A role of Lys^{614} in the sulfotransferase activity of human heparan sulfate *N*-deacetylase/*N*-sulfotransferase

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Abstract An active sulfotransferase (ST, residues 558–882) domain of the human heparan sulfate *N*-deacetylase/*N*-sulfotransferase (hHSNST) has been identified by aligning the amino acid sequence of hHSNST to that of mouse estrogen sulfotransferase (EST). The bacterially expressed ST domain transfers the 5′-sulfuryl group of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to only deacetylated heparin with an efficiency similar to that previously reported for the purified rat HSNST. Moreover, the $K_{m,PAPS}$ (2.1 μM) of the ST domain is also similar to that of the rat enzyme. Lys^{48} is a key residue in mEST catalysis. The residue corresponding to Lys^{48} is conserved in all known heparan sulfate sulfotransferases (Lys^{614} in the ST domain of hHSNST). Mutation of Lys^{614} to Ala abolishes *N*-sulfotransferase activity, indicating an important catalytic role of Lys^{614} in the ST domain. Crystals of the ST domain have been grown (orthorhombic space group $P2_12_12$) with diffraction to 2.5 Å resolution.

Key words: Lysine-614; Sulfotransferase

1. Introduction

Heparan sulfate is a member of the glycosaminoglycans that also include chondroitin, keratan and dermatan sulfates. Organic sulfates play important roles in various biological processes such as blood coagulation, cell growth, and viral infection [1–3]. Biosynthesis of heparan sulfate is catalyzed by a group of Golgi membrane enzymes called heparan sulfate sulfotransferases including *N*-deacetylase/*N*-sulfotransferase [1]. Heparan sulfate *N*-deacetylase/*N*-sulfotransferase (HSNST) is a multifunctional enzyme that catalyzes the sequential reactions of deacetylation and sulfation, converting *N*-acetylated glucosaminoglycan to the *N*-sulfated product [4]. *N*-Sulfation is obligatory for heparan biosynthesis to proceed with subsequent reactions such as 2-*O*-, 3-*O*- and 6-*O*-sulfation. Recent progress in the cloning of cDNAs has begun to provide the molecular basis for understanding the function of these enzymes, as well as the biological and therapeutic implications of these enzymes [5–9].

HSNST is an enzyme within the large sulfotransferase family. Sulfotransferases catalyze transfer of the 5′-sulfuryl group of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to various endogenous and exogenous substrates [10]. There are two major sets of the sulfotransferases: cytosolic and Golgi membrane enzymes. The membrane enzymes generally sulfate higher molecular weight substrates such as oligosaccharides and proteins and exhibit high substrate specificity. On the other hand, the cytosolic enzymes such as estrogen sulfotransferase (EST) catalyze the sulfation of low molecular weight substrates including steroid hormones, bioamines, pharmaceuticals and environmental chemicals ([10] and the subsequent series). Contrary to the membrane enzymes, these cytosolic enzymes display broad but distinct substrate specificity. Recently, the X-ray crystal structure of mouse EST has been solved for the sulfotransferase family and has identified potential catalytic residues [11,12]. Structural and mutational studies of a membrane sulfotransferase have not yet been reported.

To characterize the structure and function of the sulfotransferase activity of human heparan sulfate *N*-deacetylase/*N*-sulfotransferase (hHSNST), we have bacterially expressed the ST domain and have performed a site-directed mutagenesis study. Moreover, the ST domain has been crystallized.

2. Materials and methods

2.1. cDNA cloning, expression and purification

Using a GCG program BESTFIT, the amino acid sequence of the mouse estrogen sulfotransferase [12] was aligned to that of the human heparan sulfate *N*-deacetylase/*N*-sulfotransferase [7]. A cDNA encoding the 325 residues from positions 558 to 882 of the human enzyme was then amplified from a cDNA of RCC23 (human renal carcinoma cell line) and was verified by the nucleotide sequencing. The amplified cDNA was inserted into pGEX-4T3 vector between *Bam*HI and *Eco*RI site and transformed into *Escherichia coli* BL21 cell in order to express the sulfotransferase domain with the 325 amino acids under the conditions previously reported from this laboratory [13]. The expressed fusion protein was applied to a glutathione-Sepharose 4B (Pharmacia) column (2.6 ∅×10 cm) equilibrated with 50 mM Tris-HCl (pH 8.3) containing 0.1 M NaCl. The ST domain was eluted by cleavage with bovine thrombin solution (Sigma, 10 U/ml in the equilibration buffer) at a flow rate of 0.1 ml/min. The eluate was passed through a DEAE Sepharose column (5 ml bed volume), then an benzamidine Sepharose (Pharmacia) column (2 ml bed volume) and finally applied on a PAPS-agarose (Sigma) column (1.6 ∅×5 cm). The bound protein was eluted with the buffer containing 1 mM PAP. The purified protein was analyzed with a 10% SDS-polyacrylamide gel.

