activity was measured by its ability to hydrolyze *N*-acetylglucosamine-6-sulfate and release free *N*-acetylglucosamine. Enzyme-containing protein (or boiled protein as a control) was added to 50 μL of 2 mg/mL solution of *N*-acetylglucosamine-6-sulfate in buffer F in a 1.5-mL microfuge tube. The enzyme mixture was incubated at 37 °C for 5 h, and the reaction was stopped by the addition of 500 μL of buffer D preheated to 100 °C. The formation of the product was determined by either method 1 or 2 below. *One unit of NG6SS was defined as the amount of the enzyme that would release 1 nmol of sulfate from GlcNAc-6-sulfate in buffer D at 37 °C in 1 h.*

In method 1, we measured the release of *N*-acetylglucosamine from GlcNAc-6-sulfate following ion-exchange chromatography. The reaction mixture was applied to a 0.25-mL column of QAE-Sephadex in a Pasteur pipette in buffer D. After the reaction mixture passed through the column, the column was washed with an additional 0.5 mL of buffer D. A portion (0.2 mL) of the material not bound by QAE-Sephadex was assayed for the presence of the reducing sugar GlcNAc by the Park–Johnson assay (Park & Johnson, 1949).

In method 2, the product of NG6SS was analyzed by high-pH anion-exchange (HPAE) chromatography with pulsed amperometric detection using a Dionex system. After stopping the enzymatic digestion, 20 μL of the mixture was injected into a Dionex system with a 25-μL loop and a PA-1 column (4- × 250-mm) under the following gradient program: time 0 min, 100% 50 mM NaOH; time 5 min, 100% 50 mM NaOH; time 35 min, 100% NaOAc/100 mM NaOH; time 40 min, 100% NaOAc/100 mM NaOH; time 41 min, 100% 50 mM NaOH. No postcolumn NaOH was used for enhancement of detection. Both free GlcNAc and GlcNAc-6-sulfate were readily separable, as shown in Results.

Purification of NG6SS. All procedures were performed at 4 °C unless indicated otherwise. Throughout the purification procedure, total protein was monitored by the method of Lowry et al. (1951) and by the BCA assay (BioRad).

Step 1—Homogenization of bovine kidney. Two fresh bovine kidneys, about 500 g each, were trimmed of fat, chopped into small pieces, and washed in buffer A for 30 min. A glass Waring blender was chilled by homogenizing 50 g of ice in 500 mL of buffer A. After the buffer was chilled, the chopped tissues were added in small portions and homogenized for 15 min. Three milliliters of 100 mM PMSF was added to the homogenate, and the material was then centrifuged at 30000*g* for 1 h.

Step 2—Ammonium sulfate precipitation. The supernatant was filtered over cheesecloth and made 30% saturated with solid ammonium sulfate. The 30% precipitate was spun down at 30000*g* for 1 h, and the supernatant was saturated to 60% with solid ammonium sulfate. The 60% saturated solution was also spun down at 30000*g* for 1 h, and both precipitate and supernatant from this cut were also assayed for enzyme activity. The precipitate from the 30–60% ammonium sulfate cut was solubilized in 50 mL of buffer A containing 1 μg/mL aprotinin, leupeptin, and pepstatin-A, giving a total volume of 200 mL.

Step 3—Chromatography on a Sephacryl S-200. The 30–60% ammonium sulfate cut solution (100 mL each time) was applied (10 mL/min) to a 1-L XK-50 column of Sephacryl S-200 equilibrated in buffer A using an LKB-Pharmacia FPLC. Fractions (15 mL) were collected and assayed for NG6SS activity. Fractions containing 80% of the eluted activity were pooled. This step was necessary to remove lipid-containing material from the 30–60% ammonium sulfate cut.

Step 4—Anion-exchange chromatography on DE-52. The active enzyme from Sephacryl S-200 (300 mL) was dialyzed against buffer A (20 L) for 6 h and then applied to a 250-mL column of DE-52 (XK-50 column of LKB-Pharmacia) equilibrated in buffer A according to the directions of the manufacturer. The column was washed with 400 mL of buffer A and eluted with a 1000-mL linear gradient of NaCl (0–550 mM) in buffer A at the rate of 80 mL/h. Fractions (15 mL) were collected, and each fraction was assayed for enzyme activity. Fractions containing 80% of the eluted activity (325 mL) were pooled and dialyzed against buffer B (10 L) for 12 h. Upon dialysis against this pH 5.5 buffer, a considerable amount of protein precipitated but not the NG6SS activity. The soluble activity was termed the pH 5.5 cut.

Step 5—Cation-exchange chromatography on CM-Sepharose. The dialyzed fraction of enzyme activity from DE-52 was centrifuged at 50000*g* and the supernatant applied to a column of CM-Sepharose (1.5- × 25-cm) equilibrated in buffer B. This column was washed at the rate of 30 mL/h with 150 mL of buffer B and then eluted with a 160-mL linear gradient of NaCl (0–400 mM) in buffer B. Fractions (4 mL) were collected, and each fraction was assayed for enzyme activity. Fractions containing 90% of the eluted activity were pooled and dialyzed against buffer B (10 L) for 6 h.

Step 6—Rechromatography over CM-Sepharose. The pooled material from step 5 was reapplied to the CM-Sepharose column as above, but the elution was performed with a 210-mL salt gradient of 0–550 mM NaCl. Each fraction was assayed for NG6SS activity. Fractions containing 80% of the activity were pooled and carried to the next step. This step was included to further clarify the protein solution before continuing on the next Mono-S step. Without complete clarification, the Mono-S column flow was greatly hindered.

Step 7—Chromatography over Mono-S. The active fractions from step 6 were pooled and dialyzed against buffer E (10 L) for 5 h. This pool was applied to a column of Mono-S

HR 5/5 on a Pharmacia FPLC through a superloop. The column was washed for 10 min at 1 mL/min with the same buffer and then a salt gradient of 0–150 mM NaCl was applied in a step-gradient fashion with 10 mM increments of 10 mL at each step. Fractions (1 mL) were collected, and each fraction was assayed for NG6SS activity.

Step 8—Chromatography over Superose-12 Prep. The NG6SS was concentrated to 1 mL with a Centriprep 30 (Amicon) and then applied (1 mL/min) to a Superose-12 (prep grade) in an HR-16 column, LKB-Pharmacia, calibrated at 14 000 theoretical plates. Fractions (1 mL) were collected, and each fraction was tested for NG6SS activity.

Step 9—Chromatography over high-resolution Superose-6. The activity pooled from step 8 was concentrated to 100 μL and then applied to a Superose-6 HR (flow rate 200 μL/min), and fractions (200 μL) were collected. Each fraction was assayed for NG6SS activity.

Purity and Molecular Weight Determination by SDS/PAGE. Proteins were electrophoresed in a gradient polyacrylamide gel (10–20%) in reducing and nonreducing SDS/PAGE, as described by Laemmli (1970). Proteins in the gel were silver stained by a modification of the method of Blum et al. (1987) to facilitate staining of 0.5–0.7-mm gels.

