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Investigations on β1,4-galactosyltransferase I using 6-sulfo-GlcNAc as an acceptor sugar substrate

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Abstract 6-sulfate modified *N*-acetylglucosamine (6-sulfo-GlcNAc) is often found as part of many biologically important carbohydrate epitopes such as 6-sulfo-Le^X. In these epitopes, the 6-sulfo-GlcNAc moiety is extended by a galactose sugar in a β1-4 linkage. The β4GalT1 enzyme transfers galactose (Gal) from UDP-Gal to *N*-acetylglucosamine (GlcNAc) in the presence of manganese. Here we report that the β4GalT1 enzyme transfers Gal to the 6-sulfo-GlcNAc and 4-methylumbelliferyl-6-sulfo-*N*-acetyl-β-D-glucosaminide (6-sulfo-βGlcNAc-MU) acceptor substrates, although with very low efficiency. To understand the effect that the 6-sulfate group on the GlcNAc acceptor has on the catalytic activity of the β4GalT1 molecule, we have determined the crystal structure of the catalytic domain of bovine β4GalT1 mutant enzyme M344H-β4GalT1 complex with the 6-sulfo-GlcNAc molecule. In the crystal structure, the 6-sulfo-GlcNAc is bound to the protein in a way that is similar to the GlcNAc molecule. However, the 6-sulfate group engages in additional interactions with the hydrophobic region, residues 276–285, of the protein molecule, and this group is found wedged between the aromatic side chains of Phe-280 and Trp314 residues. Since the side chain of the Trp314 residue undergoes conformational changes during the catalytic cycle of the enzyme, molecular interaction between Trp314 and the 6-sulfate group might hinder this conformational change. Therefore, the lack of a favorable binding environment, together with hindrance to the conformational changes, might be responsible for the poor catalytic activity.

Keywords β1,4-galactosyltransferase · Acceptor specificity · 6-sulfo-GlcNAc · Crystal structure · Capillary electrophoresis

Abbreviations

β4GalT1	β1,4-galactosyltransferase 1
CE	Capillary electrophoresis
GlcNAc	*N*-acetylglucosamine
6-sulfo-βGalNAc-MU	4-methylumbelliferyl-6-sulfo-*N*-acetyl-β-D-glucosaminide TRIS tris(hydroxymethyl)aminomethane
UDP-Gal	UDP-α-D-galactose
MOPS	4-morpholinepropanesulfonic acid (MOPS)

Introduction

Cell surface glycans play important roles in several cellular functions [1]. The LacNAc disaccharide βGal1-4βGlcNAc- is the most abundantly found glycan moiety in most mammalian cells. This LacNAc moiety is further modified to synthesize several important cancer antigen epitopes, such as Lewis-type sugars, in which a fucose sugar is attached to the GlcNAc residue in an α1-3 linkage [1, 2]. In addition, the LacNAc moiety undergoes chemical modification such as sulfation. A 6-sulfo-GlcNAc-containing LacNAc moiety (6-sulfo-LacNAc) is an important antigen. The linear polymer of the 6-sulfo-LacNAc motif repeat is known as keratan sulfate and is found in several tissues [3–5].

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The glycosyltransferases are involved in the synthesis of glycoconjugates of glycoproteins and glycolipids. They are type *II* membrane proteins and found mostly in the Golgi apparatus [6]. Among them, β1,4-galactosyltransferase I transfers galactose (Gal) from UDP-Gal to a non-reducing end GlcNAc, thus synthesizing the LacNAc moiety [7]. In vertebrates, the β4GalT1 molecule exists as a family of seven enzymes with highly similar protein sequences [8]. All of these family members transfer Gal from UDP-Gal, but to a different acceptor sugar substrate. The β4GalT4 enzyme exhibits 41 % protein sequence identity with the β4GalT1 enzyme, and it has been shown to transfer Gal from UDP-Gal to a 6-sulfo-GlcNAc acceptor substrate [9]. Among the family members, only the structure and function of the β4GalT1 enzyme is well understood, although the structure of β4GalT7 from *Drosophila* is known [10, 11].

