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Engineering Sulfotransferases to Modify Heparan Sulfate*

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Abstract

The biosynthesis of heparan sulfate (HS), an essential glycan in many organisms, involves an array of specialized sulfotransferases. Here, we present a study aimed at engineering the substrate specificity of different HS 3-*O*-sulfotransferase isoforms. Based on the crystal structures, we identified a pair of amino acid residues responsible for selecting the substrates. Mutations at these residues altered the substrate specificities. Our results demonstrated the feasibility of tailoring the specificity of sulfotransferases to modify HS with desired functions.

Heparan sulfate (HS) plays a role in regulating embryonic development, inflammatory response, and assisting viral/bacterial infections. HS is a highly sulfated polysaccharide that contains repeating glucuronic acid (GlcUA, **1**) or iduronic acid (IdoUA, **2**) and glucosamine units, each capable of carrying sulfo groups. Heparin, a commonly used anticoagulant drug, is a specialized form of highly sulfated HS. The uniquely distributed sulfation pattern in HS is believed to regulate its functional specificity^{1,2}. The wide range of functions attracts considerable interest in exploiting HS or HS-like molecules for the development of anticancer, antiviral, and better anticoagulant drugs³. However, chemical synthesis of HS, especially those larger than hexasaccharides, is extremely difficult. Employing HS biosynthetic enzymes, specifically the HS sulfotransferases, for synthesizing HS has recently gained momentum^{4,5}. The substrate specificities of HS sulfotransferases control the sulfation patterns in HS. Thus, understanding the substrate recognition mechanism utilized by HS biosynthetic enzymes will aid efforts in enzyme-based synthesis of HS with unique biological functions.

The HS 3-*O*-sulfotransferases (3-OST) family is composed of seven isoforms that synthesize HS with specific biological consequences. These enzymes transfers a sulfo group (SO₃) to a 3-OH position of a glucosamine unit. Each isoform of 3-OST transfers the sulfo group to the glucosamine unit that is linked at the non-reducing end to either GlcUA (**1**)/IdoUA(**2**) unit (3-OST-1-like activity), 2-*O*-sulfated iduronic acid (IdoUA2S, **3**) unit (3-OST-3-like activity), or a combination of both (3-OST-5-like activity) (Fig. 1)¹. 3-OST-1-modified HS that is bound by antithrombin regulates the blood coagulation cascade through intrinsic anticoagulant

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Author Contributions

D.X., D.S. and J. L. prepared and characterized 3-OST mutants. A.F.M. and L.C.P. performed crystallization of 3-OST-5 and subsequent data analysis. D.X., A.F.M., L.C.P. and J.L. wrote the manuscript.

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activity. 3-OST-3-modified HS is bound by herpes simplex virus type 1 glycoprotein D, serving as an entry receptor for the virus. Interestingly, HS modified by 3-OST-5 endows both anticoagulant activity and the ability to promote herpes simplex virus type 1 entry.

The crystal structure of human 3-OST-5 (h3-OST-5) presented here (Fig. 2A and Suppl. Table 1) exhibits high structural similarity to the previously reported structures of murine 3-OST-1 (r.m.s.d of 1.2Å over 248 Cα atoms) and human 3-OST-3 (r.m.s.d of 0.95Å over 257 Cα atoms) (Suppl. Fig. 1). The core of these enzymes is comprised of an α/β motif, a structural feature common among sulfotransferases and also found in certain protein kinases^{6,7}. Central to the core is a P-loop like motif, termed the PSB-loop in sulfotransferases, which is involved in binding to the sulfo donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS, 4). The proposed position of the acceptor substrate, in a large open cleft running across the active site, is predicted based on the crystal structure of 3-OST-3 in complex with a tetrasaccharide (Suppl. Fig 1)⁸. The secondary and tertiary structures surrounding the core are quite similar in all three enzymes, suggesting similar positioning of the acceptor polysaccharide substrate. Some small structural variations are observed among isoforms as described in Suppl. Fig 1.