Analysis of the pH and Temperature Optimum. NG6SS was added to tubes containing *N*-acetylglucosamine-6-sulfate at varying pH. The buffers used were sodium acetate, pH 4.0–6.0, Tris-HCl, pH 6.0–8.0, and sodium borate, pH 8.0–10.0. The formation of the product was assayed by method 1. The optimum temperature for the enzyme was measured by the addition of NG6SS to 1-mL microfuge tubes containing *N*-acetylglucosamine-6-sulfate in 100 mM sodium acetate buffer, pH 6.5. The mixture was incubated at varying temperatures, and the formation of the product was measured by method 1.

Assay for Contaminating Enzyme Activities. NG6SS from step 6 was tested for the presence of β-hexosaminidase, β-galactosidase, α-galactosidase, α-mannosidase, β-mannosidase, and aryl sulfatase by using corresponding PNP substrates, at approximately 1 mM. One unit of enzyme was incubated with substrates for 48 h at 37 °C, and products were then determined at 490 nm after adjustment of pH to 10.

Substrate Specificity. The specificity of NG6SS was determined by incubation of 1 unit of the enzyme with the following substrates under the standard conditions: GlcNAc-6-SO_4, Glc-6-SO_4, Gal-6-SO_4, GlcNAc-3-SO_4, GluAβ1-4GalNAc-4-SO_4, and GluAβ1-4GalNAc-6-SO_4. The formation of the neutral product was determined by anion-exchange chromatography with pulsed amperometric detection using the Dionex system, as described above.

Lectin Affinity Chromatography. A portion of the activity of NG6SS was applied to a 1-mL column of either Con A-Sepharose (1 mg/mL), RCA-I-Sepharose (4 mg/mL), or WGA-Sepharose (2 mg/mL). Each column was incubated with NG6SS for 6 h and then eluted as described previously (Cummings, 1993; Cummings & Kornfeld, 1982).

Metabolic Radiolabeling of Molt-3 Cells and Preparation of Glycoproteins and Glycopeptides. Molt-3 cells (10^7 cells) growing in RPMI-1640 supplemented with 10% fetal calf-serum were radiolabeled with either [6-^{3}H]galactose or $Na_2{}^{35}SO_4$, as described previously (Shilatifard et al., 1993), and total Pronase-digested glycopeptides were prepared. The [^{3}H]galactose-labeled and $^{35}SO_4$-labeled glycopeptides were directly treated with 1 unit of NG6SS or boiled-enzyme control at 37 °C for 24 h (5000 cpm/digestion). The reaction mixture was terminated by the addition of 10 μL of saturated Na_2SO_4 and 400 μL of a saturated solution of $BaCl_2$. The insoluble product, $Ba^{35}SO_4$, was collected by centrifugation, and both precipitated and unprecipitated counts were determined by liquid scintillation counting.

The human kidney cell line 293 was metabolically labeled with 1 mCi of $^{35}S^{35}S$ protein-labeling mix ([^{35}S]methionine/[^{35}S]cysteine) (from DuPont NEN) for 12 h, essentially as described before (Cummings, 1993). The cells were harvested after 12 h and desalted over a column of Sephadex G-25. About 60 000 cpm of the $^{35}S^{35}S$-labeled proteins was treated with either no NG6SS or boiled NG6SS, and 30 000 cpm was treated with active NG6SS for 24 h. Half of each reaction mixture was applied to a gradient SDS/PAGE, and the gel was run and developed as in Shilatifard et al. (1993). The other half of the reaction mixture was applied to a column of Sephadex G-50, and fractions of 1 mL were collected and analyzed by scintillation counting.

N-Terminal Sequencing. Two micrograms of purified NG6SS was electrophoresed on the gradient SDS/PAGE and then transblotted to PVDF membranes in 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid from Sigma) with 10% methanol. The blotted protein was stained in 0.2% Coomassie blue (no acetic acid) and destained with a 50% methanol solution. The 52.5-kDa band, which was the major band in the lane, was cut, and its *N*-terminal sequence was analyzed by gas-phase sequencing by Dr. Ken Jackson at the Center for Molecular Medicine, University of Oklahoma Health Sciences Center.

RESULTS

Presence of N-Acetylglucosamine-6-sulfate Sulfatase Activity in Bovine Kidney Extracts. To demonstrate that the soluble portion of bovine kidney extract contained an NG6SS activity, we mixed a portion of the extract with *N*-acetylglucosamine-6-sulfate under the standard conditions. Hydrolysis of the sulfate ester from the C-6 hydroxyl of *N*-acetylglucosamine-6-sulfate was assessed by both methods 1 and 2 as described in Experimental Procedures. The enzyme activity was present in homogenates and was dependent on both the amount of kidney extract and the time of incubation. The purification scheme for the enzyme is detailed in Table 1 and described in Experimental Procedures.

NG6SS was purified over 200000-fold with about 1.2% recovery. Approximately 0.5–1 μg of protein from steps 1–8 of the purification scheme was applied to a gradient (10–20%) SDS/PAGE, and proteins were visualized by silver staining (Figure 1A). Analysis of the material in step 8 of the purification on Superose-12 revealed a major protein band(s) at approximately 60 kDa.

We then performed a second chromatography to assess whether this major protein(s) from step 8 contained enzyme activity and to obtain a more purified form of the enzyme. The activity pooled from the Superose-12 in step 8 was applied to an analytical Superose-6 column, and each fraction was assessed for the enzyme activity and analyzed by SDS/PAGE. Figure 1B,C demonstrates that the peak of enzyme activity elutes in fractions 83–85, coincident with a major band of protein having an apparent size of approximately 60 kDa. The precise MW was estimated by plotting log MW versus migration for this major band in both nonreducing and reducing SDS/PAGE, as shown in Figure 2A,B, which indicates that the size is 57.8 and 52.5 kDa, respectively. The size of the protein was also estimated by its size-exclusion on the Superose-6 column (Figures 1B and 2C), indicating that its apparent size is 60.2 kDa. In Figure 1C, a faint band of protein around 33 kDa (indicated by the smaller arrow) was observed. This band may be a proteolytic fragment of the

Table 1: Purification of *N*-Acetylglucosamine-6-sulfate Sulfatase from Bovine Kidney

purification step[a]	total volume (mL)	total protein (mg)	total activity (units)[b]	specific activity (units/mg of protein)	recovery (%)	purification (fold)	step purification (fold)
1. total homogenate	800	82000	2.2×10^5	3	100		
2. 30–60% cut	200	28730	1.4×10^5	5	64	1.8	1.8
3. S-200	300	11850	8.1×10^4	7	37	2.54	1.4
4. DE-52, pH 5.5 cut	325	1292	6.9×10^4	53	31	20	7.8
	325	285	8.8×10^4	307	40	113	5.7
5. CM (0–400 mM)	25	6.2	4.3×10^4	6935	20	2568	22.6
6. CM (0–550 cM)	18	3.8	2.6×10^4	6842	12	2534	0.98
7. Mono-S	4.5	0.78	1.6×10^4	19638	7	7273	3.0
8. Superose-12	4.0	0.015	7.4×10^3	500000	3	183209	25.46
9. Superose-6	1.0	0.005	2.7×10^3	540000	1	200740	1.1

[a] Results are shown for the preparation of the enzyme from 1 kg of bovine kidney. Details of the procedure are given under Experimental Procedures. [b] A unit is defined as the amount of enzyme required to release 1 nmol of sulfate ester from the C-6 hydroxyl of *N*-acetylglucosamine as determined by method 1.