Earlier structure and function studies of the β4GalT1 enzyme have led to a better understanding of both the donor and acceptor substrate specificities. For example, β4GalT1 is known to transfer Gal to an acceptor sugar GlcNAc but not to glucose. However, in the presence of α-lactalbumin, glucose becomes a preferred acceptor substrate to β4GalT1, thereby synthesizing the lactose disaccharide [12, 13]. Enzyme kinetic studies have shown that bulky substitution at the O6 position of the acceptor sugar GlcNAc makes it a poor acceptor substrate for the β4GalT1 enzyme [14–16]. In order to understand the effect of 6-sulfate modification of the GlcNAc molecule on the enzyme activity, we have undertaken the present study on β4GalT1 enzyme activity with the 6-sulfo-GlcNAc molecule.

We found that the β4GalT1 enzyme transfers Gal from UDP-Gal to the 6-sulfo-GlcNAc acceptor molecule with poor efficiency. To determine whether any steric hindrance caused by the 6-sulfate group might be responsible for the poor enzyme activity, we have determined its complex crystal structure with the bovine β4GalT1 molecule. We observed that the sulfate group interacts with the side chain of Trp314 residue, causing steric hindrance to the enzyme's ability to undergo conformational changes during the catalytic cycle of the enzyme, thus affecting the catalytic activity of the enzyme.

Experimental procedures

Crystal structure determination of bovine C342T-M344H-β4GalT1 with 6-sulfo-GlcNAc molecule

Earlier it has been shown that it is necessary to use the double mutant C342T-M344H-GalT1 enzyme for the crystallization of the acceptor substrate complex [17]. For this purpose the M344 to His mutation was introduced on the C342T-GalT1 mutant plasmid DNA. The detailed site directed mutagenesis, expression and purification of the bovine C342T-M344H-β4GalT1 protein are previously described [17]. The crystals of the protein complex with the acceptor substrate, 6-sulfo-GlcNAc, were grown by hanging drop vapor diffusion method at room temperature, 272 K by mixing 1 μl of protein solution with 1 μl of precipitating solution. The protein solution contains M344H-GalT1 protein at 20 mg/ml, 5 mM $MnCl_2$, 5 mM UDP-hexanolamine, and 5 mM 6-sulfo-GlcNAc while the precipitating solution contains 100 mM Mes-NaOH buffer pH 6.5, 1.8 M ammonium sulfate and 10 % (*v/v*) 1, 4-dioxane. The crystals were transferred into Nujol mineral oil without any mother liquid on them and flash cooled to 100 K for x-ray diffraction data collection. The three-dimensional diffraction data from the single crystals were collected in-house at a data collection facility equipped with a Mar345 area detector and processed using HKL2000 [18]. The data collection statistics are given in Table 1. The crystals are isomorphous to the previous crystals of the same protein complex with the different oligosaccharide acceptors; therefore, the crystal structures were solved by molecular placement methods using only protein atoms without any substrates. The substrates Mn^{2+}, UDP-hexanolamine, and the acceptor were located using the difference Fourier electron density maps. The structures were

Table 1 Crystal data collection and refinement statistics on the bovine C342T-M344H-β4GalT1 protein complex with UDP-hexanolamine•Mn^{2+} and 6-sulfo-GlcNAc

Crystal data	
a = (Å)	49.28
b = (Å)	89.90
c = (Å)	139.73
Space group	$P2_12_12_1$
X-ray data collection data	
Resolution (Å)	2.4
Unique reflections	21,217
Data redundancy	5.2 (5.0)
Completeness	90 (93)
I/σ(I)	14.1 (4.0)
R_{sym} (%)	10.6 (44)
Refinement parameters	
R_{final} (%)	18.8 (23)
R_{free} (%)	25.0 (31)
R.m.s. deviation on	
Bond length (Å)	0.004
Bond angle (°)	0.695
Ramachandran map	
Preferred region	87.9
Allowed region	12.1
PDB entry	4KRV

Numbers in the parentheses represent the value for 2.5–2.4 Å resolution shell

initially refined using REFMAC5, followed by PHENIX [19, 20]. WinCoot was used for model correction and solvent locations [21]. The final refinement statistics are given in Table 1. The final coordinated and the diffraction data were deposited in the protein database.

