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Production of *N*-sulfated polysaccharides using yeast-expressed *N*-deacetylase/*N*-sulfotransferase-1 (NDST-1)

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Heparan sulfate/heparin *N*-deacetylase/*N*-sulfotransferase-1 (NDST-1) is a critical enzyme involved in heparan sulfate/heparin biosynthesis. This dual-function enzyme modifies the GlcNAc-GlcA disaccharide repeating sugar backbone to make *N*-sulfated heparosan. *N*-sulfation is an absolute requirement for the subsequent epimerization and *O*-sulfation steps in heparan sulfate/heparin biosynthesis. We have expressed rat liver (r) NDST-1 in *Saccharomyces cerevisiae* as a soluble protein. The yeast-expressed enzyme has both *N*-deacetylase and *N*-sulfotransferase activities. *N*-acetyl heparosan, isolated from *Escherichia coli* K5 polysaccharide, de-*N*-sulfated heparin and completely desulfated *N*-acetylated heparan sulfate are all good substrates for the rNDST-1. However, *N*-desulfated, *N*-acetylated heparin is a poor substrate. The rNDST-1 was partially purified on heparin Sepharose CL-6B. Purified rNDST-1 requires Mn^{2+} for its enzymatic activity, can utilize PAPS regenerated *in vitro* by the PAPS cycle (PAP plus para-nitrophenylsulfate in the presence of arylsulfotransferase IV), and with the addition of exogenous PAPS is capable of producing 60–65% *N*-sulfated heparosan from *E. coli* K5 polysaccharide or *Pasteurella multocida* polysaccharide.

Key words: heparan sulfate/K5 polysaccharide/NDST-1/PAPS cycle/yeast

Introduction

Heparin/heparan sulfates play a central role in many biological processes such as blood coagulation, cell–cell communications, and cancer (Bernfield *et al*., 1999; Liu *et al*., 2002; Shriver *et al*., 2000). Sulfation of the carbohydrates is catalyzed by a group of sulfotransferases, type II membrane-bound Golgi enzymes, which use the biological high-energy sulfate donor, adenosine 3′-phosphate 5′-phosphosulfate (PAPS) to transfer a sulfate group to a specific position on a variety of sugar residues (see recent reviews, Fukuda *et al*., 2001; Esko and Lindahl, 2001; Perrimon and Bernfield, 2000).

N-deacetylase/*N*-sulfotransferase (NDST), a bifunctional enzyme in heparin/heparan sulfate biosynthesis, removes acetyl groups from GlcNAc residues, then sulfates the newly formed free amines on the GlcN residues using PAPS as a sulfate donor. *N*-sulfated glucosamine is required for the subsequent C-5 epimerization of the GlcA to IdoA by C5-epimerase, 2-*O*-sulfation of IdoA by 2-O-sulfotransferase, and 6-*O*- or 3-*O*-sulfation of glucosamine residues by 6-*O*-sulfotransferase and 3-*O*-sulfotransferase, respectively (Bame and Esko, 1989; Esko and Lindahl, 2001; Grobe *et al*., 2002; Perrimon and Bernfield, 2000).

The genes for NDST isozymes 1–4 have been cloned and expressed from different mammalian sources (Aikawa and Esko, 1999; Aikawa *et al*., 2001; Cheung *et al*., 1996; Hashimoto *et al*., 1992; Kushe-Gullberg *et al*., 1998; Orellana *et al*., 1994). The NDST isozymes share 65–80% sequence identity (Esko and Selleck, 2002) but differ in their *N*-deacetylase and *N*-sulfotransferase activities (Aikawa *et al*., 2001). *Caenorhabtidis elegans* and *Drosophila melanogaster* possess single orthologs of NDST that are involved in the development of these organisms (Grobe *et al*., 2002; Perrimon and Bernfield, 2000; Selleck, 2000). NDST-1 and NDST-2 are found in all tissues, whereas localization of NDST-3 and NDST-4 is more restricted, existing only in fetal tissues and adult brain (Aikawa *et al*., 2001; Esko and Selleck 2002; Grobe *et al*., 2003). NDST-1 is an essential enzyme for development, as its knockout in mice results in neonatal lethality (Grobe *et al*., 2002; Ringvall *et al*., 2000). NDST-2 knockout mice showed a deficiency in heparin biosynthesis (Grobe *et al*., 2002). Esko and Selleck (2002) have suggested that different isoforms may work on the same heparan sulfate (HS) chain.

NDST-1 has been demonstrated to be a single protein having both *N*-deacetylase and *N*-sulfotransferase activities (Pettersson *et al*., 1991; Wei *et al*., 1993). Berninsone and Hirschberg (1998) demonstrated that in COS-expressed NDST-1, the sulfotransferase activity was located in the carboxyl-terminal half of the protein, containing a PAPS-binding domain that is common in all sulfotransferases (Fukuda *et al*., 2001).

All NDSTs containing both activities have been expressed in mammalian cells such as COS (Wei *et al*., 1993), human kidney 293 cells (Pikas *et al*., 2000), and Chinese hamster ovary cells (Aikawa and Esko, 1999). Sueyoshi *et al*. (1998) have expressed the sulfotransferase domain of the human NDST-1 as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli*, and expression of NDST-2 was recently reported in insect cells (Kuberan *et al*., 2003). To

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date the expression of NDST carrying both *N*-deacetylase and *N*-sulfotransferase activities has not been achieved in non-glycosaminoglycan (GAG)–containing expression systems, such as yeast or bacteria.

In this work, soluble rat liver (r) NDST-1 was cloned and expressed in *Saccharomyces cerevisiae.* The lack of background enzymatic activity and endogenous proteoglycans in *S. cerevisiae* simplifies purification and analysis of the enzyme. The yeast-expressed recombinant rNDST-1 successfully achieved coupled *N*-deacetylation and *N*-sulfation on a variety of polysaccharides containing GlcNAc-GlcA repeating units.

Results

Cloning and expression of rNDST-1 in S. cerevisiae

The coding sequence for a truncated rNDST-1, lacking the first 44 N-terminal amino acids, including the membrane-spanning region, was cloned into a yeast expression vector (pYES2/NTC). The expression vector, pYES2-rNDST-1, produces a fusion protein (rNDST-1) of truncated rNDST-1 preceded by 49 amino acids, from the vector, encoding a 6 HIS tag, an Xpress epitope, and an enterokinase recognition site. Expression of rNDST-1 was carried out in InvSc1 *S. cerevisiae* host yeast cells using the galactose-inducible promoter, GAL1. Following 24 h induction, the sulfotransferase activity of the rNDST-1 in yeast lysates, using various polysaccharide substrates, was at least 1000-fold higher than background (11,240 pmol/min/L yeast culture).