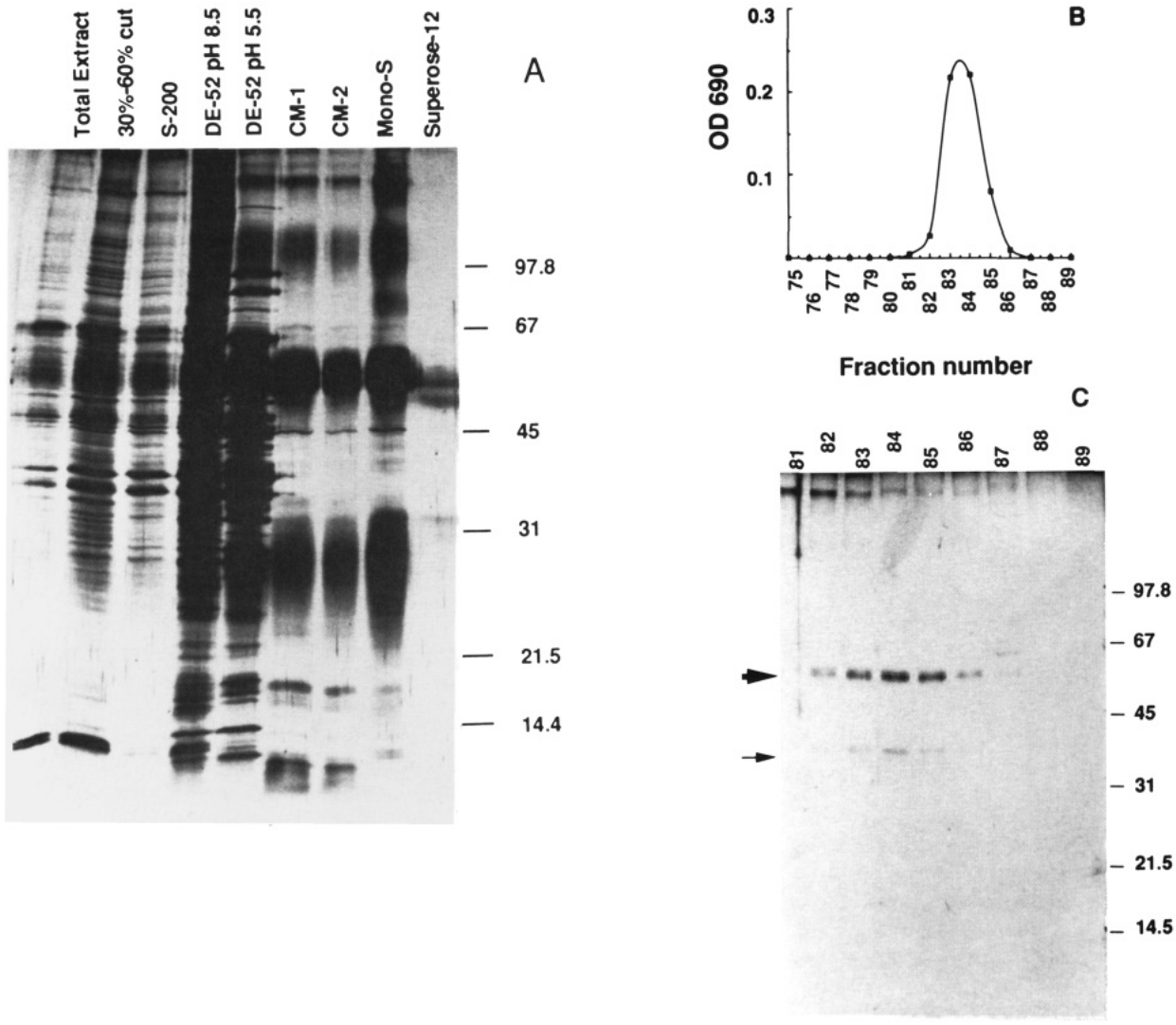

FIGURE 1: SDS/PAGE of material at each step in the purification. (A) From each step of purification, 0.5–1 μg of protein was applied to a gradient (10–20%) SDS/PAGE and analyzed by silver staining as described in Experimental Procedures. (B) Active fractions pooled from Superose-12 were concentrated to 0.1 μL and applied to the analytical Superose-6. A portion of each fraction was tested for NG6SS activity. (C) Also, a portion of each fraction from Superose-6 was analyzed by gradient (10–20%) SDS/PAGE under nonreducing conditions, and proteins were visualized by silver staining.

57.8-kDa protein, since it varied in amounts between different purifications in a fashion unrelated to the specific activity of NG6SS. As discussed below, we were able to obtain an *N*-terminal sequence for the 57.8-kDa protein, but insufficient amounts of the smaller 33-kDa protein were obtained for sequence comparison.

Assay for the Presence of Contaminating Activities. The NG6SS from step 6 following the second chromatography on CM-cellulose was tested for contaminating activity of other enzymes including aryl sulfatase, β-*N*-acetylhexosaminidase, β-galactosidase, α-galactosidase, α-mannosidase, and β-mannosidase, as described in Experimental Procedures. No significant or detectable activity of these enzymes was observed after a 48-h incubation with colorimetric substrates (data not shown, but see below).

Lectin Affinity Chromatography of NG6SS. NG6SS from step 6 of the purification scheme was applied to a column of Con-A, RCA-I, and WGA as described under Experimental