Based on the crystal structures, we attempted to engineer the specificity of the 3-OST-5 isoform by selectively removing either 3-OST-1- or 3-OST-3-like activity from 3-OST-5 using a site-directed mutagenesis approach. Initially, our efforts were focused on the catalytic site, where both the acceptor and PAPS are in close proximity. However, these mutants are all catalytic inactive, suggesting that these residues are required for catalytic function (Suppl. Table 2). We next examined the structure of the 3-OST-5 substrate-binding cleft, in regions distal to the active site, compared with the structures of 3-OST-1 and 3-OST-3. Two residues (Glu⁸⁸ and His²⁷¹) in 3-OST-1, which might contact the polysaccharide unit toward the nonreducing end of the sulfation site, appear to form a 'gate' across that end of the proposed substrate binding cleft (Fig. 2B). These residues are located at the ends of β-strands 2 and 11. This 'gate' narrows the cleft to a distance of 6.7Å. In 3-OST-3, however, the structurally corresponding residues have very small side chains (Gly¹⁸² and Gly³⁶⁵), resulting in a wider cleft, with a distance of 14.2Å (Fig. 2C). 3-OST-5 is more similar in structure to 3-OST-3 in this region, where Ser¹²⁰ and Ala³⁰⁶ also give a wide (14.2Å) cleft (Fig. 2D).

The role of this gate in conferring substrate specificity for 3-OST-5 was investigated by swapping the corresponding residues among isoforms. Three mutants were prepared, including A306H, S120E and a double mutant S120E/A306H, converting them to amino acid residues corresponding to those of 3-OST-1. We then determined the reactivity of the mutants to different polysaccharide substrates (Table 1): *N*-sulfo heparosan (NSHP) consists mainly of a disaccharide repeating unit of (–GlcUA-GlcNS–)_n (Suppl. Fig. 2B); *N*-sulfo heparosan iduronate 2-*O*-sulfate (NSHPI2S) predominantly consists of a disaccharide repeating unit of (–IdoUA2S-GlcNS–)_n (Suppl. Fig 2C). Choosing NSHP and NSHPI2S, we were able to measure the contribution of the IdoUA2S unit to the substrate reactivity of the substrate. It is known that 3-OST-3-like activity sulfates the glucosamine that is linked to an IdoUA2S unit at the nonreducing end (Fig. 1). HS is a natural substrate and is structurally more complex, consisting of six different disaccharides units (Suppl. Fig 2D). All three 3-OST-5 mutants displayed reduced reactivity to NSHPI2S (4 to 12-fold decreased compared to that of wild type enzyme, Table 1), while retaining the same reactivity to NSHP, suggesting that Ser120 and Ala306 are required for those substrates carrying the IdoUA2S unit. Our results also suggest that mutation of the gate residues greatly reduces 3-OST-3-like activity of 3-OST-5 enzyme.

"Gate" residues of 3-OST-1 were replaced by the gate residues of 3-OST-3 to create the following mutants: E88G, H271G and H271G/E88G. We observed that both 3-OST-1 H271G and 3-OST-1 H271G/E88G had increased reactivity for NSHPI2S (17- and 9-fold, respectively) (Table 1). Furthermore, both mutants had increased reactivity to NSHP. Our data

suggest that 3-OST-1 mutants H271G and H271G/E88G retain 3-OST-1 activity and have acquired enhanced 3-OST-3-like activity, which is equivalent to the substrate specificity of 3-OST-5. Indeed, both NSHP and NSHP12S are good substrates for 3-OST-5 (Table 1). Thus, the gate structure in 3-OST-1 also regulates the substrate specificity. We noted that 3-OST-1 E88G displayed no changes in reactivity to either NSHP12S or NSHP.

Replacing the gate residues of 3-OST-1 with serine (E88S), alanine (H271A) or both (E88S/H271A), which are the corresponding residues in 3-OST-5, had similar effect on reactivity to the polysaccharide substrates as the 3-OST-1 mutants (H271G and E88G/H271G) (Suppl. Table 3). Mutations at the gate residues were prepared for 3-OST-3, but did not have significant effects on substrate specificity, suggesting that there may be other factors contributing to substrate selection in 3-OST-3 (Suppl. Table 3).