Acceptor specificity of rNDST-1

rNDST-1 specificity was first tested on different sugar acceptors using yeast lysates, prepared following 24 h induction, under conditions predicted to be optimal for sulfotransferase activity (Pettersson *et al.*, 1991). Using de-*N*-sulfated heparin (DNSH) as substrate, rNDST-1 had a sulfotransferase activity of 159 pmol/min/ml lysate, whereas desulfated chondroitin, containing repeats of GalNAc instead of GlcNAc, was not a substrate (Figure 1). Using *E. coli* K5 polysaccharide (K5 or *N*-acetyl heparosan) as substrate, rNDST-1 had a sulfotransferase activity of 196 pmol/min/ml lysate (Figure 1). Because K5 did not have any free glucosamine residues, the substrate for the *N*-sulfotransferase, the activity measured indicates coupling of the *N*-deacetylase and *N*-sulfotransferase activities of rNDST-1. When a similar acceptor, completely desulfated *N*-acetylated heparan sulfate (CDSNAcHS), was tested, *N*-sulfotransferase activity of rNDST-1 was found to be 168 pmol/min/ml lysate (Figure 1). However when *N*-desulfated *N*-acetylated heparin (NDSNAcH), containing *O*-sulfates (2-*O*, 6-*O*, and/or 3-*O*), predicted to inhibit *N*-deacetylation (Brandan and Hirschberg, 1988) was used as an acceptor, *N*-sulfotransferase activity dropped dramatically to 20 pmol/min/ml lysate (Figure 1).

Purification of rNDST-1

It has been demonstrated that rNDST-1 will bind to heparin-Sepharose (Brandan and Hirschberg, 1988). To improve the characterization of enzyme activities,

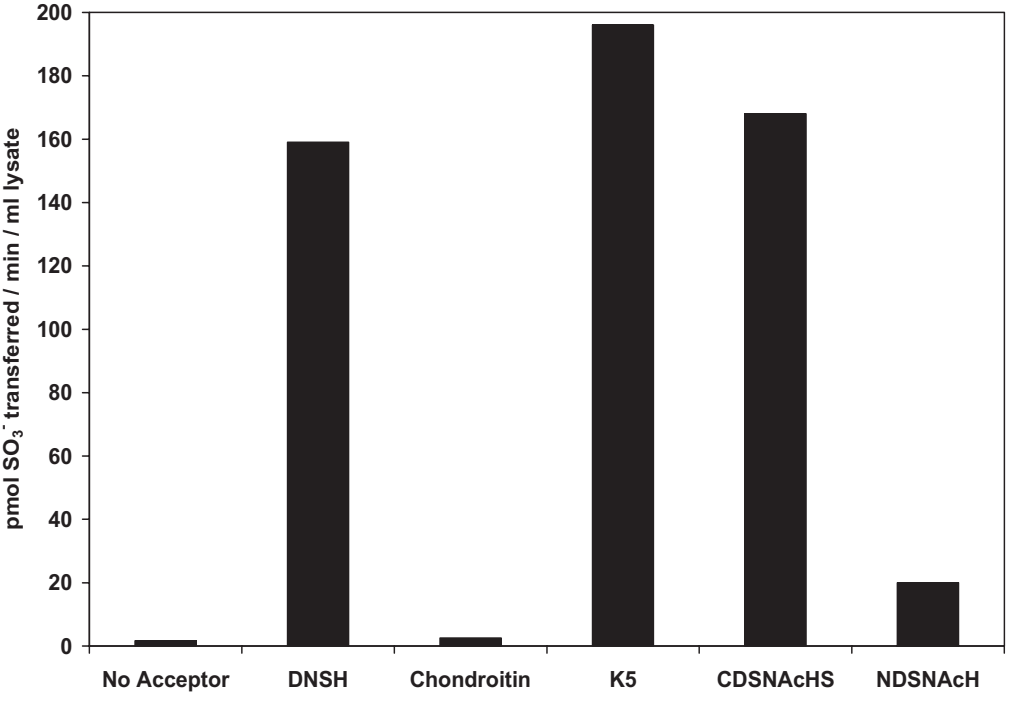

Fig. 1. Substrate specificity of the rNDST-1. A yeast cell lysate expressing rNDST-1 (INVSc1/pYES2-rNDST-1) was used in a sulfotransferase assay using DNSH, chondroitin (desulfated), K5, CDSNAcHS, NDSNAcH. Ten-microliter aliquots were analyzed for NDST activity. Results were duplicate experiments. Activities were expressed as pmol SO_3^- transferred per min per ml yeast cell lysate.

rNDST-1 was partially purified from yeast lysates using heparin–Sepharose CL-6B chromatography. All of the rNDST-1 activity bound to the column and ~48% of the activity was recovered following salt gradient elution and concentration. The partially purified rNDST-1 was stored in elution buffer containing glycerol at −20°C without significant loss of activity (data not shown). The specific activity of the purified rNDST-1 was 231 pmol/min/mg.

Optimization of a coupled enzymatic N-*deacetylation/* N-*sulfation reaction*

The ability of the rNDST-1 sulfotransferase to successfully add sulfates to a K5 polysaccharide substrate that is completely *N*-acetylated indicated that the enzyme was functioning in a coupled reaction, with the *N*-deacetylase activity creating the substrate for the *N*-sulfotransferase. Because this reaction was carried out under conditions predicted to be optimal for sulfotransferase activity (pH 7.0–7.4, in the presence of Mn^{2+}, Mg^{2+}, and Ca^{2+}) (Pettersson *et al*., 1991), we tested the coupled enzyme reaction under reaction conditions predicted to be optimal for the *N*-deacetylation reaction (pH 6.5, in the presence of Mn^{2+}) (Pettersson *et al*., 1991). *N*-sulfation of K5 polysaccharide by rNDST-1 under conditions optimized for deacetylation was sevenfold higher than *N*-sulfation in conditions optimized for *N*-sulfation (Figure 2). This suggests that *N*-deacetylation is the rate-limiting step in the coupled enzyme reaction. The data showed that Mn^{2+} was the only divalent cation required for both *N*-deacetylation and *N*-sulfation (Figure 2).

N-*deacetylation assay*

To characterize the *N*-deacetylase activity of rNDST-1, an analytical method was developed to verify and quantify activity. *E. coli K5* polysaccharide and *Pasteurella multocida* (PM) polysaccharide (PS) also containing GlcNAc-GlcA repeating units (DeAngelis and White, 2002) were used in overnight *N*-deacetylation reactions using rNDST-1. The reaction products were digested with heparinase I, heparinase II, and heparitinase I, creating disaccharides with UV-absorbing unsaturated uronic acids (ΔUAs) at their non-reducing termini. The disaccharide products were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) on a Chromolith Performance RP-18e column, with the ΔUA-GlcN representing the product of the deacetylation reaction and the uronic acid (ΔUA)-GlcNAc disaccharide representing unreacted substrate. Recovery of disaccharides following digestion of *N*-deacetylated K5 polysaccharide and control K5 polysaccharide by heparinase I, heparinase II, and heparitinase I was found to be equivalent based on total disaccharide HPLC peak areas (data not shown). Both *N*-deacetylated K5 polysaccharide (Figure 3A) and *N*-deacetylated PM PS (Figure 3B) exhibited similar levels of deacetylation in overnight reactions. Although the initial rate of deacetylation of K5 at 37°C was twice that at 23°C ± 2°C, the reactions at both temperatures reached a maximum of 20–23% deacetylation of K5 following an overnight reaction (Figure 4).