Site-directed mutagenesis to generate octa-mutant of β4GalT1 enzyme

Site-directed mutagenesis was performed using the PCR method. Plasmid pEGT-d129 was used as the template for constructing the mutants; this template contains a BamH I/ EcoR I fragment inserted into pET23a vector, coding for residues 130 to 402 of bovine β4GalT1 [22], and has a Cys342- to Thr- mutation. The mutation primers corresponding to the upper DNA strand are:

(1) GAT TCG ACT G**GA** ***TAT C****GC* CTA *CGT* TAC GTG CAG TAT TTT GGA GGT GTC TCT
(2) AGG TAG GC**G** ***ATA*** **TC**C *AGT CGA* ATC *CCT TCC* TAC AGA AAT GTG CCG TGG CTG

The restriction sites are shown in bold and the mutation codon in italicized letters. The entire C342T-GalT1 DNA was PCR-amplified as two fragments using the terminal cloning primers and two mutagenesis primers. The fragments were then cut with the restriction enzyme EcoR V and ligated. The full C342T-GalT1 DNA with the mutation was amplified from the ligation mixture using the cloning primers and then inserted into the pET23a vector. Mutants were screened for the incorporated mutations based on alterations in the restriction enzyme digestion patterns, and then sequenced. The positive clones were transformed into BL21(DE3)pLysS cells as described previously [22]. The mutant proteins were expressed and purified according to the published method [22].

Determination of catalytic activity using the phosphatase-coupled method

The catalytic activity of the C342T-GalT1 enzyme using 6-sulfo-GlcNAc as the acceptor substrate was determined using the phosphatase-coupled glycosyltransferase activity kit according to the manufacturer's instructions (R&D systems, USA) [23]. The assay was performed using 20 mM Tris–HCl, 5 mM $MnCl_2$, 1 mM UDP-Gal, and 1 μg of enzyme at various concentrations of the acceptor substrate, at 37 °C for 30 min. The specific enzyme activity using 6-sulfo-βGlcNAc-MU as the acceptor substrate was also determined using the same kit [23].

Determination of catalytic activity by capillary electrophoresis (CE)

The potassium salt of 6-sulfo-βGlcNAc-MU, tris(hydroxymethyl) aminomethane (TRIS), and UDP-α-D-galactose, disodium salt (UDP-Gal) were obtained from EMD Millipore (Billerica, MA, USA). 4-morpholinepropanesulfonic acid (MOPS) was obtained from Alfa Aesar (Ward Hill, MA, USA). Deionized water was obtained from an Elga Purelab ultra water system (Lowell, MA, USA).

A concentration of 1.4 μg mutant enzyme (*i.e.*, 1.8 μL of 0.75 μg/μL enzyme stock) or 1.8 μg C342T-GalT1 enzyme (*i.e.*, 1.8 μL of 1.0 μg/μL enzyme stock) was used in each 55-μL reaction volume (*i.e.*, 0.025 μg/μL octa-mutant, or 0.033 μg/μL C342T-GalT1) to measure enzyme velocity at different substrate concentrations. Each enzyme reaction contained 20 mM TRIS buffered at pH 8.0 and 1.8 mM $MnCl_2$. To determine the effect of UDP-Gal concentration, velocity was measured using 1 mM 6-sulfo-βGlcNAc-MU at different concentrations of UDP-gal (10, 20, 50, 100, 150, 250, 300, 400, or 500 μM). To determine the effect of 6-sulfo-βGlcNAc-MU concentration, velocity was measured using 0.363 mM UDP-Gal at different concentrations of 6-sulfo-βGlcNAc-MU (45, 91, 164, 272, 364, 454, 545, or 1,000 μM). Capillary electrophoresis (CE) separations were performed using a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a UV-visible absorbance or photodiode array detector. Substrate and product were monitored at 214 nm. The fused silica separation capillary had a 25-μm inner diameter and 360-μm outer diameter (Polymicro Technologies, Phoenix, AZ, USA), with an effective length of 10.0 cm and a total length of 60.2 cm. The cartridge temperature was held at 25 °C. The separation capillary was subject to the following flushing sequence daily: 1 M NaOH for 30 min at 170 kPa (25 psi); deionized water for 15 min at 170 kPa (25 psi); methanol for 15 min at 170 kPa (25 psi); deionized water for 15 min at 170 kPa (25 psi). Between each electrophoretic separation, the capillary was flushed briefly with 1 M NaOH, deionized water, methanol, and 100 mM MOPS. Reaction progress was monitored by measuring the appearance of the disaccharide product in the electropherogram. Separations were performed at 400 V/cm using a background electrolyte of 100 mM MOPS buffered at pH 7. As the reaction progressed, the reaction vial was sampled repeatedly by CE with 6-s hydrodynamic injections at 14 kPa (2.0 psi). The area of the disaccharide product obtained under each reaction condition was measured at each time point using 32 Karat Software version 5.0 (Beckman Coulter). The reaction velocity, estimated by the change in product area with time, was then determined using the "linest" function of Micosoft Excel (Redmond, WA, USA).

