Published in final edited form as:
Biochem Biophys Res Commun. 2008 May 23; 370(1): 67–71. doi:10.1016/j.bbrc.2008.03.029.

Identification and analysis of novel functional sites in human GD3-synthase

Yihua Gu and **Robert K. Yu***
Institute of Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912, USA

Abstract

GD3-synthase is a sialyltransferase that catalyzes the synthesis of ganglioside GD3 leading to the b- and c-series gangliosides. It contains four common sequence regions of vertebrate sialyltransferases, referred to as the L, S, III, and VS sialylmotifs, which have been identified in all vertebrate sialyltransferases that play important roles in spatial structure maintenance and protein functions. No 3D structural information, however, is currently available for vertebrate sialyltransferases. Using primary sequence of human GD3-synthase, we identified the structure of a prokaryotic sialyltransferase (CstII, also known as an α2,3/ α2,8-sialyltransferase) as the template for protein homology modeling. Secondary structural alignment between these two proteins identified several conserved amino-acid residues. The functions of four conserved residues (Asn^{188}, Pro^{189}, Ser^{190}, and Arg^{272}) between the L and S sialylmotifs in human GD3-synthase were investigated using mutational analysis and molecular modeling, and it was found that these sites are involved in determining the α2,8-linkage specificity of GD3-synthase.

Keywords

Sialyltransferase; GD3-synthase; Functional site; Site-directed mutagenesis; Molecular modeling

The sialyltransferases form an important glycosyltransferase family whose members catalyze the transfer of a sialic acid residue from a donor molecule, CMP-NeuAc, to different acceptor oligosaccharides on glycoproteins and glycolipids. According to the specificity of the linkage formed and the acceptor carbohydrates, sialyltransferases can be classified into ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia subfamilies. Based on pair-wise sequence similarity of all the known vertebrate sialyltransferases, four conserved sequence regions have been identified, referred to as sialylmotifs L [1], S [1], III [2,3], and VS [4]. Using a series of mutational analyses with different vertebrate sialyltransferases as models, these common sequence regions have been demonstrated to participate in binding of substrates, structure maintenance, and catalytic process [5–8]. Lack of structural information for the vertebrate sialyltransferases retards a clear understanding of the detailed catalytic mechanisms. Protein modeling is required to build 3D structures. Notably, two sialyltransferases from *Campylobacter jejuni*, CstI and CstII [9,10], can be identified as the homologs of vertebrate sialyltransferases, using fold-recognition methods. CstI is a mono-functional sialyltransferase catalyzing an α2,3-sialic acid transfer, while CstII is a bi-functional sialyltransferase catalyzing the transfer of sialic acid with an α2,3- or α2,8-

*Corresponding author. Fax: +1 706 721 8727. ryu@mcg.edu (R.K. Yu)..

Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbrc.2008.03.029.

linkage specificity [11]. Using CstII as a remote template, 3D structures of the human ST3Gal subfamily were modeled and acceptor-binding sites thoroughly studied by computer analysis [12,13].

According to substrate specificity, our study suggests that CstII is a remote homolog for structure building of human ST8Sias, although both CstI and CstII have a structure similar to the vertebrate sialyltransferases with high confidence. Secondary and tertiary structures of human GD3-synthase were predicted. Using secondary structure alignment of human GD3-synthase with CSTII, several highly conserved amino-acid residues between sialylmotif L and S were identified. Using the protein sequence alignment of human sialyltransferases, all conserved residues identified in our study showed ST8Sia subfamily specificity. Site-directed mutational analysis and enzymatic assay of human GD3-synthase were performed. The predicted 3D structure of human GD3-synthase with docking of substrates supports the mutation data and elucidated the contribution of these functional sites to the enzyme properties.

Materials and methods

Template searching for GD3-synthase and protein sequence analysis

PSI-BLAST [14] was used to search for homologs of human GD3-synthase. PSIPred [15], FUGUE [16], and HHPred [17] servers were also used to search for homologs of human GD3-synthase by fold-recognition methods. Retrieved from GenBank [18], amino-acid sequences of human sialyltransferases and vertebrate ST8Sias were used for multiple sequence alignment with the ClustalW program [19].

3D structure modeling and molecular docking for human GD3-synthase

Using the 3D structure of CstII (PDB ID 1RO7, chain A) as a template, the MODELLER 7.0 program [20] was used to build the structure of the residue 136–356 of human GD3-synthase. The resulting model was then evaluated using VERIFY3D [21], SolvX [22], and ANOLEA [23].

Using Autodock 3.0 [24], CMP-NeuAc was docked into the binding site of human GD3-synthase in a conformation and location similar to what has been observed in CstII. Sialyllactose, the oligosaccharide group of GM3, was docked into the GD3-synthase complex with CMP-NeuAc. Hydrogen bonds between functional sites and substrates were computed by Swiss-Pdb Viewer 3.7.