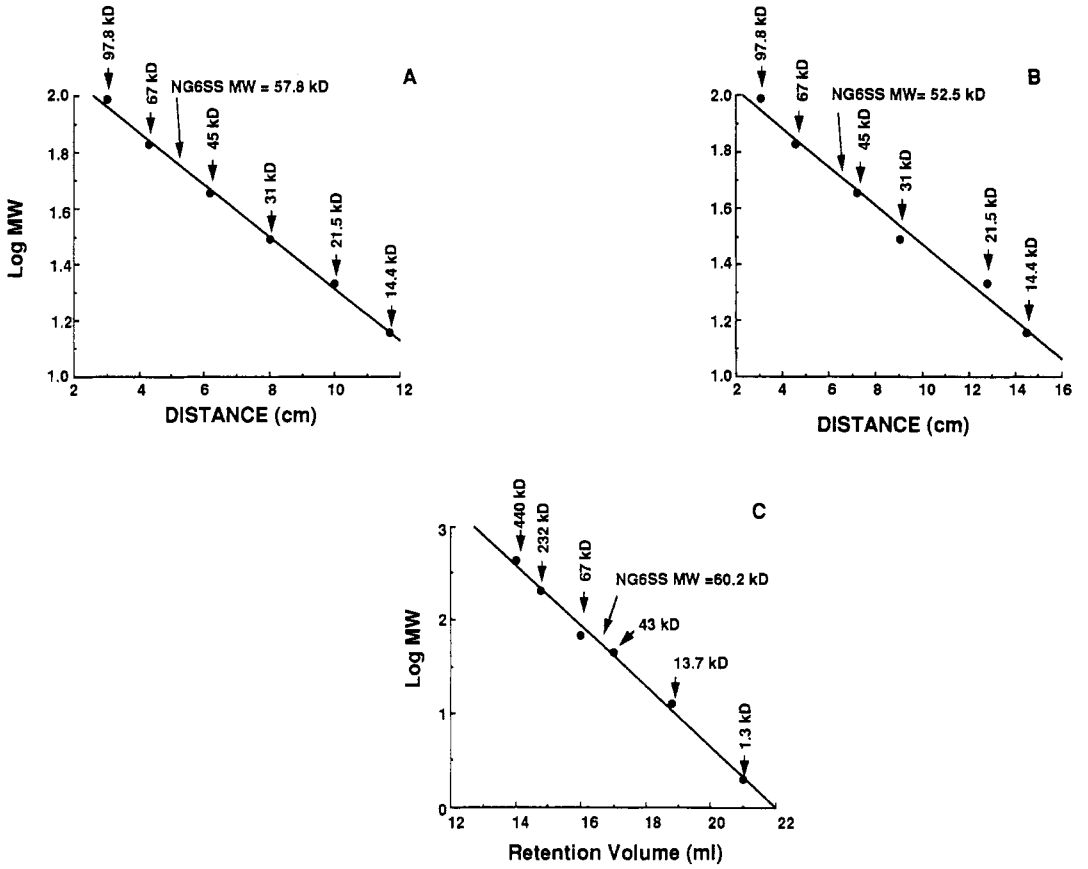

FIGURE 2: Molecular weight estimates of NG6SS. The log of the molecular weight of standard proteins versus migration in cm and the NG6SS are plotted for both (A) reducing and (B) nonreducing SDS/PAGE conditions. (C) The log of the molecular weight versus the retention volume of authentic standards on an analytical Superose-6 column from Figure 1B,C was plotted as described under Experimental Procedures.

Procedures. The NG6SS activity was bound by both Con-A and WGA but not RCA-I (data not shown). These results indicate that the enzyme contains *N*-linked oligosaccharides and it may have a mixture of high mannose/hybrid- and/or complex-type chains.

Temperature and pH Optima. Using GlcNAc-6-sulfate as a substrate, we found that NG6SS has a broad pH range with optimum activity at pH 6.5 and no activity either below pH 4 or above pH 9 (data not shown). The optimum temperature for the enzyme was 37 °C, and the enzyme had no observed activity at either 60 or 15 °C.

Stability and Storage. The purified enzyme was stable at 4 °C for about 1 week when stored in buffer B. However, pure NG6SS was unstable to freezing/thawing. NG6SS purified from step 6 following the second chromatography on CM-cellulose was stable at 4 °C for at least 1 month and could be frozen in buffer B at −80 °C for several months without significant loss of activity.

pI Measurement. We attempted to determine the p*I* of the enzyme using available ampholytes between a pH range of 6 and 9.5. However, the purified enzyme had a p*I* slightly greater than 9.0. The high basicity of this enzyme may contribute to its ability to attract acidic substrates containing *N*-acetylglucosamine-6-sulfate.

The N-Terminus of NG6SS Is Homologous to a Human-Liver-Derived N-Acetylglucosamine-6-sulfate Sulfatase. To obtain the *N*-terminal sequence of purified bovine kidney NG6SS, 2 mg of the enzyme from step 9 was electrophoresed in 10–20% polyacrylamide SDS-PAGE. The 57.8-kDa band visualized after staining was excised, and its *N*-terminus determined by gas-phase sequencing. The sequence is shown in Figure 3. The first residue could not be determined, but residues 2–11 demonstrate homology to the published *N*-terminal sequence of *N*-acetylglucosamine-6-sulfate sulfatase deduced from a partial cDNA clone for the enzyme from human liver (Robertson et al., 1988).

	1 * 11
Bovine kidney *N*-terminus:	X -Glu-Pro-Phe-Phe-Met-Met-Ile- X -Thr-Pro
Human liver NG6SS *N*-terminus:	Phe-Glu-Pro-Phe-Phe-Met-Met-Ile-Ala-Thr-Pro

FIGURE 3: *N*-Terminal sequence obtained from bovine *N*-acetylglucosamine-6-sulfate sulfatase. About 2 μg of bovine NG6SS was applied to a gradient SDS/PAGE (10–20%) and then blotted to immobilon as described in Experimental Procedures. The *N*-terminal sequence of the 52.8-kDa band was determined by gas-phase sequencing and is compared to the *N*-terminus of the 48-kDa human-liver-derived NG6SS (Robertson et al., 1988). The asterisk indicates that the amino acid was detected but, because of the poor recovery at this cycle, we are not certain. The X indicates that no specific amino acid could be identified at that cycle.

NG6SS Is Specific for the Hydrolysis of Sulfate Ester from the C-6 Position of N-Acetylglucosamine-6-sulfate. The substrate specificity of NG6SS toward several commercially available and simple sulfated mono- and disaccharides was determined (Table 2). Among all the tested substrates, the enzyme preferentially cleaved GlcNAc-6-sulfate, although there was slight activity toward Glc-6-sulfate. These results indicate that a primary sulfate ester, equatorial hydroxyl at C-4, and an acetylated primary amine at the C-2 position of the hexosamine promote efficient recognition by this enzyme. The observation that only 40% of the GlcNAc-6-sulfate was hydrolyzed with exhaustive enzyme treatment led us to consider whether free sulfate could inhibit the enzyme. This was confirmed when the enzyme was incubated with GlcNAc-6-sulfate in the presence of different concentrations of Na_2SO_4 or Na_2HPO_4 ranging from 1 to 20 mM. More than an 80% inhibition of activity was observed at 5 mM or higher of

Table 2: Substrate Specificity of *N*-Acetylglucosamine-6-sulfate Sulfatase

substrate[a]	sulfate ester cleaved (%)
GlcNAc-6-SO_4	39
Glc-6-SO_4	4
Gal-6-SO_4	<1
GlcNAc-3-SO_4	<1
GluAβ1-4GalNAc-4-SO_4	<1
GluAβ1-4GalNAc-6-SO_4	<1
PNP-SO_4[b]	<1

[a] Each substrate at a concentration of 5 mM was incubated with 1 unit of NG6SS (purified up to step 6, Table 1) for 24 h at 37 °C. The release of sulfate from substrate was determined by HPAE chromatography on a PA-1 Dionex column equipped with PAD, as described in Experimental Procedures. [b] Cleavage of sulfate was monitored by a colorimetric assay for PNP, as described in Experimental Procedures.