The MALDI-TOF analysis at negative ion mode was performed as described earlier [24] at the Protein Characterization Laboratory (PCL), SAIC-Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD.

Results and discussion

Catalytic activity of the C324T-GalT1 enzyme using 6-sulfo-βGlcNAc

Using a recently developed phosphatase-coupled glycosyltransferase assay [23], we have determined the catalytic activity of the bovine C342T-GalT1 enzyme using 6-sulfo-GlcNAc as the acceptor substrate (Fig. 1a). The C342T-GalT1 enzyme exhibits the best catalytic activity with the acceptor substrate GlcNAc molecule, while as very poor catalytic activity with the 6-sulfo-GlcNAc which is nearly 1,000 fold lower than with the GlcNAc molecule. The apparent K_m value for the 6-sulfo-GlcNAc is 1.7±0.2 mM, suggesting that the presence of the 6-sulfate group slightly enhances the affinity of the acceptor substrate, while severely hindering its catalytic activity.

CE has been utilized to determine the catalytic activity of the C342T-GalT1 enzyme [25] using 6-sulfo-GlcNAc-MU as acceptor substrate. While the advantage of this method is that it does not use radioactive-labeled UDP-Gal as a donor substrate, the disadvantage is that the acceptor substrate has to be labeled with methylumbelliferone to facilitate detection. We used CE to measure the catalytic activity of the C342T-GalT1 enzyme with 6-sulfo-βGlNAc-MU as an acceptor substrate, and we used the phosphatase-coupled method to determine its specific activity (Fig. 1b). The apparent K_m value for the 6-sulfo-GlcNAc molecule is comparable with the that of GlcNAc. The apparent K_m value derived for the acceptor substrate 6-sulfo-βGlcNAc-MU and UDP-Gal molecules are 90±10 μM and 230±90 μM, respectively. The enzyme's transfer of the Gal to the 6-sulfo-βGlcNAc-MU

