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Biochemistry

A single protein catalyzes both N-deacetylation and N-sulfation during the biosynthesis of heparan sulfate

(*N*-sulfotransferase/*N*-deacetylase/recombinant enzyme)

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ABSTRACT **Heparan sulfate is a highly sulfated carbohydrate polymer that binds to and modulates the activities of numerous proteins. The formation of these protein-binding domains in heparan sulfate is dependent on a series of biosynthetic reactions that modify the polysaccharide backbone; the initiating and rate-limiting steps of this process are the N-deacetylation and N-sulfation of *N*-acetylglucosamine residues in the polymer. We now report that in the rat liver, biosynthesis of heparan sulfate utilizes a single protein that possesses both *N*-deacetylase and *N*-sulfotransferase activities. This was accomplished by demonstrating that both activities resided in a purified soluble fusion protein containing the Golgi-lumenal portion of the enzyme. We propose that this protein be renamed the rat liver Golgi heparan sulfate *N*-deacetylase/*N*-sulfotransferase.**

Heparan sulfate (HS) and heparin are large polymers that are unique among glycoconjugates in the animal kingdom in that they contain N-sulfated glucosamine residues in their carbohydrate backbones. The presence of these modified residues is a major element in the production of polymers possessing a diverse range of biological activities; for instance, N-sulfated residues participate in the binding of these polymers to proteins such as basic fibroblast growth factor (1–3). In addition, further modification of these polymers requires the initial formation of N-sulfated products; thus, C5 epimerization of D-glucuronic acid to iduronic acid and the many O-sulfation reactions cannot occur until N-sulfation has been initiated (4).

The N-deacetylation of *N*-acetyl-D-glucosamine residues is required for the N-sulfation reactions to occur. On structural grounds, these two reactions appear to be tightly coupled; free glucosamine residues are rarely found in HS or heparin. This is true even in the case of a Chinese hamster ovary (CHO) cell mutant where the *N*-sulfotransferase is significantly reduced in activity (5). Pettersson *et al.* (6) have recently observed that an *N*-sulfotransferase, purified from a mast cell tumor that produces large amounts of heparin, acquired *N*-deacetylase activity upon addition of a mast cell-derived crude factor that, by itself, had neither activity. This crude factor may be replaced by a basic protein such as histone or a polycationic compound such as Polybrene (7), suggesting that both activities may reside in the same protein.

We have previously reported on the purification (8), molecular cloning, and *in vitro* expression of a rat liver HS *N*-sulfotransferase (N-HSST) (9). We have now investigated whether N-HSST expressed in COS cells possesses both activities by using the full-length transmembrane form and a soluble recombinant form. It was found that a single protein possesses both the N-deacetylation and N-sulfation activities.

MATERIALS AND METHODS

Construction of a Plasmid Encoding a Soluble N-HSST. A soluble form of the N-HSST corresponding to amino acids 42–882 of the full-length transmembrane molecule (9) fused at its amino terminus to a portion of protein A was produced by use of the polymerase chain reaction. For this purpose, 30 pmol of each primer (5′-GGTGTCGACACTCGAGCCCTCGGCAGATG-3′ and 5′-GGCGTCGACCAAGCGTTCTGGCTGGCTGTG-3′) was added to 10 ng of pCMVST DNA (9) in a total volume of 100 μl containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 μmol of each dNTP, and 2.5 units of Amplitaq (Perkin–Elmer/Cetus, Norwalk, CT). The following amplification protocol was used: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 45 sec, 55°C for 30 sec, and 72°C for 3 min; and 1 cycle of 72°C for 10 min. The product was digested with *Sal* I, agarose gel purified, and ligated into the eukaryotic expression vector pRK5F10Protein-A, derived from the protein A-encoding vector pPROTA (10).