Although we did not observe significant differences in the substrate reactivity among 3-OST mutants to HS, the products displayed different binding profiles to antithrombin (AT) and herpes simplex virus glycoprotein D (gD) (Table 1). HS modified by 3-OST-5 mutants S120E, A306H, and S120E/A306H increased binding to AT from 15% to 25%, 22% and 25%, respectively. In contrast, the percentage of gD-binding HS was decreased among the HS modified by the mutant proteins (from 13.4% to 5.7–7.5%). For 3-OST-1 mutants, both H271G and E88G/H271G showed a significant decrease in the binding to AT (from 44% to about 18%), and a modest increase in gD-binding HS for 3-OST-1 mutants H271G and E88G/H271G was observed (from 6.4% to 8–11%). 3-OST-3 mutants showed no significant difference in the synthesis of AT- and gD-binding HS. The decrease in gD binding by products of 3-OST-3 mutants G365H G182E/G365H was likely due to substantially decreased 3-*O*-sulfotransferase activity (Suppl. Table 3). Disaccharide composition analysis of HS [³⁵S]sulfated by 3-OST mutants confirmed the transformation of 3-OST isoforms (Suppl. Table 4 and Suppl. Fig 3). Results from kinetic analysis suggested that mutation did not alter the enzyme catalytic efficiency (Suppl. Table 5).

Our results have given insight into the mechanism used by 3-OST isoforms, especially for 3-OST-5 and 3-OST-1, to determine the substrate specificity. It appears that the enzyme employs two sites, namely the catalytic site and the gate, to select the appropriate polysaccharide substrate as depicted in Suppl. Fig. 4. The catalytic site interacts with the acceptor glucosamine unit (S-site) as well as one saccharide unit to either side (+1 site and −1 site). The catalytic sites are highly homologous among isoforms, and the amino acid residues involved in binding to the substrate are essential for 3-*O*-sulfotransferase activity. However, the entire substrate recognition motif that dictates different selectivity among the isoforms likely incorporates at least five sugar units (expanding from −3 site to +1 site, Suppl. Fig 4). Although the exact structure of the substrate recognized by this motif is unknown, the gate residues appear to be involved in the selective process, discriminated for or against the presence of IdoUA2S (Suppl. Fig. 4). Alterations of the gate residues could allow the isoform to gain substrate selectivity by replacing them with larger amino acid residues, as demonstrated for 3-OST-5 S120E, 3-OST-5 A306H, and 3-OST-5 S120E/A306H. Alternatively, replacing the gate residues of 3-OST-1 with smaller amino acids, such as glycine, resulted in loss of substrate selectivity, as demonstrated for 3-OST-1 H271G and 3-OST-1 E88G/H271G.

One explanation for the substrate selectivity for IdoUA2S is based on sugar conformations within the chain. It is known that glucuronic acid and glucosamine are stably found in the ⁴C₁, while IdoUA2S is present in both ¹C₄ and ²S₀ conformations⁹. The conformational flexibility of the IdoUA2S residue, compared to that of GlcUA, can contribute to local changes in polysaccharide chain conformation, and altered distribution of sulfate groups. Such difference could enhance binding selection by different 3-OST isoforms. The overall charge from the sulfo groups may also play a role in binding. However, the precise involvement of

charge-charge interactions is currently unknown. Additional factors may also be involved in fine tuning the synthesis of the polysaccharides with specific functions because the level of AT-binding HS in the products modified by 3-OST-5 mutants is not as high as that made by 3-OST-1 wild type protein.

Because the substrate specificity of HS sulfotransferases is inherently high, it is believed that the number of the saccharide structures generated by naturally occurring enzymes is limited. Substrate specificity engineering could expand the scope of the saccharide structures generated by a given enzyme. The structural diversity of the polysaccharides could be further expanded by utilizing polysaccharide substrates with different sulfation patterns. Our findings may someday be utilized for enzymatic synthesis of HS libraries or to prepare more homogenous polysaccharides for developing polysaccharide-based drugs⁴.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The substrate specificity of 3-OST isoforms and the biological functions of the HS modified by different 3-OST isoforms