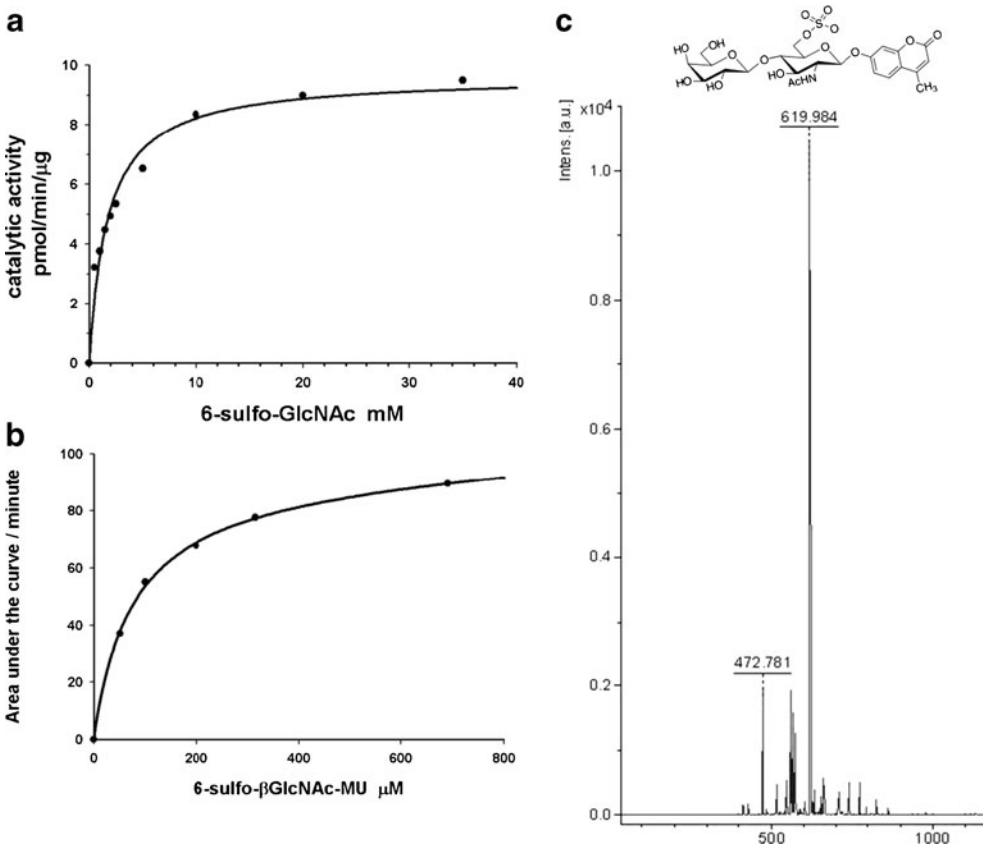

Fig. 1 Catalytic activity of C342T-GalT1 enzyme at varying concentrations of 6-sulfo-GlcNAc (**a**) and 6-sulfo-βGlcNAc-MU (**b**) 6-sulfo-βGlcNAc-MU. Each point in the graph is an average of three separate experiments. The catalytic activity using 6-sulfo-GlcNAc was measured using a phosphatase-coupled assay method [23], while the 6-sulfo-GlcNAc-MU was measured using capillary electrophoresis (CE) [25]. In the CE method, the catalytic activity was measured by the area of disaccharide product produced in the electropherogram. The catalytic activity curve was fitted using Sigma plot and using an equation defining a single-substrate binding mode. (**c**) MALDI-TOF spectrum of the reaction mixture at the negative ion mode. The molecular weight of the acceptor substrate and the product disaccharide were 458.1 Da and 620 Da, respectively. The peak, at 472.8 Da, represents the mono-oxygenated form of the acceptor. Oxygenation of aromatic compounds is known to form during the ionization of the sample in the MALDI-TOF experiment

acceptor substrate was also confirmed by mass spectrometry (MALDI-TOF) in the negative ion mode (Fig. 1c). Although the apparent K_m values are comparable with the true values for the βGlcNAc-MU and UDP-Gal, as determined previously by the CE methods [25], the poor catalytic efficiency is comparable to that of the less-preferred donor substrates, such as UDP-Glc and UDP-GalNAc [26, 27]. In order to understand the poor catalytic activity, it is essential to understand the binding of 6-sulfo-GlcNAc to β4GalT1 at the molecular level. Therefore, we have determined the crystal structure of the catalytic domain of bovine β4GalT1 in complex with 6-sulfo-GlcNAc.

Crystal structure of C342T-M344H-β4GalT1 in complex with the 6-sulfo-GlcNAc molecule

Earlier we reported the crystal structure of the catalytic domain of bovine mutant protein C342T-M344H-β4GalT1 in the presence of Mn^{2+} and UDP-hexanolamine with chitobiose (βGlcNAc1-4βGlcNAc) acceptor substrates [17]. The mutation of residue Cys342 to Thr342 is found to enhance the *in vitro* folding efficiency and also gives better stability to the enzyme [22]. The mutation of the metal-binding residue Met344 to His344 increased its affinity for the metal ion and also readily crystallized the bovine β4GalT1 in the closed conformation in the presence of Mn^{2+} and UDP-hexanolamine. This allowed the binding of the oligosaccharide acceptor substrates and the crystallization of their complexes [28]. Here we report the crystal structures of the bovine mutant protein C342T-M344H-β4GalT1 in complex with the acceptor substrate 6-sulfo-GlcNAc molecule.