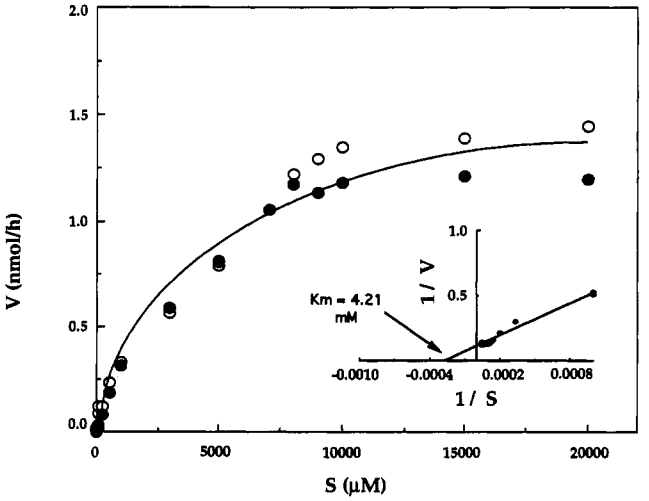

FIGURE 4: K_m and V_{max} of NG6SS for *N*-acetylglucosamine-6-sulfate. *N*-Acetylglucosamine-6-sulfate sulfatase from step 7 of the purification scheme was incubated with the indicated concentrations of *N*-acetylglucosamine-6-sulfate (100 μM–100 mM). Formation of the product was measured by HPLC on Dionex by method 2 in Experimental Procedures. For each reaction tube, 0.5 mg of total protein was used. The K_m and the V_{max} were determined from the Lineweaver–Burk plot of the Michaelis–Menten saturation kinetics as shown in the inset.

free sulfate or phosphate (data not shown). Preliminary studies also suggest that free GlcNAc can inhibit NG6SS activity.

K_m and V_{max} for N-Acetylglucosamine-6-sulfate Sulfatase. The K_m and the V_{max} of NG6SS toward *N*-acetylglucosamine-6-sulfate was determined from double-reciprocal plots of $1/V$ versus $1/S$ as shown in Figure 4. The enzyme from step 7 of the purification was assayed under linear conditions for time and protein. The K_m of this enzyme for *N*-acetylglucosamine-6-sulfate was determined to be 4.2 mM, and its V_{max} was approximately 3 nmol/mg h.

Ability of NG6SS To Release Sulfate from Glycoproteins Containing GlcNAc-6-sulfate within a Complex-Type N-Linked Oligosaccharide. We recently demonstrated that glycoproteins from Molt-3 cells add sulfate to the C-6 hydroxyl of internal *N*-acetylglucosaminyl residues within the sequence NeuAcα2-3(6)Galβ1-4GlcNAcβ1-R of complex-type *N*-linked oligosaccharides (Shilatifard et al., 1993). All of the sulfate esters found on the *N*-acetylglucosamine of the sulfated oligosaccharides synthesized by Molt-3 cells were shown to be penultimate to galactose, as evidenced by methylation analysis, hydrazine/nitrous acid fragmentation, and differential hydrolysis. To test whether NG6SS could release sulfate from these glycopeptides, we performed several types of experiments.

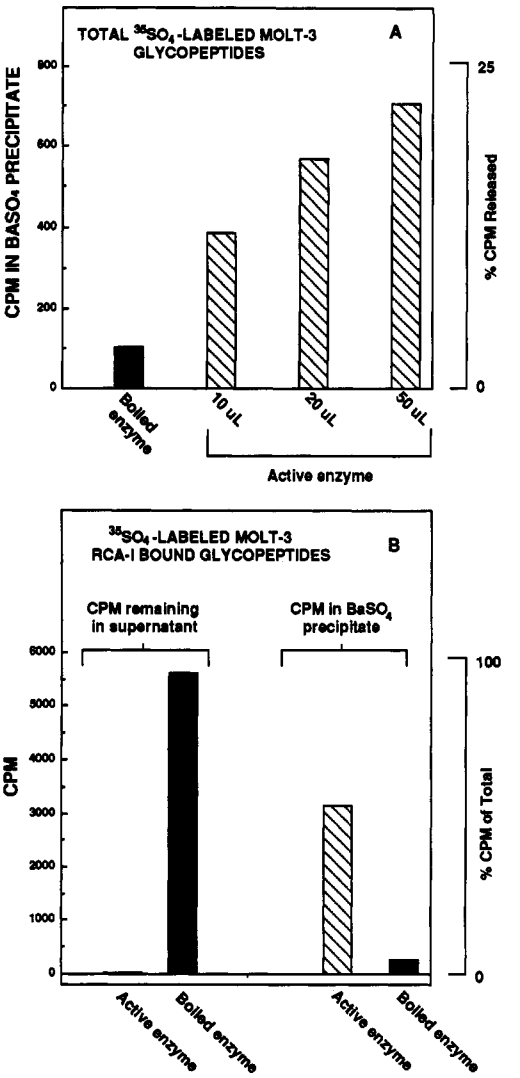

FIGURE 5: Release of sulfate by NG6SS from internal GlcNAc-6-sulfate residues in glycopeptides. Molt-3 cells were metabolically labeled with $^{35}SO_4$, and glycopeptides were obtained by Pronase digestion as described in Experimental Procedures. (A) $^{35}SO_4$-labeled glycopeptides were treated with increasing amounts of NG6SS (or boiled enzyme) from step 7 for 2 h at 37 °C. (B) A portion of the $^{35}SO_4$-labeled glycopeptides was treated with 10 mM HCl to remove sialic acid and then passed over a column of RCA-I-Sepharose. $^{35}SO_4$-labeled glycopeptides bound by RCA-I, as described in Experimental Procedures, were incubated with 1 unit of NG6SS (or boiled enzyme) for 24 h at 37 °C. To each reaction mixture were added 10 μL of a saturated solution of $NaSO_4$ (as carrier) and 400 μL of a saturated solution of $BaCl_2$. Both precipitate ($Ba^{35}SO_4$) and supernatant were analyzed by liquid scintillation counting. Approximately 5000 cpm of $^{35}SO_4$-labeled glycopeptides was used for each of the incubations described in (A) and (B).

First, we prepared $^{35}SO_4$-labeled glycopeptides from Molt-3 cells and incubated them with NG6SS. The release of $^{35}SO_4$ was determined by the barium sulfate precipitation assay described in Experimental Procedures. A preparation of NG6SS from step 6 released $^{35}SO_4$ from these glycopeptides, but boiled enzyme was inactive (Figure 5A). In addition, we desialylated a portion of the $^{35}SO_4$-labeled Molt-3 cell-derived glycopeptides and applied them to a column of RCA-I-Sepharose, which we showed previously binds to the sulfated complex-type *N*-linked oligosaccharides from these cells (Shilatifard et al., 1993). These galactose-terminating $^{35}SO_4$-labeled glycopeptides were also efficiently cleaved by NG6SS (Figure 5B). Again, the boiled enzyme was inactive. In this experiment, there was nearly quantitative release of $^{35}SO_4$, as evidenced by the loss of radiolabel in the supernatant

(Figure 5B). However, the barium sulfate precipitate is extremely insoluble and not all of the material could be resolubilized.