Transient Expression of Full-Length and Soluble N-HSST cDNA in COS Cells and Purification of Soluble N-HSST. COS cells (5×10^5 cells per 10-cm dish) were transfected with 5 μg of pCMVST DNA containing the cDNA encoding the full-length transmembrane N-HSST or pCMV5 vector DNA alone (control) in a 10-cm dish using the DEAE-dextran transfection method (11, 12). After 72 hr of incubation, the monolayers were rinsed, scraped off the dishes, and collected by centrifugation. Cell pellets were resuspended in homogenate buffer [250 mM sucrose, leupeptin at 1 μg/ml, pepstatin at 0.4 μg/ml, 0.2 mM phenylmethylsufonyl fluoride, and 50 mM Tris·HCl (pH 7.5)] and disrupted by a Dounce homogenizer (20 strokes). To purify and immobilize the soluble N-HSST on IgG-agarose, medium (10 ml) from one dish of cells transfected with the plasmid containing the soluble N-HSST was collected 72 hr after transfection and incubated with 10 μl of rabbit IgG-agarose (Sigma) at 4°C for 24 hr. To elute the enzyme from the IgG-agarose, the beads (10 μl) were washed with 10 ml of 50 mM Tris·HCl, pH 7.4/20% glycerol, incubated with 50 μl of 100 mM citrate buffer at pH 4.0, vortexed for 10 sec, and centrifuged; the supernatant fluid was neutralized with 20 μl of 1 M Tris·HCl, pH 8.2/20% glycerol. The preparation was adjusted to a final concentration of 20% glycerol and stored at −20°C. The purity of these preparations was assayed by SDS/PAGE using a silver stain method for detection.

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Abbreviations: HS, heparan sulfate; N-HSST, HS *N*-sulfotransferase; WGA, wheat germ agglutinin; PAPS, adenosine 3′-phosphate 5′-phosphosulfate.

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***N*-Deacetylase and *N*-Sulfotransferase Assays.** The *N*-deacetylase activity was measured by determining the release of [^{3}H]acetate from N-[^{3}H]acetylated polysaccharide derived from *Escherichia coli* K5-derived capsular polysaccharide with a specific activity of 400 cpm/ng (dry weight) after a 1-hr incubation as described (5). *N*-Sulfotransferase and *O*-sulfotransferase activities were measured by determining $^{35}SO_4$ incorporation into N-desulfated heparin and completely desulfated, N-resulfated heparin, respectively, from ^{35}S-labeled adenosine 3′-phosphate 5′-phosphosulfate (PAPS) after a 30-min incubation as described (13). Twenty-five micrograms of total protein from each cell homogenate was included in the enzyme reaction mixtures.

Affinity Chromatography. Ten micrograms of purified soluble enzyme was loaded onto a 1-ml 3′,5′-ADP-agarose (Sigma) column equilibrated with buffer A (50 mM Tris·HCl, pH 7.5/20% glycerol/50 mM NaCl). The column was washed with 10 ml of buffer A. Elution was accomplished with buffer containing an eight-step gradient of 3′,5′-ADP (0–400 μM). Fractions of 200 μl were collected, and 5 μl of each was assayed for both enzymatic activities. Conditions for the wheat germ agglutinin (WGA)-Sepharose column (Pharmacia) were identical except that buffer A additionally contained 0.15 M NaCl and that elution was initiated with a step gradient of *N*-acetylglucosamine (0–400 mM).

RESULTS AND DISCUSSION

To investigate if N-HSST possessed both *N*-deacetylase and *N*-sulfotransferase activities, a recombinantly derived N-HSST was transiently expressed in COS cells. Transfection of COS cells with a vector encoding the full-length, membrane-bound form of N-HSST resulted in a substantial increase (over control) in both activities from cell extracts (Table 1). No increase in *O*-sulfotransferase activity was detected (Table 1).

To directly examine if both activities were derived from the recombinant protein, a plasmid was constructed to facilitate purification of N-HSST after transfection. This plasmid DNA encoded a soluble protein consisting of the predicted Golgi-lumenal portion of the N-HSST fused at its amino terminus to the IgG binding portion of protein A. As expected, the majority of this soluble N-HSST produced by COS cells was secreted into the culture medium, which allowed for its purification by affinity chromatography on IgG-agarose. The purified protein migrated as a single species at 130 kDa (Fig. 1). The apparent molecular mass of the soluble protein agreed with that expected based on the open reading frame encoded by the plasmid DNA. The immobilized soluble protein also displayed both the *N*-deacetylase and *N*-sulfotransferase enzymatic activities (Table 1).