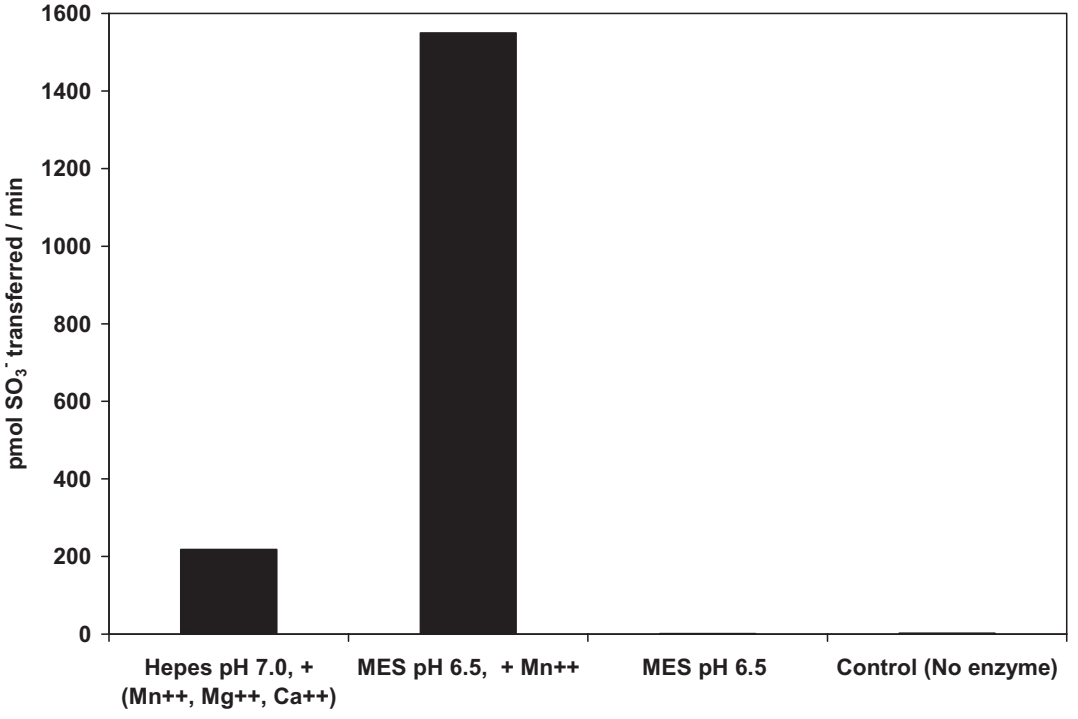

Fig. 2. Activity of rNDST-1 using K5 polysaccharide under different reaction conditions. Partially purified rNDST-1 was tested in the sulfotransferase assay, separately, under conditions predicted to be optimal for sulfotransferase activity and *N*-deacetylase activity. Activities were expressed as pmol SO_3^- transferred per min per ml purified enzyme.

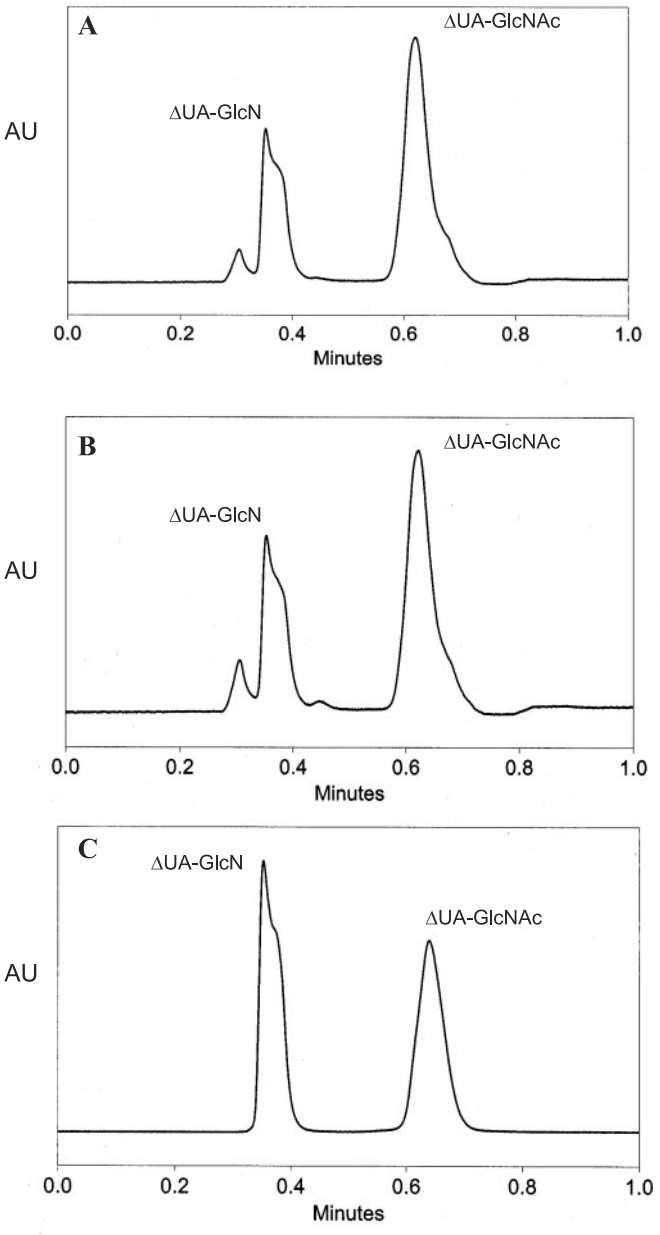

Fig. 3. HPLC profiles of *N*-deacetylated polysaccharides. Following *N*-deacetylation, polysaccharides were enzymatically digested and the resultant uronic acid–containing disaccharides were analyzed on HPLC. The ΔUA-GlcN peak represents deacetylase activity (see *Materials and methods* for details) (**A**) K5; (**B**) PM PS; (**C**) standards: ΔUA-GlcN, 0.35 min, ΔUA-GlcNAc, 0.64 min (AU: Absorbance units).

N-*sulfation assay*

A time course of *N*-sulfation of K5 polysaccharide by rNDST-1, as measured by the radioactive sulfotransferase assay, was determined using the optimized coupled reaction conditions (pH 6.5, in the presence of Mn^{2+}) (Figure 5). The reaction exhibited a sigmoidal curve, with a short 10-min lag in sulfation, during which the GlcN substrate is formed by the deacetylase activity, followed by a linear increase in *N*-sulfation (Figure 5). To better identify the *N*-sulfated residues in the polysaccharide substrates, the HPLC method developed for the *N*-deacetylase activity was modified. Following *N*-deacetylation and *N*-sulfation of K5 or PM PS with rNDST-1, the polysaccharide products were digested with heparinase I, heparinase II, and heparitinase I, creating sulfated and nonsulfated disaccharides with UV-absorbing unsaturated UAs (ΔUA) at their nonreducing termini. The disaccharide mixtures were labeled with the fluorescent tag anthranilamide (2-AB) and separated on ion exchange HPLC. When the *N*-deacetylation/*N*-desulfation reactions were optimized, the reaction yields of *N*-sulfated K5 polysaccharide was 60% (Figure 6C), whereas the reaction yields of *N*-sulfated PM PS was 65% (Figure 6D).

N-*sulfation in PAPS cycle*

The development of a useful industrial process for sulfation of polysaccharides is inhibited by the high cost of the sulfate donor, PAPS. A PAPS cycle has been developed that transiently produces PAPS from adenosine 3′-phosphate 5′-phosphate (PAP) and paranitrophenol sulfate (PNPS) (Burkart *et al.*, 2000). The PAPS cycle enzymes require 23°C ± 2°C for optimum activity *in vitro* (Burkart *et al.*, 2000). To explore the possibility that rNDST-1 could be used with a PAPS cycle, the *N*-deacetylation/*N*-sulfation of K5 polysaccharide and PM PS by rNDST-1 were tested using a modification of the published PAPS cycle conditions (Burkart *et al.*, 2000) (see *Materials and methods*). First, a coupled *N*-deacetylation/*N*-sulfation reaction, using modified PAPS cycle components, was performed at 23°C ± 2°C. Although the *N*-sulfation of K5 polysaccharide was successful, the reaction yield was low based on *N*-sulfation analysis of the digested products by HPLC (data not shown).