Second, we considered the possibility that release of sulfate ester from the C-6 hydroxyl of N-acetylglucosamine from Molt-3 glycopeptides by NG6SS was due partly to the presence of contaminating activity of β-galactosidase or some other glycosidase which could remove terminal galactose and generate a terminal GlcNAc-6-sulfate residue. To test this possibility, we performed two experiments. In the first experiment, we incubated the preparation of enzyme from step 6 with GlcNAc-6-sulfate, LNFP III (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc), and lactose for 24 h and analyzed the reaction products by HPAE chromatography on a Dionex system. The enzyme efficiently cleaved GlcNAc-6-sulfate, but the other two substrates were not substantially cleaved (data not shown). No galactose was released under the same conditions from LNFP III, and less than 1% of galactose was released from lactose after 24-h incubation with NG6SS. These results demonstrate that there are negligible levels of exoglycosidase activities in the preparation of NG6SS.

As a second method to assess the integrity of glycopeptides after treatment with NG6SS and the ability to release sulfate from intact glycopeptides, we prepared [^{3}H]galactose-labeled glycopeptides from metabolically radiolabeled Molt-3 cells. We previously demonstrated that a significant fraction of these glycopeptides are both sulfated and sialylated (Shilatifard et al., 1993). Desialylation results in the production of glycopeptides whose only charge under these conditions is contributed by sulfate. We desialylated the [^{3}H]galactose-labeled glycopeptides by treatment with 10 mM HCl, 100 °C, 1 h, and purified the charged, sulfated material over a column of QAE-Sephadex. The rechromatography of these [^{3}H]galactose-labeled, sulfated glycopeptides after treatment with boiled NG6SS is shown in Figure 6A, and the results are as predicted by the studies of Shilatifard et al. (1993). The QAE-Sephadex elution profile in Figure 6A demonstrates that a majority of glycopeptides have a single sulfate (eluted with 20 mM pyridine acetate) while some others have two to three sulfates and are eluted with 70 and 140 mM pyridine acetate, respectively. Treatment of these glycopeptides with active NG6SS caused a quantitative loss of charged species (Figure 6B).

We were concerned that NG6SS might contain a small amount of exoglycosidase that could degrade these glycopeptides, resulting in the release of [^{3}H]galactose and the exposure of terminal GlcNAc-6-sulfate residues, which might then become substrates for NG6SS. To assess the integrity of the glycopeptides following these treatments, the glycopeptides were analyzed by chromatography on Sephadex G-25 in order to determine whether any significant [^{3}H]galactose was released. Compared to the profile on Sephadex G-25 of glycopeptides treated with boiled NG6SS (Figure 6C), there was no significant [^{3}H]galactose released by NG6SS treatment (Figure 6D). To confirm that all the radioactivity in the [^{3}H]-galactose-labeled glycopeptides was indeed in galactose, the [^{3}H]galactose-labeled glycopeptides from Figure 6 were hydrolyzed and the released monosaccharides characterized by their retention time following high-pH anion-exchange chromatography on a Dionex PA-1 column (Figure 6E). The results demonstrate that more than 90% of the recovered radioactivity is present in [^{3}H]galactose. These combined results, shown in Figures 5 and 6, prove that the glycopeptides were not degraded by some contaminating activity in the enzyme preparation and NG6SS efficiently and quantitatively removed sulfate from GlcNAc-6-sulfate moieties within these [^{3}H]galactose-labeled glycopeptides without release of free [^{3}H]galactose.

Ability of NG6SS To Release $^{35}SO_4$ from $^{35}SO_4$-Labeled Glycoproteins. To test the ability of NG6SS to release sulfate from intact glycoproteins, we grew Molt-3 cells in media containing $Na_2{}^{35}SO_4$. An extract of the cells was prepared, and the total $^{35}SO_4$-labeled material was applied to a column of Sephadex G-25 (Figure 7). All of the radioactivity was contained in the void fractions. Treatment of the sample with boiled NG6SS did not cause release of free $^{35}SO_4$ into the included fractions, whereas treatment with active NG6SS was effective in releasing 23% of the total radioactivity. Since this represented a total cell extract, it is to be expected that $^{35}SO_4$ is present also in material other than GlcNAc-6-sulfate, such as sulfated proteins and glycosaminoglycans, and thus not susceptible to release by NG6SS. To confirm that the release of $^{35}SO_4$ by the enzyme was specific, the same reaction was carried out in the presence of 10 mM sodium sulfate, which inhibits the activity of NG6SS. Inclusion of this inhibitor markedly reduced the amount of free $^{35}SO_4$ released (Figure 7). These results demonstrate that NG6SS can release sulfate from GlcNAc-6-sulfate, glycopeptides containing GlcNAc-6-sulfate, and glycoproteins containing GlcNAc-6-sulfate.

The results in Figure 7 showing that free sulfate inhibits the release of sulfate indicated that release is caused by the specific action of NG6SS and not some contaminating enzyme, such as a protease which might degrade proteins resulting in the release of free glycopeptides. To confirm that the NG6SS preparation was free of proteases, we prepared [^{35}S]methionine/[^{35}S]cysteine-labeled proteins from human 293 cells and incubated a portion of this material with active NG6SS and boiled NG6SS. The SDS/PAGE and autoradiogram of these proteins revealed that NG6SS treatment did not result in any significant degradation of proteins (data not shown). To further confirm that no significant proteolysis occurred, the [^{35}S]methionine/[^{35}S]cysteine-labeled proteins were examined by size-exclusion chromatography on a column of Sephadex G-50 before and after treatments. The profiles of eluted radioactivity on these columns showed that no significant release of [^{35}S]methionine/[^{35}S]cysteine-labeled peptides occurred and no material was released into the included volume of the column (data not shown). The results demonstrate that NG6SS can not only release sulfate from glycopeptides but can also efficiently release sulfate from intact glycoproteins.

DISCUSSION

We have reported the purification and characterization of NG6SS from bovine kidney. The purification was facilitated by the high p*I* of the enzyme, which allowed us to effectively use cation- and anion-exchange chromatography as main steps in the purification without need for affinity chromatographic methods. On gel filtration, NG6SS behaves as a single subunit enzyme with a solution molecular weight of 60.2 kDa. The enzyme is glycosylated and contains Asn-linked oligosaccharides; thus, the precise size of the core protein is not known. In regard to small, sulfated mono- and disaccharides, the enzyme is able to hydrolyze sulfate esters specifically from N-acetylglucosamine-6-sulfate and it is less active toward Glc-6-sulfate and not active toward the other simple sulfated monosaccharides tested. These preliminary studies indicate that the minimum determinants contributing to enzyme recognition are the C-6 primary sulfate ester, the C-4 equatorial hydroxyl, and the N-acetyl at the C-2 of the hexosamine.