In the crystal structure, the bovine C342T-M344H-β4GalT1 molecule is found in the closed conformation with a bound Mn^{2+} ion, UDP-hexanolamine and 6-sulfo-GlcNAc molecules. The Mn^{2+} ion coordination and the molecular interactions of the UDP-hexanolamine with the protein molecule are similar to the previously studied crystal structures. Although the binding of the 6-sulfo-GlcNAc molecule to the protein molecule is similar to the binding of the GlcNAc molecule with similar molecular interactions, the 6-sulfate moiety engages in additional interactions with the protein molecule (Fig. 2). The orientation of the 6-sulfate group, with respect to the sugar hexose ring, is similar to the orientation of the O6 atom of the GlcNAc molecule. The 6-sulfate group interacts primarily with the hydrophobic region, residues 276 to 285, and residue Trp314 of the protein molecule. The 6-sulfate group is found wedged between the aromatic side chains of Phe280 and Trp314 residues, exhibiting van der Waals interactions with them. In addition, two protein molecules are in the asymmetric unit, and in one of them the side chain amino group of Lys279 forms a hydrogen bond with the 6-sulfate oxygen atom (Fig. 2).

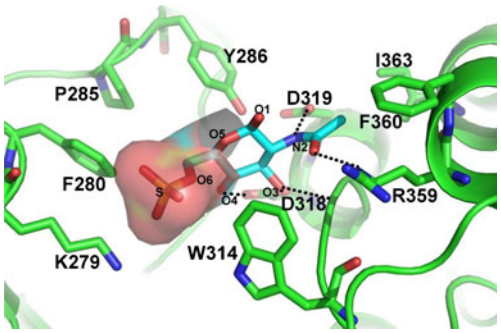

Fig. 2 Crystal structure 6-sulfo-GlcNAc bound to the catalytic domain of bovine C342T-M344H-β4GalT1 protein. The protein molecule is shown in green cartoon diagram, with the side chain of the interacting residues and 6-sulfo-GlcNAc molecules shown in stick diagram. The 6-sulfo-GlcNAc molecule is shown in cyan, with its 6-sulfate group alone shown in the surface diagram. The hydrogen bonds are shown as black dotted lines. The molecular interactions between the GlcNAc moiety and the β4GalT1 molecule are similar to the binding of the GlcNAc molecule to the β4GalT1 molecule [12, 17]. The 6-sulfate group interacts mostly with the hydrophobic region, residues 276 to 285, and the W314 residue of the protein molecule. The 6-sulfate group is found wedged between the aromatic side chains of F280 and W314 residues, undergoing van der Waals interactions with them

Thus, it seems that the presence of the sulfate group does not influence the conformation of the GlcNAc moiety or the binding of GlcNAc to the protein molecule. On the other hand, the unfavorable hydrophobic binding environment for the 6-sulfate group found in the crystal structure may reduce the affinity of the 6-sulfo-GlcNAc molecule for the protein molecule, as compared to the GlcNAc molecule.

The enzymatic catalysis of the β4GalT1 enzyme follows an S_N2 mechanism, in which the side chain carboxylate oxygen atom of Asp318 residue forms a strong hydrogen bond with the O4 hydroxyl group of the bound GlcNAc molecule, initiating the catalytic mechanism. In the present structure, the molecular interactions of the Asp318 with the 6-sulfo-GlcNAc molecule are similar to those found with the GlcNAc molecule. Also, the 6-sulfate group is well away from the catalytic pocket of the protein molecule. Therefore, the presence of the 6-sulfate group is not likely to hinder the catalytic mechanism of the enzyme.

The interactions of the 6-sulfate group with the aromatic side chain of Trp314 can affect protein conformation change in a manner that reduces the catalytic activity of the enzyme. During the catalytic cycle, the β4GalT1 molecule undergoes conformational changes involving two flexible loops: a short loop containing Trp314 residue and a long loop with residues 345 to 365. The apo-enzyme exists in an open conformation with the aromatic side chain of Trp314 residue outside the catalytic pocket to facilitate the binding of the Mn^{2+} ion and the UDP-Gal molecule. Upon binding, conformational changes occur, in which the side chain of the Trp314 moves from outside to inside the catalytic pocket, followed by the conformational change in the long loop that creates the