2.2. Site-directed mutagenesis

We mutated the codon Lys^{614} to that of Ala using a QuickChange site-directed mutagenesis kit (Stratagene). The primers used were 5′-CATCATCGGCCCCCAGGCAACTGGCACCACTGCCCTC-3′ and 5′-GAGGGCAGTGGTGCCAGTTGCCTGGGGGCCGATGATG-3′.

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2.3. Sulfotransferase assay and other analytical methods

The *N*-sulfotransferase activity determination was based on the reported method [5] using desulfated heparin as substrate. The reaction mixture (total volume of 50 μl) consisting of 50 mM HEPES-NaOH (pH 7.0), 0.15 M NaCl, 0.75 mg/ml protamine, 0.1 mg/ml or 0.2 mg/ml desulfated heparin, 10 ng enzymes and ^{35}S-PAPS was incubated for 10 min at 37°C. The reaction was stopped by immersing for 2 min in boiling water. The products were co-precipitated with 20 μg chondroitin sulfate by adding 150 μl ethanol containing 1.3% potassium acetate. The precipitates were washed with 70% ethanol and dissolved in 50 μl H_2O then applied on Micro-spin G25 columns (Pharmacy). The radioactivities of the eluates were determined by liquid scintillation. $K_{m,PAPS}$ was determined by the direct linear plot [14]. The enzyme concentrations were determined using a molar absorbance coefficient (ε_{280} = 60 790) calculated from the content of tryptophan and tyrosine in the enzyme.

2.4. Crystallization and data collection

The purified bacterially expressed ST domain was dialyzed against 100 mM Tris-HCl buffer (pH 8.3) containing 100 mM NaCl. The resulting solution was then adjusted to a concentration of 18 mg/ml and 4 mM PAP (3′-phosphoadenosine-5′-phosphate). Crystals were grown in the presence of 200 mM NaCl, 30% PEG3000 and Bis-Tris-propane (pH 7.0). The diffraction data were collected at room temperature on an RAXIS IV area detector system and a Rigaku RV-300 rotating anode generator. The data were processed using DENZO and scalepack [15].

3. Results and discussion

3.1. Sequence identification of the ST domain

We have aligned the sequence of 878 amino acids in hHSNST with that of mEST. The 294 residue sequence of mEST aligns to residues 558–882 of hHSNST (Fig. 1). Thus, the sulfotransferase activity of hHSNST appears to be restricted to this carboxy-terminal region of 325 residues (now designated the ST domain). The ST domain is 42 residues shorter from its N-terminus than the putative sulfotransferase domain previously defined [16]. Although the ST domain shares only 18.6% sequence identity with the mEST, it contains the conserved sequence motifs for PAPS binding [17]: 5′PSB (5′-phosphosulfate) and 3′PB (3′-phosphate) (indicated by boxes in Fig. 1). In the X-ray crystal structure of the ternary EST-PAP-E2 complex, Lys^{48} of the 5′PSB motif is directly coordinated to the 5′-phosphate group of PAP and is in a position to function as a catalytic residue [12,17]. In addition, Lys^{48} is conserved in all known sulfotransferases (aligned to Lys^{614} in the ST domain). Intriguingly, other possible catalytic residues (Lys^{106} and His^{108}) of EST are not conserved in the ST domain.

A.

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mEST:     1 METSMP---- -EYYEVFGEF RGVL-----M DKRFTKYWED VEMFLARPDD  40
hHSNST: 558 LQTLPPVQLA QKYFQIFSEE KDPLWQDPCE DKRHKDIWSK -EKTCDRFPK 606

                    5′PSB
mEST:    41 LVIATYPKSG TTWISEVVYM IYKEGDVEKC KEDAIFNRIP YLECRN----  86
hHSNST: 607 LLIIGPQRTG TT--ALYLFL GMHPDLSSNY PSSETFEEIQ FFNGHNYHKG 645

mEST:    87 ----EDLI-- --NGIKQLKE KESPRIVKTH LPPKVLPASF WEKNCKMIYL 128
hHSNST: 655 IDWYMEFFPI PSNTTSDFYF EKSANYFDSE VAPRRAAALL --PKAKVLTI 702

          3′PB
mEST:   129 CRNAKDVAVS YYY------- -FLLMITSYP NPKSFSEFVE KF--MQGQVP 168
hHSNST: 703 LINPADRAYS WYQHQRAHDD PVALKYTFHE VITAGSDASS KLRALQNRCL 752

mEST:   169 YGSWYDHVKA WWEKSKNSRV LFMFYEDMKE DIRREVVKLI EFLERKPSAE 218
hHSNST: 753 VPGWYATHIE RWLSAYHANQ ILVLDGKL-- -LRTEPAKVM DMVQKFLG-- 797

mEST:   219 LVDRIIQHTS FQEMKNNPST NYTMMPEEMM NQKVSPFMRK --GIIGDWKN 266
hHSNST: 798 -VTNTIDYHK TLAFDPKKGF WCQLLEGGKT KCLGKSKGRK YPEMDLDSRA 846

mEST:   269 HFPEALRERF DEHYKQQMKD C---TVKFRM EL 295
hHSNST: 847 FLKDYYRDHN IELSKLLYKM GQTLPTWLRE DLQNTR 882
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B.