Different 3-OST isoforms transfer a sulfo group (SO₃) to the 3-OH position of glucosamine present in different disaccharide units. 3-OST-1-like activity transfers a sulfo group to the glucosamine residue that is either linked to the glucuronic acid (GlcUA) or the iduronic acid (IdoUA), yielding the product containing disaccharides –GlcUA-GlcNS3S±6S- or –IdoUA-GlcNS3S±6S-. HS modified by 3-OST-1 binds to antithrombin and has the anticoagulant activity. The 3-OST-3-like activity transfers a sulfo group to the glucosamine residue that is linked to the 2-*O*-sulfated iduronic acid (IdoUA2S) to yield the product containing the disaccharide of –IdoUA2S-GlcNR±3S6S- (R = –H or –SO₃⁻). HS modified by 3-OST-3 binds to herpes simplex virus type 1 gD and serves as an entry receptor for herpes simplex virus type 1. 3-OST-5 has both 3-OST-1-like and 3-OST-3-like activities, thus, the HS modified by 3-OST-5 binds to both antithrombin and gD and has both the anticoagulant activity and serves as an entry receptor for herpes simplex virus.

Fig. 2. Structural characterization of 3-O-sulfotransferase isoforms 1, 3, and 5

A. Ribbon diagram of the crystal structure of human 3-OST-5 with bound PAP (5). Secondary structural elements are shown as α -helices (blue, labeled as A#), β -strands (green, labeled as B#), and random coils (yellow). The bound PAP molecule is drawn as ball-and-stick (cyan). This figure was created using Molscript¹⁰ and Raster3D¹¹. Panels **B–D** show substrate recognition by 3-OST enzymes via a gating structure. The bound PAP molecule is shown as ball-and-stick, though only the phosphate moieties (orange) are visible due to their buried location. Amino acid residues are shown on the surface in red, and are labeled in white text. Distances measured are shown as black dotted lines, and are labeled in white. The position of the substrate binding cleft is shown as a dashed green arrow. **B.** Key amino acid residues

forming a 'gate' on the nonreducing end of the substrate binding cleft of m3-OST-1. **C.** Structurally corresponding gate residues in h3-OST-3. The bound tetrasaccharide substrate (Δ UA2S-GlcNS6S-IdoUA2S-GlcNS6S, **6**) is drawn as ball-and-stick, with the glucosamine residue to be sulfated circled in yellow. **D.** Structurally corresponding gate residues in h3-OST-5. These molecular surfaces were generated using PyMOL¹².

Table 1
Determinations of the reactivity of polysaccharide substrates to 3-OST mutants and the AT and gD binding profiles of the HS modified by mutants

Mutants	Susceptibilities to different polysaccharides (pmoles of [³⁵ S]sulfo groups/ μg of protein) ^a			The bindings of 3-O-sulfated HS to antithrombin (AT) and HSV-1 glycoprotein D (gD) ^d	
	NSHP ^b	NSHPI2S ^b	HS ^b	AT binding ^d (%)	gD binding ^e (%)
3-OST-5 WT	87 ± 6	161 ± 1	155 ± 13	15.0 ± 0.3%	13.4 ± 1.4%
3-OST-5 S120E	80 ± 2	20 ± 1	90 ± 1	25.1 ± 0.6%	7.3 ± 0.6%
3-OST-5 A306H	62 ± 10	47 ± 2	325 ± 5	21.9 ± 0.2%	7.5 ± 2.2%
3-OST-5 S120E/A306H	94 ± 6	14 ± 1	195 ± 6	25.2 ± 0.9%	5.7 ± 0.4%
3-OST-1 WT	21 ± 3	10 ± 1	111 ± 3	43.6 ± 0.9%	6.4 ± 0.8%
3-OST-1 E88G	20 ± 3	13 ± 0	93 ± 16	37.9 ± 1.8%	8.8 ± 1.1 %
3-OST-1 H271G	187 ± 30	168 ± 3	190 ± 2	18.8 ± 1.1%	11.0 ± 2 %
3-OST-1 H271G/E88G	103 ± 20	88 ± 9	160 ± 11	18.4 ± 1%	8.0 ± 2 %
N-sulfotransferase modified HS ^c	--	--	--	0.8 ± 0.2%	3.2 ± 0.2 %

^aData presented are the average of two determinations ± range. Additional data are shown in Suppl. Table 5.

^bNSHP represents N-Sulfo heparosan (-GlcUA-GlcNS-)n. NSHPI2S represents N-Sulfo heparosan iduronate 2-O-sulfate (-IdoUA2S-GlcNS-)n, and HS represents heparan sulfate.

^cThe HS modified by N-sulfotransferase was used as a negative control for the binding of the HS to AT and gD.