Previously, an N-acetylglucosamine-6-sulfate sulfatase was purified 136-fold from 80 L of human urine (Basner et al.,

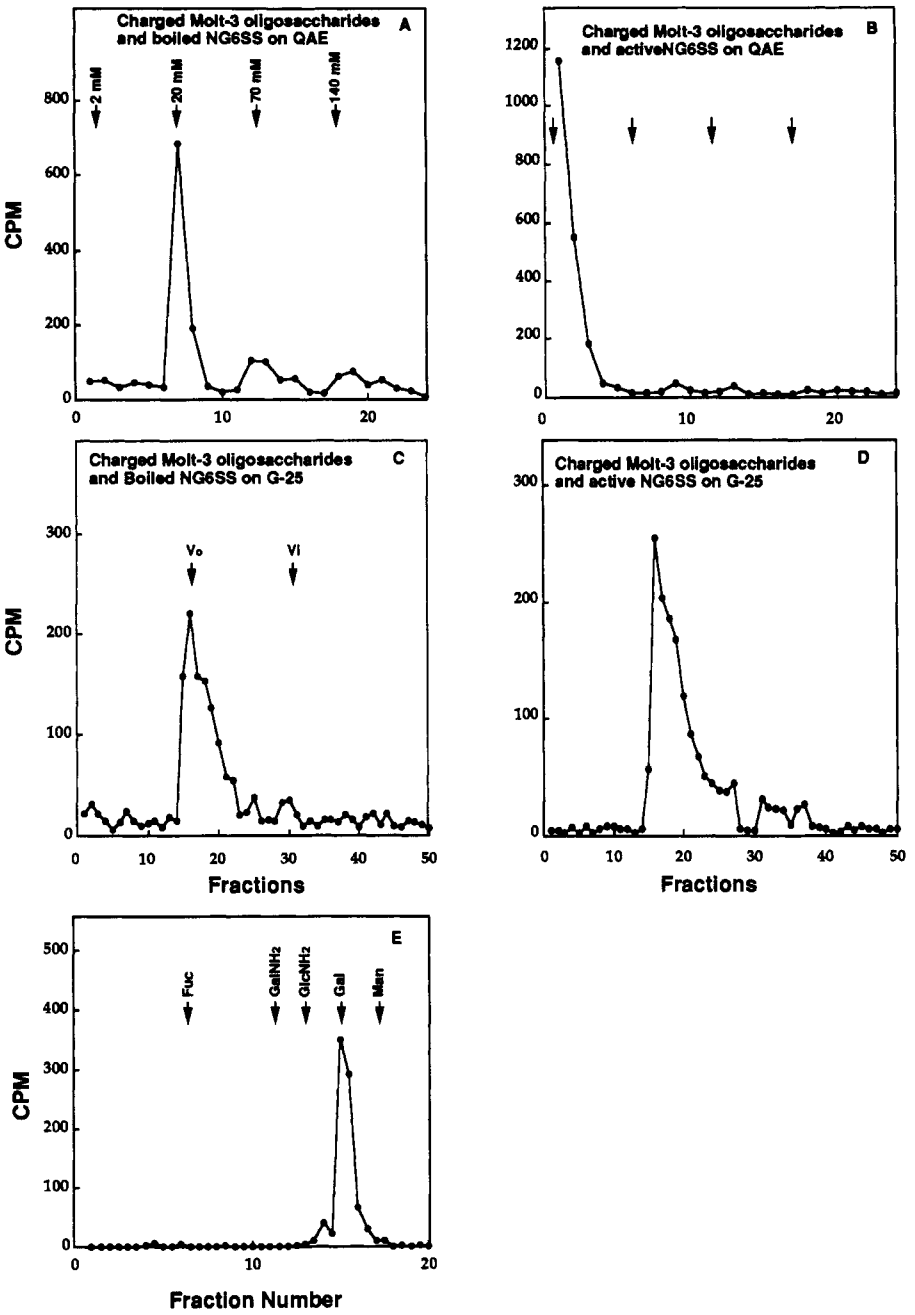

FIGURE 6: Release of sulfate by NG6SS from [^{3}H]galactose-labeled Molt-3 glycopeptides. Molt-3 cells were metabolically radiolabeled with [^{3}H]galactose, and total glycopeptides were prepared following Pronase digestion, as described in Experimental Procedures. Sulfated [^{3}H]-galactose-labeled glycopeptides were prepared by treatment with mild acid and purification over QAE-Sephadex. These glycopeptides were applied to a column of QAE-Sephadex before (A) and after (B) treatment with 1 unit of NG6SS to remove sulfate residues. The bound glycopeptides in (A) and (B) were eluted by a step gradient of pyridine–acetate (pH 5.5) as shown in the figure, from 20, 70, and 140 mM. Fractions were collected (1 mL), and 0.5 mL was removed for determining radioactivity by liquid scintillation counting. Glycopeptides treated with either boiled (C) or active (D) NG6SS were applied to a column of Sephadex G-25. Fractions were collected (1 mL), each fraction was analyzed for the presence of radioactivity by liquid scintillation counting. (E) Portions of the glycopeptide were also hydrolyzed with 2 N HCl for 2 h (100 °C), dried, resuspended in water, and applied directly to a PA-1 column on a Dionex HPLC for analysis by HPAE. Fractions were collected, and the cpm in each fraction was determined by liquid scintillation counting. The elution positions of authentic standards are indicated.

1979). The urinary enzyme is a monomer with an apparent molecular weight of 97 000 Da and thought to be important in heparin sulfate degradation. In regard to substrate specificity, the urinary enzyme is more active toward a monosulfated trisaccharide with a nonreducing, terminal GlcNAc-6-sulfate residue than it is toward free GlcNAc-6-sulfate (Basner et al., 1979). The p*I* value of the urinary enzyme was measured to be in multiple forms between 5.4 and 8.3 with a maximum at 7.7. There are no reported amino acid compositions or cloned cDNA sequences for the urinary enzyme as of yet. An *N*-acetylglucosamine-6-sulfatase from human liver has been purified at least 50000-fold and found to occur in three forms (A, B, and C) (Freeman & Hopwood, 1987; Freeman et al., 1987). Form A is a single polypeptide of 75 kDa and believed to be the proprotein to forms B and C, which are 48 and 32 kDa, respectively. There is strong indirect evidence that the human-liver-derived GlcNAc-6-sulfate sulfatase might be the same enzyme which is missing or malfunctioning in Sanfilippo D syndrome patients (Siciliano et al., 1991). A partial cDNA sequence for the human-liver-derived *N*-acetylglucosamine-6-sulfatase has been reported, but the full-length cDNA has not been determined (Robertson et al., 1988). These studies on *N*-acetylglucosamine-6-sulfatases from human sources have indicated that the enzymes