To eliminate the possibility that a small molecular weight cofactor was required for either of these enzymatic activities, a series of experiments was executed. The enzymatic properties and SDS/PAGE profile of soluble N-HSST, immobilized on IgG-agarose beads, were unaffected by successive treatments with 0.5 M NaCl, 0.2 M EDTA, and 1% Triton X-100 (data not shown). Furthermore, no alterations in either

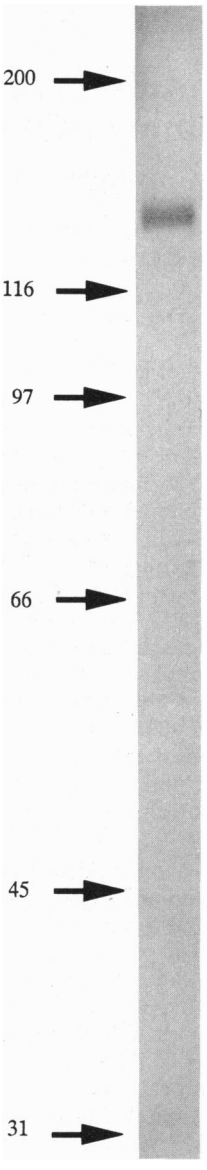

FIG. 1. Purification of a soluble form of the N-HSST. One hundred nanograms of soluble enzyme that had been purified and eluted from a rabbit IgG-agarose affinity chromatography column was subjected to gradient SDS/PAGE (8–20%). The gel was stained with silver nitrate. The molecular masses of marker proteins are indicated (in kDa).

Table 1. Overexpression of N-HSST in COS cells

Sample	*N*-Deacetylase activity, ^{3}H released (cpm)	*N*-Sulfotransferase activity, ^{35}S incorporated (cpm)	*O*-Sulfotransferase activity, ^{35}S incorporated (cpm)
Full-length N-HSST	52,000	61,000	8900
pCMV5 vector control	8,200	18,200	9100
Immobilized soluble N-HSST	59,000	82,500	ND
Immobilized control	860	750	ND

Transfected COS cell extracts were prepared, and *N*-deacetylase, *N*-sulfotransferase, and *O*-sulfotransferase activities in each cell extract were measured as described in *Materials and Methods*. The soluble enzyme was isolated by using 10 μl of rabbit IgG-agarose to bind the enzyme derived from the culture medium (10 ml) of one dish of transfected cells. The enzyme was used while still immobilized to the IgG-agarose. IgG-agarose exposed to an equivalent amount of medium from cells transfected with a plasmid containing the insert for the soluble enzyme in the reverse orientation served as the immobilized control. All of the determinations are the average values obtained from a minimum of duplicate cultures. ND, not determined.

enzymatic activity were observed after the protein treated in this fashion was next eluted from the IgG-agarose and dialyzed in a 30-kDa molecular mass cut-off membrane (data not shown).

The eluted enzyme was also subjected to two chromatographic procedures previously utilized for the purification of the full-length transmembrane form of N-HSST from rat liver (8). Both activities bound to and were coeluted from a 3′,5′-ADP agarose column and a WGA-Sepharose column (Fig. 2). Again, no change was seen in the migration of the protein by SDS/PAGE analysis (data not shown). In contrast, it has been reported that the mast cell-derived *N*-sulfotransferase, which bound to WGA-Sepharose, was devoid of *N*-deacetylase activity until an unbound fraction was added back to the eluted enzyme (6). The chromatographic properties of the soluble N-HSST were also observed for the full-length transmembrane form of the N-HSST partially purified from a transfected COS cell-derived extract (data not shown). This excluded the possibility that the protein A portion of the soluble enzyme might be serving as a cofactor for the *N*-deacetylase activity. These results, taken together, demonstrate that a single protein possesses both enzymatic activities independent of other proteins or cofactors.

The sensitivity of either activity to *N*-ethylmaleimide, NaCl, 3′,5′-ADP, and PAPS was also studied by using purified soluble N-HSST, as shown in Fig. 3. The *N*-deacetylase activity was very sensitive to NaCl, with >90% activity lost at physiological concentrations. Significant loss in *N*-sulfotransferase activity was seen only at concentrations >200 mM. *N*-Ethylmaleimide inhibited 80% of the *N*-deacetylase activity at a 1 mM concentration, whereas the *N*-sulfotransferase was unaffected at all concentrations tested (up to 20 mM). The *N*-sulfotransferase was inhibited by 3′,5′-ADP (Fig. 3), in agreement with previous results obtained with the heparin-related mast cell-derived enzyme (6, 14, 15). Judging from the differential sensitivity to inhibitors, the two catalytic sites appear to behave somewhat independently.