To test the potential incompatibility of the PAPS cycle components with the *N*-deacetylase activity of rNDST-1, the reaction was uncoupled. *N*-deacetylation of *N*-acetyl heparosan was first carried out overnight at 23°C ± 2°C, followed by *N*-sulfation of the partially *N*-deacetylated polysaccharides, using the PAPS cycle components, at 23°C ± 2°C. Under these conditions, the *N*-sulfation yield increased to 24% (Table I), suggesting incompatibility of the *N*-deacetylase activity of rNDST-1 with the PAPS cycle components. To maximize the uncoupled reaction, the deacetylation reaction was carried out on *N*-acetyl heparosan at 37°C, followed by *N*-sulfation of the partially *N*-deacetylated polysaccharide, using the PAPS cycle components, at 23°C ± 2°C. Under these conditions the *N*-sulfation yield increased to 38% (Table I). By comparison, coupled reactions carried out with exogenously added PAPS resulted in an *N*-sulfation yield of 37% at 23°C ± 2°C and 60–65% at 37°C (Table I).

Discussion

There has been a dramatic increase in the past decade in the understanding of HS/heparin structure, biosynthesis, assembly, regulation, and role in processes from development to cancer. Many excellent reviews have been published detailing the advances in this fast growing field of research (Esko and Lindahl, 2001; Esko and Selleck, 2002; Forsberg

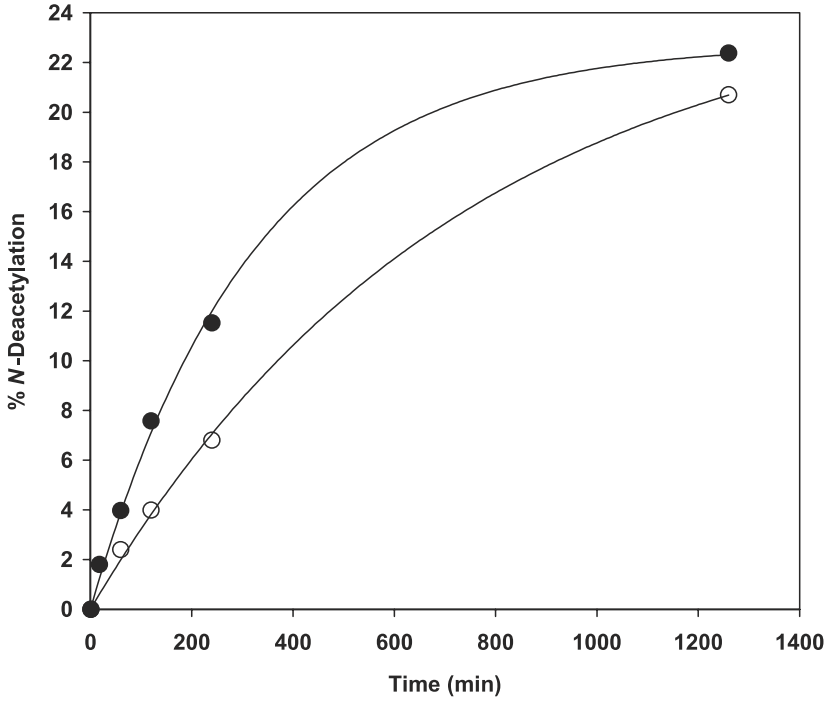

Fig. 4. Time course of *N*-deacetylation of K5 polysaccharide by rNDST-1. Percent *N*-deacetylation was calculated from the HPLC profiles of *N*-deacetylated products. Reactions were performed at 37°C (closed circles) and 23°C ± 2°C (open circles).

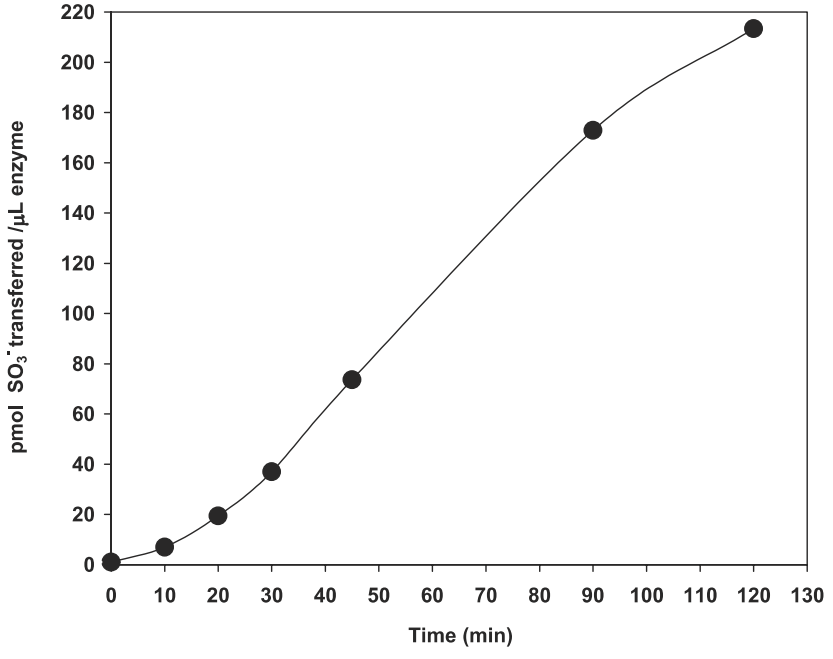

Fig. 5. Time course of *N*-sulfation of K5 polysaccharide by rNDST-1. Sulfotransferase activity of rNDST-1 was measured over time using a radioactive assay under optimal conditions (pH 6.5, 37°C). Activities were expressed as pmol SO_3^- transferred per µl purified enzyme.

and Kjellen, 2001; Perrimon and Bernfield, 2000; Shriver *et al.*, 2000).

Of the enzymes involved in the synthesis and modification of HSs, the NDST isozymes have the unique role of defining areas on the polysaccharide backbone where subsequent modifications can occur (reviewed in Grobe *et al.*, 2002). The bifunctional NDST has been studied extensively in natural and cloned forms from mammalian sources, but

analysis of this enzyme has been complicated by the existence of endogenous GAGs, as well as the enzymes that synthesize and modify these polysaccharides in all mammalian tissues and cell lines. Here we report for the first time expression of rNDST-1 in *S. cerevisae* and the demonstration of both *N*-deacetylase and *N*-sulfotransferase activities of the expressed enzyme. The use of *S. cerevisae* offers a clear advantage over mammalian expression systems in that a yeast expression system contains a background free of GAGs and GAG-synthesizing and -modifying enzymes. Although bacterial expression systems provide a similar background to yeast, the intracellular environment of bacteria is not conducive to the recovery of a bifunctionally active NDST-1. The *N*-sulfotransferase domain of human NDST-1 has been successfully expressed in *E. coli* (Sueyoshi *et al.*, 1998), but expression of both *N*-deacetylase and *N*-sulfotransferase activities of NDST-1 in bacteria was not successful (Kusche-Gullberg and Kjellen, 2003). Our attempts to express the rNDST-1 in bacteria resulted in inclusion