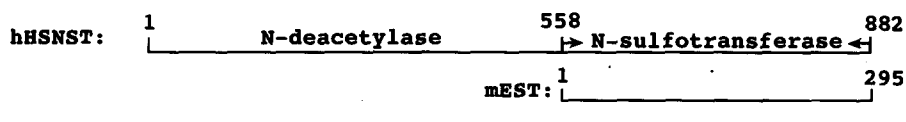

Fig. 1. Amino acid sequence alignment and ST domain. A: The alignment of EST with hHSNST was produced using the GCG BESTFIT program. Numbers indicate the residue positions in each enzyme. The 5′-phosphosulfate binding motif (5′PSB) and 3′-phosphate binding motif (3′PB) are boxed. Lys^{614} is shadowed. B: A possible domain structure of hHSNST is depicted. Arrows indicate the regions from which oligonucleotide primers were generated for PCR amplification.

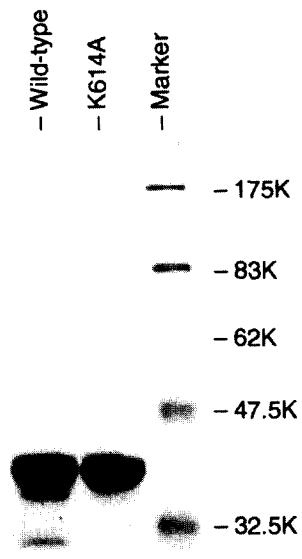

Fig. 2. SDS-gel electrophoresis of the wild-type ST domain and K641A mutant. 5 μg of protein and protein molecule weight markers (NEB) were electrophoresed on a 10% SDS polyacrylamide gel. The gel was then stained by Coomassie blue.

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                5'PSB
mEST      47  PKSGTTWI
hHSNST   613  QKTGTTAL
haHS2OST  82  PKTASTSF
hHS3OST   63  RKGGTRAL
hHS6OST   85  QKTGGTTF
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Fig. 3. Multiple sequence alignments of heparan sulfate sulfotransferases. This alignment was made by extending our previous work using Multiple [17]. The amino acid sequences used here: mouse estrogen sulfotransferase (mEST, S78182), human heparan sulfate *N*-deacetylase/*N*-sulfotranseferase (hHSNST, U18918), hamster heparan sulfate 2-*O*-sulfotramsferase (hHS2OST, D88811), human heparan sulfate 3-*O*-sulfotransferase (haHS3OST, AF019386) and human heparan sulfate 6-*O*-sulfotransferase (hHS6OST, AB006179). The numbers in parentheses indicate their accession numbers in the NCBI/GenBank protein sequence database, whereas those in the figure show the positions of the first residues of the sequences. The Lys^{48} and its corresponding lysines are in bold. 5'PSB represents the sequence motif that binds to the 5'-phosphosulfate of PAPS [12,16,20].

3.2. Characterization of the ST domain

The ST domain was successfully expressed in BL21 cells transformed by the pGEX vector containing the amplified cDNA that encodes the 325 residues of the domain. Approximately 15 mg of the pure domain protein can be obtained from a 1 l bacterial culture. An apparent molecular weight of the bacterially expressed ST domain is 38 kDa, in agreement with the 37861 Da calculated from the amino acid sequence (Fig. 2). The ST domain displays a high heparan sulfotransferase activity of 231 nmol sulfate incorporated/min/mg protein (Table 1). After correcting for the substrate concentration, the specific activity of the ST domain is comparable to the activity previously reported for purified rat HSNST [18]. As expected, the ST domain does not sulfate *N*-acetylated substrates. The low sulfotransferase activity of the ST domain toward *O*-desulfated/*N*-sulfated substrate may be due to an incomplete *N*-sulfation of this substrate. Alternatively, the ST domain may have broadened its substrate specificity with sulfating the 2-, 3- and/or 6-positions. This is unlikely, however, since the ST domain has exhibited no sulfation activity toward NDSNAc in that the 3-*O*-, 2-*O*- and/or 6-*O*-positions are partially desulfated (Table 1). $K_{m,PAPS}$ of the ST domain is similar to that of rat HSNST (1 μM) [19] and also to the corresponding K_m values of heparan sulfate 2-*O*-sulfotransferase and heparan sulfate 6-*O*-sulfotransferase [5,6]. The bacterially expressed ST domain thus appears to have retained the original enzymatic properties for the hHSNST.