The *N*-deacetylase activity of the soluble enzyme was not stimulated by the addition of PAPS (Fig. 3*C*), although Riesenfeld *et al.* (15) found that the *N*-deacetylase activity in microsomal preparations derived from mast cells was stimulated by the presence of PAPS. They attributed this effect to a stimulatory effect of newly incorporated N-sulfate groups on the N-deacetylation reaction in heparin synthesis. In this respect, the N-HSST is similar to the activity in CHO cells that directs HS biosynthesis (5), although the inclusion of PAPS in *in vitro* assays of crude preparations of the CHO cell N-deacetylase lowers the K_m for the polysaccharide substrate. Additionally, the *N*-deacetylase activity of the

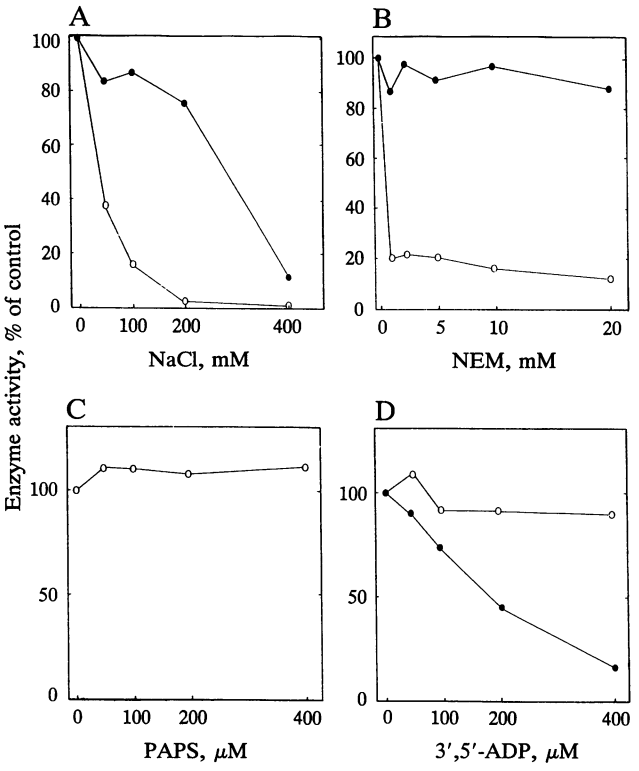

FIG. 3. Sensitivity of the N-HSST to various chemical agents. (*A*) Sensitivity of activities to NaCl. (*B*) Sensitivity of activities to *N*-ethylmaleimide (NEM). (*C*) Dependence of *N*-deacetylase activity on PAPS. (*D*) Sensitivity of activities to 3′,5′-ADP. ●, *N*-sulfotransferase activity; ○, *N*-deacetylase activity. Twenty-five nanograms of purified soluble N-HSST was used in each reaction. Reactions were carried out at 37°C for 1 hr for the *N*-deacetylase assay and for 30 min for the *N*-sulfotransferase assay.