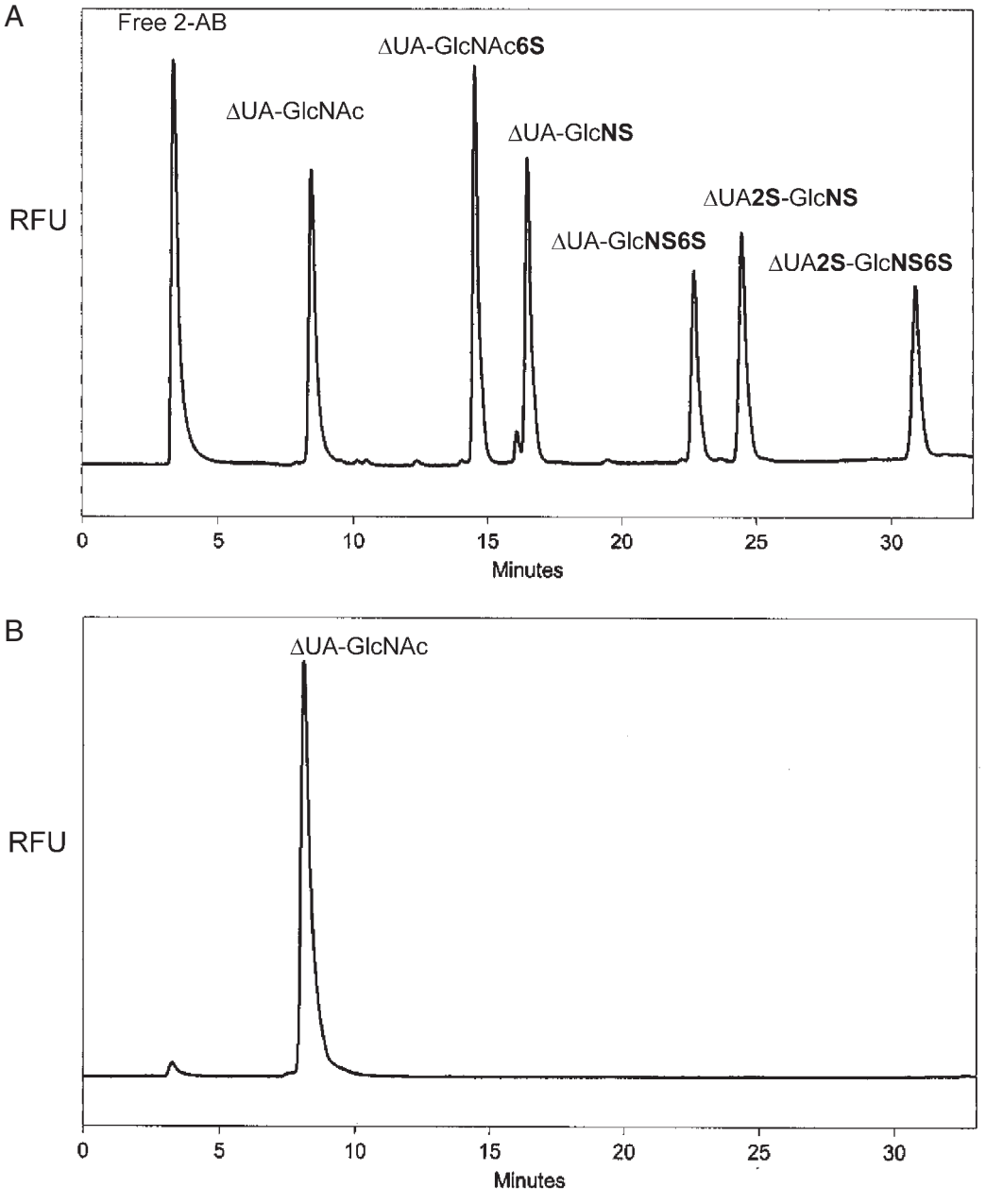

Fig. 6. HPLC profiles of *N*-deacetylated, *N*-sulfated polysaccharides. Following *N*-deacetylation and *N*-sulfation, polysaccharides were enzymatically digested, labeled with 2-AB, and analyzed on ion exchange HPLC. ΔUA-GlcN coelutes with the reagents (unincorporated 2-AB fluorescent tag) at 3.3 min. (**A**) Sulfated and nonsulfated unsaturated disaccharides labeled with 2-AB (standards, ΔUA-GlcNAc, 8.1 min, ΔUA-GlcNS, 16.3 min); (**B**) 2-AB-derivatized disaccharides obtained from unmodified K5 polysaccharide; (**C**) 2-AB-derivatized disaccharides obtained from *N*-acetylated/*N*-sulfated K5 polysaccharide; (**D**) 2-AB-derivatized disaccharides obtained from *N*-acetylated/*N*-sulfated PM PS (RFU: Relative Fluorescence units).