The potential catalytic residue Lys^{48} of EST is conserved in all known sulfotransferases including heparin sulfate sulfotransferases. Previous site-directed mutagenesis studies have shown that mutation of the conserved Lys residues (Lys^{48} in EST; Lys^{59} in flavonol sulfotransferase) elicits a profound effect on transferase activity [20,21]. To examine whether the Lys^{48}-corresponding Lys^{614} of the ST domain is also critical for the *N*-sulfotransferase activity, this Lys residue was mutated to Ala (K614A mutant) and expressed in BL21 cells. The purified mutant enzyme exhibits the same apparent molecular weight on a SDS-polyacrylamide gel electrophoresis as the wild-type ST domain (Fig. 2). Most importantly, the mutated enzyme shows no detectable heparan sulfotransferase activity (Table 1), indicating that Lys^{614} plays a key role in conferring heparan *N*-sulfotransferase activity to hHSNST.

3.3. Crystallization of the ST domain

Since our bacterially expressed ST domain is an active enzyme, its X-ray crystal structure may provide useful information for understanding substrate specificity and catalytic mechanism of hHSNST. Crystals of the ST domain were grown to $0.05 \times 0.05 \times 0.2$ mm in the presence of the inactive cofactor PAP. These crystals are suitable for X-ray crystallographic analysis and diffract to 2.5 Å resolution. The space group and unit cell dimensions have been determined (Table 2). There is one molecule in the asymmetric unit giving a V_m of 2.2 $Å^3$/Da, that corresponded to a solvent content of 44% [22]. We have collected a nearly complete set of native data and are now searching for heavy metal derivatives.

Table 1
Sulfotransferase activity of the ST domain and mutant enzymes

Enzyme	Substrate			
	ADS	CDSNAc	CDSNS	NDSNAc
Wild-type	231 ± 14	11 ± 7	57 ± 8	n.d.
K641A	n.d.	n.d.	n.d.	n.d.

The activity was measured as described in Section 2 and is expressed as μmole of sulfate/min/mg protein. ADS represents completely desulfated heparin at all positions; CDSNAc completely desulfated (the 2-, 3-, 6-positions)/*N*-acetylated heparin; CDSNS completely desulfated (the 2-, 3-, and 6-positions)/*N*-sulfated heparin; NDSNAc *N*-desulfated/*N*-acetylated heparin in that the 2-, 3- and 6-positions are partially sulfated. n.d., activity not detectable.

Table 2
Crystallographic data

Space group	$P2_12_12$
Unit cell parameters (Å)	$a = 89.2$
	$b = 55.4$
	$c = 66.3$
# of crystals	1
Resolution (Å)	2.5
% Completeness	98

3.4. General discussion

PAPS is the ubiquitous sulfate donor of all known mammalian sulfotransferases, regardless of whether membrane or cytosolic enzymes. The recently solved EST-PAP-E2 structure has revealed PAPS binding motifs and the key residues that interact with the 5′- and 3′-phosphate groups, respectively [12]. The 5′PSB motif of mEST consists of the carboxy-terminal five residues of the PSB loop and the first two residues of the following helix 3 [17]. The PSB loop resembles the P-loop structure motif often conserved as a nucleotide binding site in many kinases and phosphatases [23]. Our previous multiple sequence alignment of the cytosolic and membrane sulfotransferases has provided sequence evidence that these motifs are also conserved in the membrane ST enzymes including hHSNST [17]. In fact, the catalytic residue Lys^{48} of EST is conserved in the heparan sulfate 2-*O*-, 3-*O*- and 6-*O*-sulfotransferases (Lys^{83}, Lys^{64} and Lys^{86}, respectively), in addition to Lys^{614} of the *N*-sulfotransferase (Fig. 3). The present finding (no detectable sulfotransferase activity in the K614A mutant) has now shown that the 5′PSB motif and this lysine residue are functionally conserved in hHSNST, as well as in the other heparan sulfate sulfotransferases.

Until very recently the lack of sequence similarity between cytosolic and membrane sulfotransferases was enigmatic, since they catalyze the same sulfuryl transfer reaction using PAPS as the donor. In addition to the previous sequence motif analysis and structural correlation based on the EST crystal [18], the present mutational analysis of Lys^{614} has revealed structural and functional similarities of the PAPS binding site in all sulfotransferases. It has appeared that the sulfotransferase family enzymes conserve the structure and function of the PAPS binding site, in spite of their immensely diverse substrate specificities. The determination of the X-ray crystal structure of the ST domain of hHSNST and its comparison to the EST structure may provide clues for understanding the structural basis for the substrate specificity of the sulfotransferase family.

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