acceptor substrate binding site. Once the acceptor molecule binds to the enzyme, catalysis takes place. Then the two loops reverse their conformation, returning to the open form, and the products are released. Since the 6-sulfo moiety interacts with the aromatic side chain of the Trp314 residue in the closed conformation, the protein may not readily return to open conformation during the product release step, thus affecting the catalytic activity. A similar phenomenon involving the long loop was previously reported in the bovine β4GalT1 enzyme, in which the metal-binding Met344 residue was mutated to a His residue [17]. Previous studies have shown that mutation of Trp314 to Alanine residue results in loss of catalytic activity [29] and to a His residue results in a ten fold loss of activity (data not shown). Therefore, if a 6-sulfate binding site is created away from the Trp314 residue, the mutant enzyme is expected to transfer Gal to the 6-sulfo-GlcNAc molecule more efficiently than the C342T-GalT1 molecule. The orientation of the sulfate group is determined by the torsion angle about the C5-C6 bond (O5-C5-C6-O6) of the sugar molecule. There are two possible values observed (g^- or g^+) for this torsion angle in the GlcNAc molecule. In the present 6-sulfo-GlcNAc molecule, the values for this torsion angle are −56° (g^-) and 60° (g^+), which would place the sulfate moiety away from the Trp314 residue.

In humans, the β4GalT1 exists as a family of seven enzymes (β4GalT1 to T7) with a high degree of similarity of protein sequences. Among these enzymes, β4GalT4 has been shown to transfer Gal from UDP-Gal to an acceptor sugar 6-sulfo-GlcNAc molecule [9]; however, active recombinant β4GalT4 protein could not be produced from *E coli*. In addition, a detailed structure–function study on the human β4GalT4 molecule is not available [9]. A comparison of the protein sequence of all the family members for the 6-sulfate binding region of bovine β4GalT1 (residues 276 to 285) shows that polar amino acids with three Arg residues are present in the corresponding region of β4GalT4 only in the β4GalT4 protein. In all other family members, mostly hydrophobic amino acids are present (Fig. 3a and b). Since the catalytic domains of bovine β4GalT1 and human β4GalT4 show a protein sequence similarity of nearly 66 %, a homology modeling of the β4GalT4 structure based on the β4GalT1 structure is possible. Such modeling shows that, in the acceptor GlcNAc binding site near the O6 atom, a cavity surrounded by three Arg residues is present (Fig. 3a). This 6-sulfate binding cavity in β4GalT4 is generated by the residues that correspond to residues 276 to 285 in the bovine β4GalT1 molecule (Fig. 3b). When a 6-sulfo-GlcNAc is modeled in place of the GlcNAc molecule in the β4GalT4 model structure, the 6-sulfate group fits into the cavity away from the Trp residue in the short loop, with a torsion angle of 60° about its C5-C6 bond (Fig. 3a). This suggests that mutation of residues 276–285 in the bovine β4GalT1 enzyme with the corresponding polar residues from human β4GalT4 might create a 6-sulfate binding cavity well away from the Trp314 residue, and such a mutant enzyme is expected to transfer Gal to a 6-sulfo-GlcNAc molecule (Fig. 2). Of the ten amino acids between residues 276 and 285 in the bovine β4GalT1 molecule, only seven were considered for the mutation since two are conserved residues; Asp278 remains unchanged because its side chain carboxylate group forms intra-strand hydrogen bonds (Fig. 3b). Mutation of the bovine single mutant enzyme C342T-GalT1 with these seven mutations results into an octa-mutant of β4GalT1 enzyme. Compared to C342T-GalT1 enzyme this octa-mutant enzyme exhibits twenty fold reduced galactosyltransferase activity using GlcNAc as the acceptor substrate. However, using 6-sulfo-βGlcNAc-MU as the acceptor, the octa-mutant enzyme exhibited no enhancement of catalytic activity over the C342T-GalT1 enzyme. This finding suggests that additional mutations are necessary to enhance the catalytic activity towards the 6-sulfo-GlcNAc as an acceptor substrate. When the catalytic activity was measured by CE using 6-sulfo-βGlcNAc-MU as the acceptor substrate, the octa mutant enzyme shows activity that is similar to the C342T-GalT1 enzyme, with an apparent K_m value for the 6-sulfo-βGlcNAc-MU and UDP-Gal of 500±200 μM and 70±20 μM, respectively.