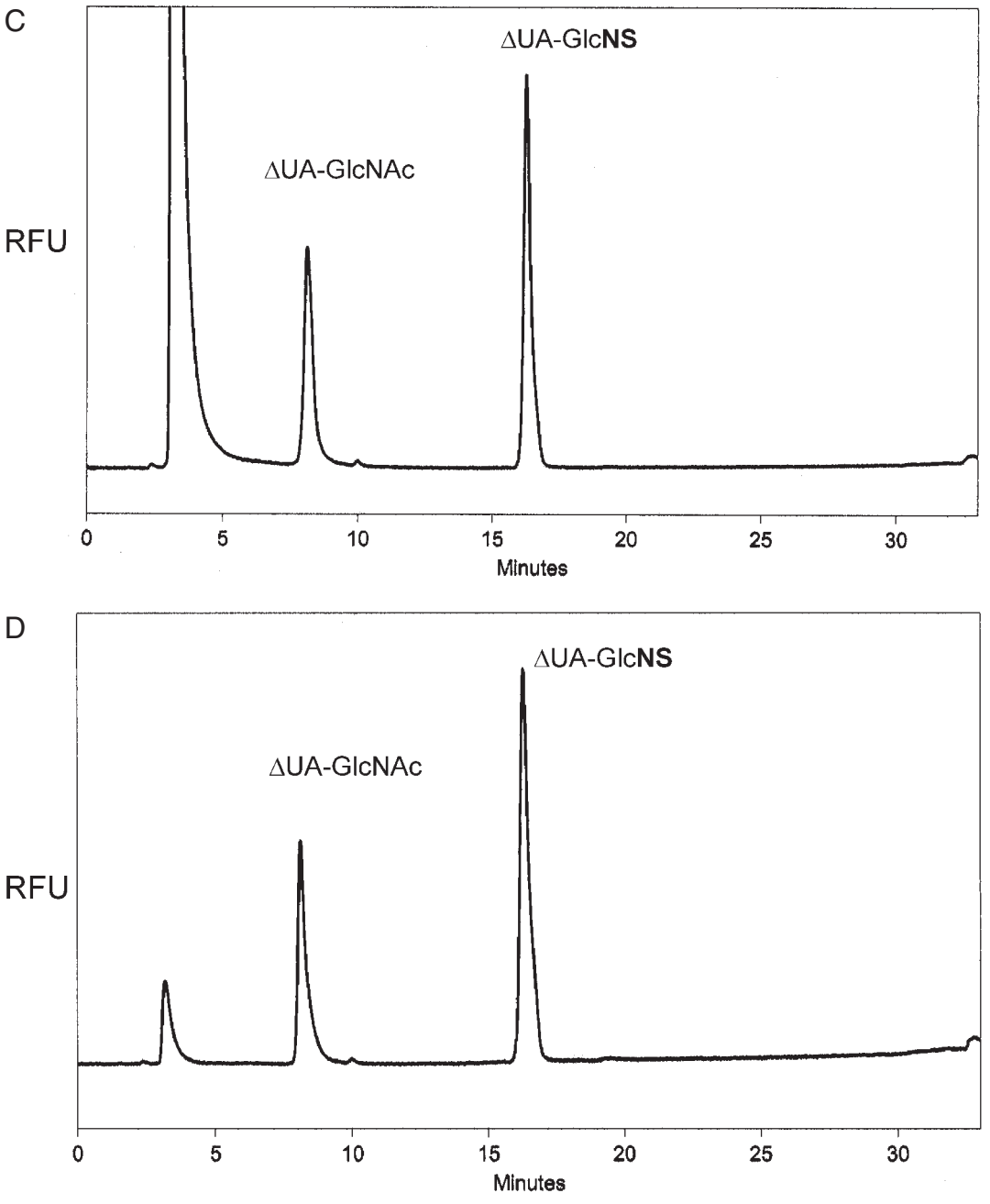

Fig. 6. Continued

body formation, and attempts at refolding of the inclusion bodies resulted in a soluble enzyme with *N*-sulfotransferase activity but lacking *N*-deacetylase activity (unpublished data).

The *N*-deacetylation and *N*-sulfation activities of NDST-1 are coupled *in vivo*, with *N*-deacetylation a prerequisite for *N*-sulfation. We chose to focus on enzymatic studies using unmodified K5 polysaccharide as substrate. This polysaccharide is identical to the heparan backbone and this substrate is sufficient for demonstrating the bifunctional activity of NDST-1. Kusche *et al.* (1991) showed that incubation of mastocytoma microsomal fractions with K5 polysaccharide resulted in *N*- and *O*-sulfated sugars resembling heparin/HS, indicating that NDST-1 would use this polysaccharide substrate. Most reports in the literature have investigated the individual enzyme activities, optimizing reactions for either the *N*-deacetylase or *N*-sulfotransferase functions. It was reported that addition of PAPS to microsomal preparations from mast cell tumors (Silbert, 1967) or rat liver (Riesenfeld *et al.*, 1982) controlled the extent of *N*-deacetylation of HS precursors, suggesting that increase in *N*-deacetylation was caused by concomitant *N*-sulfation due to a higher affinity of *N*-deacetylase for partially

Table I. *N*-deacetylation and *N*-sulfation of *N*-acetyl heparosan (K5 polysaccharide or PM PS) using endogenous PAPS or PAPS cycle

Reaction	Temp (°C)	PAPS cycle	% *N*-sulfation
N-deacetylation	35	−	
N-sulfation	35	−	60–65
N-deacetylation	23 ± 2	−	
N-sulfation	23 ± 2	+	24
N-deacetylation	37	−	
N-sulfation	23 ± 2	+	38
N-deacetylation	37	−	
N-sulfation	23 ± 2	−	37

Each step was carried out in one pot overnight using purified rNDST-1. *N*-sulfation yields were determined from HPLC chromatograms of 2-AB-derivatized disaccharides as described in *Materials and methods*.

N-sulfated substrates. Bame *et al*. (1991) have found that adding exogenous PAPS did not increase the *N*-deacetylation in microsomal preparations from wild-type or mutant Chinese hamster ovary cells containing threefold lower *N*-sulfotransferase activity. The extent of *N*-deacetylation was found to be equal to the extent of *N*-sulfation.

Our results using *S. cerevisae*–expressed rNDST-1 clearly demonstrated the coupled nature of the two activities as suggested by early *in vivo* work by Silbert (1967) and Riesenfeld *et al*. (1982). *In vitro N*-deacetylation of *N*-acetyl heparosan (K5 polysaccharide or PM PS) in the absence of *N*-sulfation reached a maximum of 30–35%, but addition of exogenous PAPS to this reaction, initiating *N*-sulfation, allowed the recovery of 60–65% *N*-sulfated products. These results demonstrate that *N*-sulfation of *N*-deacetylated residues in the K5 polysaccharide (or PM PS) allowed further *N*-deacetylation of neighboring residues. Bengtsson *et al*. (2003) have recently demonstrated that introducing single mutations in each domain of NDST-1 created mutants having either *N*-deacetylase or *N*-sulfotransferase activities. The experiment with these mutants has shown that rate-limiting step of the overall reaction was *N*-deacetylation, which also determined the degree of *N*-sulfation, agreeing with our studies.

We developed a coupled reaction incorporating *N*-deacetylation and *N*-sulfation of *N*-acetyl heparosan (K5 or PM PS) in a one-pot reaction. It was discovered that optimal *N*-sulfation of *N*-acetyl heparosan in a coupled reaction occurred when the reaction was optimized for the *N*-deacetylation reaction (Pettersson *et al*., 1991), and required only the presence of Mn^{2+} and PAPS. We also attempted to develop a coupled reaction of *N*-deacetylation/*N*-sulfation of *N*-acetyl heparosan with NDST-1 using the PAPS cycle (Burkart *et al*., 2000). It was discovered that the *N*-sulfation yields were extremely low when coupled reactions using the PAPS cycle were performed at 23°C ± 2°C, as required for optimum PAPS cycle enzyme activities. However, if *N*-deacetylation was performed first at 37°C, followed by *N*-sulfation at 23°C ± 2°C with the PAPS cycle enzymes, 37–38% *N*-sulfated polysaccharide was obtained. Further optimization of the PAPS cycle coupled reaction will be required to make this a viable large-scale process. Figure 7 summarizes production of *N*-sulfated heparosan using coupled NDST reactions in a pH 6.5 buffer containing Mn^{2+} and endogenous PAPS. It is also shown that the PAPS cycle can be used for auxiliary sulfate regeneration. We established a simple one-pot production of *N*-sulfated polysaccharides that are ready for the subsequent steps in HS and heparin biosynthesis.

Yeast expression of rNDST-1 offers an alternative to chemical preparation of *N*-deacetylated and *N*-sulfated heparosan polysaccharide (Naggi *et al*., 2001) for providing substrates, free of contaminating HS, for evaluation of other HS modification enzymes. The yeast system also allows the development of the large-scale enzymatic biosynthesis of *N*-sulfated heparosan, starting from a K5 polysaccharide backbone, following further modifications of heparin/HS (Sarıbaş *et al*., unpublished data).

Materials and methods

Materials

NDSNAcH and CDSNAcHS, unsaturated GAG disaccharide kit [heparan/heparin-disaccharide kit (H kit)] and heparitinase I were from Seikagaku (Falmouth, MA). Quick Spin Columns (G-25 Sephadex) were from Roche (Indianapolis, IN). Rat liver cDNA was obtained from Clontech (Palo Alto, CA). Yeast-PE LB yeast protein extraction kit was purchased from Genotech (St. Louis, MO). pCR-Blunt, pYES2/NTC vectors, and Sc EasyComp Transformation Kit were from Invitrogen (Carlsbad, CA). Heparinase I, heparinase II, and PAP, DNSH, desulfated chondroitin, disaccharide standards for *N*-deacetylation, and PNPS were from Sigma (St. Louis, MO). PAPS was from Calbiochem (La Jolla, CA). ^{35}S-PAPS 1 mCi was from PerkinElmer Life Sciences (Boston, MA). K5 polysaccharide and PM PS were prepared according to the protocol of Rodriguez *et al*. (1988). BCA protein assay kit was purchased from Pierce (St. Louis, MO). LB agar plates and growth media for bacteria and yeast were obtained from Teknova (Half Moon Bay, CA). The other molecular biology materials were obtained from various suppliers. All chemicals are of reagent grade.