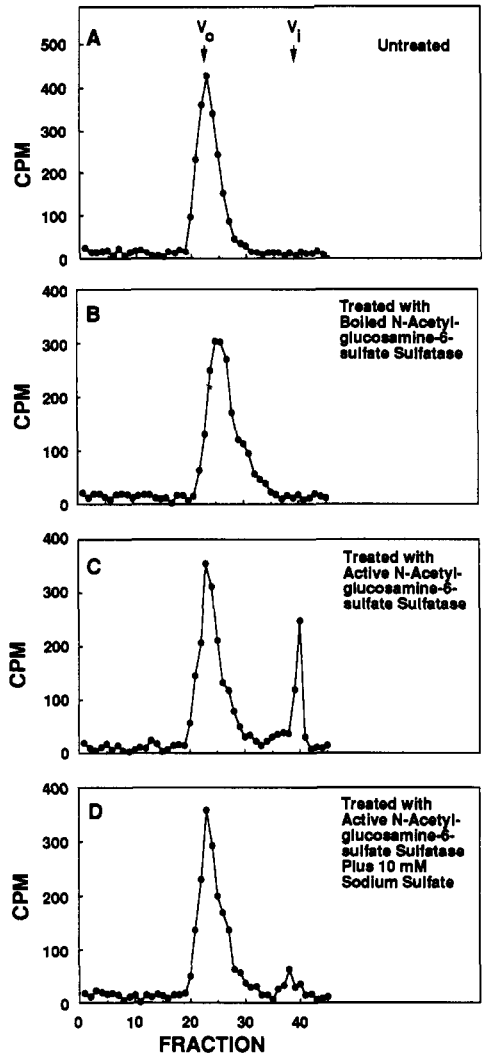

FIGURE 7: Release of sulfate by NG6SS from intact glycoproteins. Molt-3 cells were metabolically radiolabeled with $^{35}SO_4$ and the $^{35}SO_4$-labeled glycoproteins prepared by sonication and extraction into 0.05% Triton X-100 in PBS. (A) A portion (5000 cpm) of $^{35}SO_4$-labeled glycoproteins was passed over a column of Sephadex G-25 without treatment with NG6SS. (B) Equivalent portions were treated with boiled NG6SS, (C) active NG6SS, or (D) active NG6SS in the presence of 10 mM sodium sulfate, and these samples were also analyzed as in (A). Treatments were performed overnight at 37 °C using 1 unit of enzyme in a 0.1-mL total volume. After treatment, the samples were diluted to 0.5 mL in 7% *n*-propanol in water and applied to the column of Sephadex G-25. Fractions (1 mL) were collected, and all of each fraction was removed for determining radioactivity by liquid scintillation counting.

have exosulfatase activity, but they were not tested for their potential endosulfatase activity, i.e., the ability to remove sulfate from GlcNAc-6-sulfate residues within an oligosaccharide or glycoprotein.

Although much is known, there are still many unanswered questions about the pathway of degradation of sulfated glycosaminoglycans; even less is known about the pathway of degradation of sulfated *N*- and *O*-linked oligosaccharides. In regard to glycosaminoglycans, it is generally believed that they are degraded by the combined action of exo- and endoglycosidases and sulfatases (Neufeld & Muenzer, 1989). It is felt that hexosaminidase A is responsible for releasing GlcNAc-6-sulfate from the nonreducing termini of glycosaminoglycans. Both this released GlcNAc-6-sulfate and terminally sulfated oligosaccharides may be substrates for NG6SS. The finding of GlcNAc-6-sulfate in the urine of patients with Sanfilippo D syndrome and the lack of *N*-acetylglucosamine-6-sulfatase activity in their tissues have supported the possibility that this NG6SS is primarily an exosulfatase (Hopwood, 1983a,b; Hopwood, 1985; Freeman & Hopwood, 1987; Freeman et al., 1987). However, the possibility that NG6SS normally aids in the removal of sulfate from internal domains of glycosaminoglycans has not yet been rigorously tested.

In regard to sulfated glycoprotein oligosaccharides, the pathway of removal of sulfate may also be complex. To date, no known β-galactosidase has been described to be active toward terminal galactosyl residues linked to 6-sulfated GlcNAc residues. On the basis of our results, it is possible that NG6SS removes sulfate from these sulfated oligosaccharides, making them accessible substrates for the known exoglycosidases β-galactosidase and β-*N*-acetylhexosaminidase. Further studies will now be required to define more precisely the endosulfatase activity of NG6SS toward sulfated glycoproteins, oligosaccharides, and glycosaminoglycans.

Much remains to be learned about the overall biosynthesis and catabolism of sulfated sugars within glycoproteins and glycosaminoglycans and the functions of these modifications. For example, we do not know whether there are many other sulfatases that prefer as substrates complex sulfated glycans; such enzymes would not necessarily be identified by our purification procedures utilizing only a monosaccharide GlcNAc-6-sulfate substrate. Also, it is possible that different sulfatases exist to cleave sulfated glycoprotein as opposed to sulfated glycosaminoglycan substrates. It is also interesting that so many different sulfatases exist that utilize different sulfated substrates, as evidenced by the diversity of mucopolysaccharidoses and sulfatidoses in humans apparently caused by specific deficiencies in different sulfatases. These include the sulfatidase deficiency (aryl sulfatase A) in patients with metachromatic leukodystrophy (Austin et al., 1965) which causes accumulation of the glycosphingolipid sulfatide (SO_4-3-Gal-Cer) (Brady, 1978; DuBois et al., 1980), the aforementioned GlcNAc-6-sulfate sulfatase deficiency in patients with Sanfilippo D syndrome causing the accumulation of heparin sulfate (Coppa et al., 1983; Kaplan & Wolfe, 1987), *N*-acetylgalactosamine-6-sulfate sulfatase deficiency in patients with Marquio syndrome causing the accumulation of chondroitin sulfate (Matalon et al., 1974; Singh et al., 1976), and the L-iduronosulfate sulfatase deficiency in patients with Hunter syndrome causing the accumulation of heparan sulfate and dermatan sulfate (Brante, 1952). These studies suggest that a great number of highly specific sulfatases exist.

The functions of sulfate residues in glycoproteins in particular are not well understood, although many studies have documented their existence (Sundblad et al., 1988a,b; Poduslo, 1990; Von Wurtemburg & Fries, 1989; Weaver et al., 1987; Braulke et al., 1987; Colburn & Bounassasi, 1987; Freeze & Varki, 1989; Griswold et al., 1986; Freeze & Wolgast, 1986; Stadler et al., 1983; Heifetz et al., 1980). In addition, sulfate is found in many viral glycoproteins including HIV-1 (Shilatifard et al., 1993; Bernstein & Compans, 1993), herpes simplex virus (Hope & Marsden, 1983), paramyxovirus SV5 grown in bovine kidney cells (Prehm et al., 1979), influenza virus (Ward et al., 1980), and other enveloped viruses (Pinter & Compans, 1975). Although the prominence and the biochemical significance of sulfation of glycoconjugates are not well understood, there is increasing evidence that sulfation of carbohydrates may play important roles in biochemical recognition systems (Fiete et al., 1991; Jones, 1991; Urch & Patel, 1991; Lerouge et al., 1990; Lindahl et al., 1989; Lederman et al., 1989; Mitsuga et al., 1988; Parsons & Pierce, 1980). It is anticipated that studies on the structure and function of GlcNAc-6-sulfate residues within sulfated

glycans will be considerably aided in the future by the availability of NG6SS from bovine kidney.

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