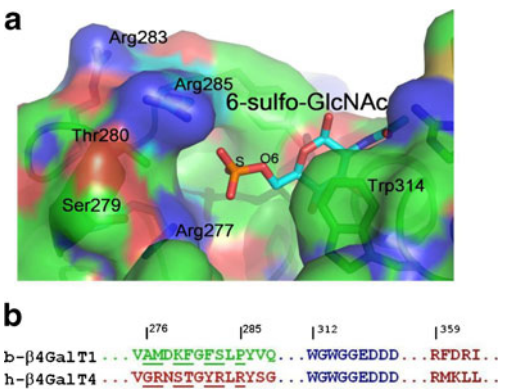

Fig. 3 A homology model of human β4GalT4 structure based on the bovine β4GalT1 crystal structure is shown in cartoon diagram embedded in the surface diagram. The possible interacting residues of the 6-sulfate group are shown in the stick diagram and the numbering corresponds to the residues in bovine β4GalT1 enzyme. The modeled, bound 6-sulfo-GlcNAc molecule is shown in the cyan stick diagram. The model shows that, in the acceptor GlcNAc binding site near the O6 atom, a cavity surrounded by three Arg residues is present. When a 6-sulfo-GlcNAc is modeled in place of the GlcNAc molecule in the β4GalT4 model structure, the 6-sulfate group fits into the cavity away from the Trp314 residue. **b** Comparison of the acceptor binding residues in bovine β4GalT1 with the human β4GalT4 protein. The 6-sulfate binding pocket forming residues in the β4GalT4 structure and their corresponding amino acids in bovine β4GalT1 are underlined. The 6-sulfate binding cavity in β4GalT4 is generated by the residues (underlined) that correspond to residues 276 to 285 in the bovine β4GalT1 molecule (underlined). Therefore, the underlined residues in bovine β4GalT1 are mutated to the corresponding underlined residues in β4GalT4 molecule

The effect of substitution on the O6 hydroxyl group of the GlcNAc moiety on the β4GalT1 enzyme activity has been reported earlier [14–16]. In these studies, when the O6 hydroxyl group of the GlcNAc molecule was replaced by a hydrogen or fluorine atom, these substrates remained as good acceptor substrates with little or no loss of catalytic activity. However, when it was replaced by a sulfhydryl (SH) group, the acceptor molecule 6-thio-GlcNAc is a very poor acceptor substrate [16]. Furthermore, loss of enzyme activity was observed only when the O6 hydroxyl group of the GlcNAc moiety was modified with either O-methylation or O-glycosylation [15]. If the O6 modification carries a charged group, it completely abolishes the activity of the enzyme. In keeping with this observation, under the enzyme assay condition (pH>7.0), the thiol group (SH) of 6-thio-GlcNAc molecule might exist as a deprotonated, negatively charged S^- form; therefore, β4GalT1 enzyme would be expected to show poor activity using this substrate. However, it is not clear how the presence of a negative charge at the sixth position of the acceptor substrate influences the catalytic activity of the enzyme. Since the O6 oxygen atom is located in the vicinity of the side chain aromatic ring of the Trp314 and Phe280 residues, there is a possibility that the anion-Pi interactions, particularly with the Trp314 side chain, would influence the conformational flexibility of the residue and thereby affect the turnaround number of the enzyme catalysis [30].

Although the C342T-GalT1 enzyme transfers Gal to the 6-sulfo-GlcNAc residue poorly, considering the difficulty in producing the recombinant β4GalT4 protein, the present study shows that β4GalT1 can be used for the synthesis of the 6-sulfo-LacNAc that is important for sulfo-Le^X carbohydrate synthesis. Many laboratories have been designing acceptor substrate–based inhibitors for the β4GalT1 molecule [31, 32]. In their design, the *N*-acetyl moiety of the GlcNAc molecule, and the extended oligosaccharide binding sites have been considered. In the present study, we find that chemical modification at the sixth position of the acceptor substrate, GlcNAc, affects the catalytic activity of the enzyme without compromising its affinity for the enzyme (as judged by the K_m value of 6-sulfo-βGalNAc-MU). Thus, the present study leads to a better understanding of the binding of the 6-sulfo-GlcNAc molecule as an acceptor substrate and also makes it possible to design better inhibitors for the β4GalT1 molecule.

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