Yeast and bacterial strains

InvSc1 yeast cells were from Invitrogen. *E.coli* K5 (ATCC #23506) and *P. multocida* (ATCC #43019) bacterial strains were from ATCC (Manassas, VA).

Cloning rNDST-1 into the pYES2/NTC yeast expression vector

To make a 132-bp truncation in the 5′ end of the rNDST-1 gene, the following polymerase chain reaction (PCR) primers were designed (restriction sites introduced are underscored): sense, NDASTXH1(5′-TTATTCTGGAGCCCTC GGCAGATGCTTCTGAG-3′), antisense, NDASTXB-1 (5′-CGGCAGATCTCTACCTGGTGTTCTGGAGGTCT T-3′). One nanogram of rat liver cDNA (Clontech) was

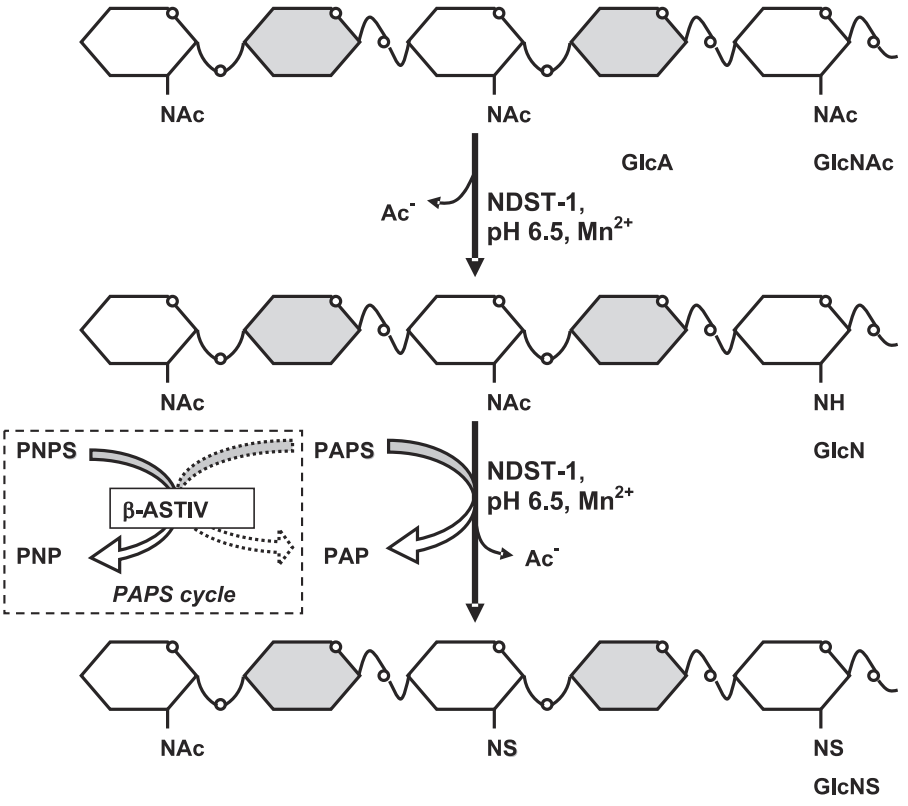

Fig. 7. *N*-deacetylation and *N*-sulfation by yeast expressed rNDST-1. The scheme describes the coupling of the two enzyme activities in rNDST-1. Sulfate donor PAPS can be added exogenously or by incorporation of the PAPS cycle, although the overall *N*-sulfation yield is lower than indicated when the PAPS cycle is used.

amplified with the primers NDASTXH1 and NDASTXB1 using Stratagene's Herculase high-performance PCR system. The 2.5-kb PCR product, containing the truncated rNDST-1 coding sequence was excised and purified from an agarose gel and cloned into the pCR-Blunt vector (Invitrogen). pCR-Blunt-rNDST-1 was digested with *Xba* I and *Xho* I and the resultant insert was cloned into *Xba* I and *Xho* I–digested pYES2/NTC, creating pYES2-rNDST-1.

Expression of rNDST-1 in yeast cells

S. cerevisiae InvSc1 cells were made competent, and pYES2-rNDST-1 DNA was transformed into the competent cells using the S.c. EasyComp Transformation kit (Invitrogen) according to the manufacturer's instructions. Transformed colonies, InvScl/pYES2-rNDST-1, were selected on glucose minus uracil agar plates (Teknova). Single colonies were picked and inoculated in 5-ml CM-glucose minus uracil media (Teknova) and grown at 30°C with shaking overnight. Overnight cultures were inoculated in 100 ml CM-glucose minus uracil and grown until the OD_{620nm} was 0.5. The cultures were harvested by centrifugation ($3000 \times g$ for 5 min), and the pellets were resuspended in 90 ml induction media (CM-galactose minus uracil, Teknova). Five-milliliter aliquots were taken at 0 h induction, centrifuged ($1500 \times g$ at 4°C) and washed with dH_2O (1/10 culture volume). The pellet was stored at −80°C until it was processed. The remaining culture was grown for 16 h at 30°C. After 16 h, 45 ml of the culture ($OD_{620nm} = 1.12$) was withdrawn, centrifuged, and washed as described and stored at −80°C. The remainder of the culture was allowed to grow for 24 h after induction ($OD_{620nm} = 1.2$). The cultures were then harvested, washed, and stored at −80°C as described.

Preparation of yeast cell extracts

The frozen yeast cell pellets were thawed and treated with yeast-PE LB yeast protein extraction kit (Genotech). An equal volume of yeast suspension buffer supplemented with β-mercaptoethanol (143 mM) was added to the pellets. The yeast cell suspension was vortexed to obtain a homogenous suspension. Longlife Zymolyase enzyme (Genotech) was added to this suspension. The contents were mixed gently followed by incubation at 37°C for 1 h. At the end of the incubation, the suspension was centrifuged at $10{,}000 \times g$ for 5 min. Supernatants were discarded and the pellets were treated with 5–10 volumes of yeast-PE LB containing 1 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride. The suspensions were vortexed and incubated on ice 30 min followed by 1–2-min incubation at 37°C to obtain yeast cell lysates. The lysates were centrifuged at maximum speed in an Eppendorf microcentrifuge for 1 h at 4°C. Clarified lysates were used for assays and purification.

Purification of rNDST-1 on a heparin-Sepharose CL-6B column

Yeast culture, induced for 24 h, was used for preparation of yeast cell extracts. Approximately 1.4 g yeast pellet was obtained, and 6 ml yeast cell extracts were prepared as described. Heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) was packed into a 1×10 cm column, washed extensively with dH_2O, and equilibrated with buffer A (10 mM Tris–HCl, pH 7.2, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 20% glycerol). Clarified yeast extracts containing the enzyme rNDST-1 were loaded onto the column, and the column was washed with buffer A until the baseline returned to absorbance return to zero at 280 nm. Linear gradient elution was carried out in buffer A from 0.15 to 0.65 M NaCl. The fractions were tested for NDST activity using the radioactive sulfotransferase assay. The fractions exhibiting highest NDST activity were pooled and concentrated using Apollo7 centrifugal concentrators (Orbital Biosciences, Topsfield, MA).