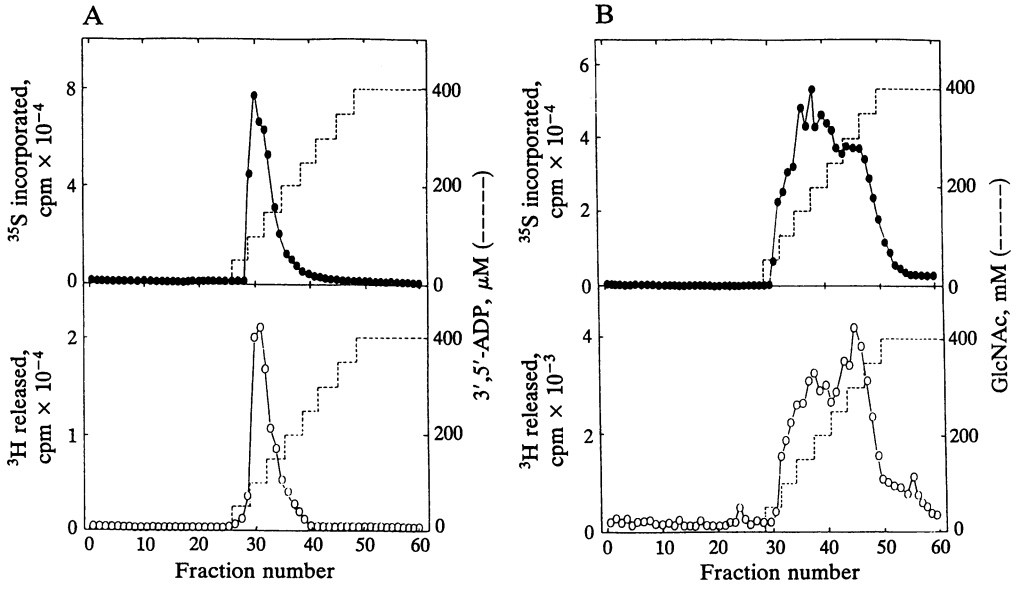

FIG. 2. Coelution of *N*-deacetylase and *N*-sulfotransferase activities of the soluble form of N-HSST on 3′,5′-ADP-agarose and WGA-Sepharose columns. (*A*) Coelution of *N*-Sulfotransferase (*Upper*) and *N*-deacetylase (*Lower*) activities on a 3′,5′-ADP-agarose column. (*B*) Coelution of *N*-sulfotransferase (*Upper*) and *N*-deacetylase (*Lower*) activities on a WGA-Sepharose column. GlcNAc, *N*-acetylglucosamine.

soluble enzyme was not increased by the addition of histone or Polybrene; these effectors were reported to substitute for a crude fraction necessary for requisition of *N*-deacetylase activity of the mast cell *N*-sulfotransferase (7). From the above findings, we conclude that the rat liver N-HSST participating in HS synthesis and the mast cell-derived *N*-sulfotransferase proposed to participate in heparin synthesis have quite distinct biological properties.

HS and heparin display the same type of modifications of their carbohydrate backbone (4); however, differences in sulfate content, clustering of sulfate within regions of the polymer, and iduronic acid content suggest that different mechanisms control their overall biosynthesis. At the cellular level, heparin is made only in connective tissue-type mast cells and is stored in intracellular granules, whereas HS is synthesized by virtually all animal cells and is found on large extracellular and cell surface proteoglycans (16). At the molecular level, a number of studies employing microsomal preparations suggest that a large part of the microheterogeneity found in both of these molecules is probably the result of incomplete processing by the enzymatic machinery (4). N-deacetylation and N-sulfation are hypothesized to be key steps in the biosynthesis of HS and heparin in that C5 epimerization and O-sulfation occur only within or adjacent to N-sulfated domains (17). In fact, we have found that expression of N-HSST activity can control the formation of the large extended N-sulfated and highly O-sulfated clusters in the polysaccharides that are necessary for high-affinity binding of HS to basic fibroblast growth factor (18).

Although our observations show that the N-deacetylation and N-sulfation reactions require different conditions *in vitro* to obtain optimal activities, the two reactions are likely to be coupled *in vivo* (5). A CHO mutant cell line partially deficient in N-HSST showed decreased N-sulfation of HS but no corresponding increase in the proportion of N-unsubstituted glucosamine residues when compared with the wild-type cells. This is so despite the fact that the *N*-deacetylase activity was higher in an *in vitro* reaction than that observed in the wild-type cells (5).

On the basis of the evidence provided in our study, we propose renaming the previously designated N-HSST heparan sulfate *N*-deacetylase/*N*-sulfotransferase. This enzyme joins a group of eukaryotic enzymes with the ability to carry out multiple enzymatic reactions effected with a single polypeptide chain (19–22). The availability of nucleic acid sequence information for the rat liver-derived enzyme and the soluble enzyme should now make it possible to dissect mechanisms for the regulation of modifications of HS at the molecular level. It will also be necessary to account for the differences in the activities of the rat liver and mast cell-derived enzymes in terms of the polysaccharide modifications displayed by HS and heparin, respectively.

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