N-*deacetylation and* N-*sulfation reactions*

N-deacetylation of K5 or PM PS by rNDST-1 (5–10 μU) was carried out in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.5, in the presence of 10 mM $MnCl_2$ at either $23°C \pm 2°C$ or 35–37°C. *N*-sulfation of K5 or PM PS by rNDST-1 was carried out in 10 mM HEPES buffer, pH 7.0, with 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, except during optimized, coupled reactions, where *N*-sulfation was carried out in the same buffer as *N*-deacetylation. In the coupled reactions, *N*-sulfation was initiated by addition of excess PAPS (200–400 μM) and additional rNDST-1 (5–10 μU). All reactions were stopped either by heating the reaction mixture at 98°C or by storage at −20°C.

The rate of *N*-sulfation was monitored using a radioactive sulfotransferase assay. In each reaction, 10 μg acceptor sugar, 10 μM PAPS, and 400,000–500,000 cpm ^{35}S-PAPS were incubated with 10–30 μl of cell lysate or 1–10 μl (1–10 μU) purified enzyme in 100 μl final volume at 37°C or $23°C \pm 2°C$, as indicated. The reactions were stopped by the addition of 10 μl chondroitin sulfate (20 mg/ml) and 480 μl 100% EtOH, followed by overnight incubation at −20°C to allow precipitation of the sugars. The following day, the tubes were centrifuged in a microcentrifuge at maximum speed for 10 min. The supernatants were removed completely and the pellets were dissolved in 50 μl TE buffer (10 mM Tris–HCl, pH 8.8, 0.1 M NaCl, 1 mM ethylenediamine tetra-acetic acid). Thirty-five microliters of resuspended reaction products were applied to prespun Quick Spin Columns (G-25 Sephadex, Roche). The columns were centrifuged at $1100 \times g$ for 2 min at $23°C \pm 2°C$, the flow-through fractions were collected and mixed with liquid scintillation fluid, and radioactivity was determined in a liquid scintillation counter.

The *N*-deacetylation rate was monitored by HPLC analysis of the formation of GlcNH-containing disaccharides following enzymatic digestion of polysaccharides (see next section).

HPLC analysis of N-*deacetylated and* N-*sulfated polysaccharides*

The method was based on the work by Kinoshita and Sugahara (1999). Reaction samples (50–200 μl), containing 5–100 μg modified/unmodified polysaccharide, were heated to 98°C for 2 min and centrifuged for 5 min at maximum speed (microcentrifuge) to deactivate and precipitate enzymes. The supernatants were dialyzed against 100 ml water on V Series Membranes (0.025 μm, Millipore) for 1 h and then evaporated to dryness. The samples were then digested to disaccharides by incubating first at 30°C for 2 h and then at 37°C overnight in 60 μl 20 mM Tris–HCl buffer, pH 7.1, containing heparinase I (5 U), heparinase II (1 U), heparitinase I (0.002 U), 50 mM NaCl, and 4 mM $CaCl_2$. The resulting disaccharides contain UV-absorbing unsaturated UAs at their nonreducing termini.

Following digestion the solutions were heated to 98°C for 2 min and centrifuged (microcentrifuge) for 5 min at maximum speed to remove the digestion enzymes. The supernatants were analyzed by RP-HPLC on a Chromolith Performance RP-18e column (10 cm × 4.6 mm) (Merck KGaA, Darmstadt, Germany). The separation was performed in an isocratic mode using 5% acetonitrile (ACN) in 5 mM Tetrabutylammonium dihydrogen phosphate, pH 6.75, as a mobile phase running at 5 ml/min. The analytes were monitored at 232 nm.

Alternatively, the supernatants were removed and evaporated to dryness. The resulting variously sulfated disaccharides were labeled with fluorescent tag 2-AB by adding 5 μl 2-AB (0.7 M) in 30% HOAc/70% dimethyl sulfoxide and 5 μl $NaBH_3CN$ (1 M) in tetrahydrofuran and incubating at 60°C for 2 h. The labeled disaccharides were suspended in 1 ml 95% ACN, and passed through a filter (MF Support Pad, 13 mm, Millipore), which was prewetted by passing 1 ml water followed by 3 ml 95% ACN. The samples were then washed with 3 ml ACN and eluted with 1 ml 20% ACN. The solutions were dried, redissolved in 100 μl water, and analyzed by ion exchange HPLC on a YMC-Pack, Polyamine II column (25 cm × 4.6 mm, 5 μm, Waters, Milford, MA). A linear gradient was employed from 0 to 100% B (A: 15 mM NaH_2PO_4, B: 800 mM NaH_2PO_4) in 30 min. The flow rate was 1 ml/min.

The analytes were detected by fluorescence with excitation and emission wavelengths of 330 nm and 420 nm, respectively. Retention times of different disaccharides were determined by processing commercially obtained disaccharide standards (ΔUA-GlcNAc, ΔUA-GlcNAc6S, ΔUA-GlcNS, ΔUA-GlcNS6S, ΔUA2S-GlcNS, ΔUA2S-GlcNS6S) following the same procedure (Kinoshita and Sugahara, 1999).

ΔUA-GlcN coelutes with the unincorporated 2-AB fluorescent tag at 3.3 min. There was also high degree of variation in the amount of free 2-AB from sample to sample.

N-*sulfation of* N-*deacetylated heparosan in PAPS cycle*

The PAPS cycle buffer Bis-tris-propane, pH 7.0 (Burkart *et al.*, 2000), was substituted with MES buffer, pH 6.5, to accommodate NDST reactions. $MnCl_2$ was also included in the cycle to ensure NDST activity. *N*-deacetylation of K5 polysaccharide was carried out overnight as described.

The *N*-deacetylated reaction mixture was added to an equal volume of 50 mM MES (pH 6.5) buffer containing 10 mM $MnCl_2$, 186 μM PAP, 100 mM PNPS, 0.5 μl dithiothreitol, and 10 μl refolded PAP-free β-arylsulfotransferase IV (GST-β-ASTIV, Sarıbaş, unpublished data). Several minutes after the PAPS cycle was initiated, additional rNDST-1 (5–10 μU) was added. The reactions were allowed to run overnight and the products were kept frozen at −20°C until analysis. The cycle was monitored by measuring *p*-nitrophenol formation at 405 nm as described earlier (Burkart *et al*., 2000). In parallel, *N*-deacetylated heparosan was *N*-sulfated by addition of PAPS and partially purified rNDST-1 at 23°C ± 2°C. This reaction was run along with the PAPS cycle reaction. Analyses of *N*-deacetylated and *N*-sulfated products were done as described.

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Abbreviations

2-AB, 2-aminobenzamide (anthranilimide); ACN, acetonitrile; CDSNAcHS, completely desulfated *N*-acetylated heparan sulfate; DNSH, de-*N*-sulfated heparin; HS, heparan sulfate; GAG, glycosaminoglycan; GST, glutathione-S-transferase; HPLC, high-performance liquid chromatography; NDSNAcH, *N*-desulfated *N*-acetylated heparin; NDST, *N*-deacetylase/*N*-sulfotransferase; PAP, adenosine 3′-phosphate 5′-phosphate; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PCR, polymerase chain reaction; PNPS, paranitrophenyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PM PS, *Pasteurella multocida* polysaccharide; PNP, paranitrophenol; RP, reverse phase; UA, uronic acid.

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