Construction of plasmid for protein expression

A DNA fragment containing mouse interleukin-2 (IL-2) signal sequence and transmembrane domain truncated human GD3-synthase cDNA fragment (amino-acid residues 49–356) was amplified by two-step PCR with two forward primers 5′-ATTGCACTAAGTCTTGCACTTGTCACGAATT CGTACCGGCTGCCCAACGA-3′, 5′-AAGCTTATGTACAGGATGCAACTCCTGTCTTGCATTG CACTAAGTCTT-3′ and one reverse primer 5′-TCTAGACTGGAAGTGGGCTGGAGT-3′, using full-length cDNA of human GD3-synthase (GeneStorm™ cDNA clone, Accession No. X77922, Invitrogen, Carlsbad, CA) as template. Two restriction sites, HindIII/XbaI, were added as underlined sequences in the primers at each side of gene. According to the manufacturer's instruction, the reaction solution contained: 5 ng plasmid DNA as template, 5.0 μl 10 × *Pfx* amplification buffer, 1.5 μl of 10 mM dNTP, 1 μl of 50 mM $MgSO_4$, 1 pmol sense and antisense primers, 1 U *Pfx* DNA polymerase (Invitrogen), and water added to the final volume of 50 μl. PCR amplification was performed 1 cycle at 94 °C for 5 min, and 30 cycles at 94 °C for 1 min, at 55 °C for 1 min, and at 68 °C for 1.5 min. After restriction digestion of

HindIII/XbaI (NEB, Ipswich, MA), the PCR fragment was inserted into pcDNA3.1 topo vector (Invitrogen) to give the plasmid pcDNA-STII49, using Fast-Link™ ligation kit (Epicentre Tech, Madison, WI). The transformants were selected for ampicillin resistance in Luria–Bertani (LB) plates. The clones were picked and amplified in LB medium with ampicillin.

Site-directed mutagenesis

Using pcDNA-STII49 as a template, mutants of human GD3-synthase were prepared by PCR-based mutagenesis with QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer pairs used for preparation of mutants are described in Supplementary Material. The recombinant plasmids were propagated in XL-10 Gold™ cells (Stratagene). All wild-type human GD3-synthase and mutants were verified with DNA sequencing.

Expression of human GD3-synthase in CHO-K1 cells and Western blot analysis

Using Lipofectamine 2000 (Invitrogen), plasmids of the wild-type human GD3-synthase and mutants were, respectively, transfected into CHO-K1 cells. Between 36 and 48 h after transfection, 100 μl culture medium was harvested, centrifuged to remove cell residue, precipitated by trichloroacetic acid, and washed with 1× PBS. The precipitate was dissolved in 15 μl Laemmli denaturing buffer (Sigma, St. Louis, MO). The amount of protein in the cell pellet and the culture medium was determined by a modified Lowry assay [25], using RC DC™ protein assay kit (Bio-Rad, Hercules, CA). The protein sample was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then electrotransferred to a nitrocellulose membrane (Bio-Rad). The membrane was developed by incubating first with anti-V5 antibody (1:5000, Invitrogen) and then with peroxidase-conjugated goat anti-mouse secondary antibody (1:3000, Santa Cruz Biotech, Santa Cruz, CA). Immunoreaction products were visualized using ECL solution kit (GE Healthcare, Piscataway, NJ).

Sialyltransferase assay

Sialyltransferase assays were performed as described previously [26]. In brief, the reaction mixtures were incubated at 37 °C for 2–3 h in a volume of 100 μl, buffered by 25 mM sodium cacodylate, pH 6.5. The reaction solution contained 10 mM MgCl, 0.15% Triton CF-54, 10 nmol donor substrate CMPNeu5Ac (Sigma) and acceptor substrate GM3 (Matreya Inc., Pleasant Hill, PA) 55,000 cpm of CMP-[14C] NeuAc (Perkin-Elmer, Waltham, MA), and 10–20 μl culture media. The reaction was terminated on ice. After partitioning with ether, the aqueous phase containing radioactive glycolipid was applied to a Sep-Pak C18 cartridge (Waters, Milford, MA) previously equilibrated with 0.1 M KCl, the column was washed with 25 ml of distilled water, and the sample was eluted with 5 ml of chloroform/methanol, 2:1 (v/v). The eluent was dried under nitrogen, and the radioactivity was measured by liquid scintillation counting. The apparent K_m of human GD3-synthase for CMP-NeuAc was obtained using 2.5–200 μM CMP-Neu5Ac with 250 μM GM3; for GM3, using 2.5–200 μM GM3 with 250 μM CMP-Neu5Ac. All enzyme assays were done in triplicate.

Results

Template identification for human GD3-synthase

Using the sequences of the full-length or catalytic domain of GD3-synthase, PSI-BLAST cannot identify a homolog whose structure is determined. When full-length human GD3-synthase was used for template searching, PSIPred and FUGUE identified CstII as a homolog at a level of “guess”, and HHPRed identified CstII as a top hit. When the sequence

of the catalytic domain of human GD3-synthase was used, all severs gave a top "hit" to CstII with high confidence.

Sequence analysis of sialyltransferases

Based on predicted structures, secondary structure alignments between CstII and human GD3-synthase were obtained from fold-recognition servers (Fig. 1). Catalytic domain of human GD3-synthase was assigned secondary structure to its sequence. The catalytic regions beginning with sialylmotif L of human GD3-synthase are shown. There are a few insertions or deletions in the regions corresponding to secondary structures. Loop regions display more variability, but their sizes are comparable, thus facilitating the modeling study. The sequence identities were determined as 16–20% by different servers. Several residues of CstII were found to be conserved or replaceable in human GD3-synthase. Some of them are located in sialylmotifs L, S, and VS, suggesting that, as a bacterial sialyltransferase, CstII also has sialylmotifs L, S, and VS, although a sialylmotif III was not present. Some other amino-acid residues were located between sialylmotifs L and S. The circled sequence "NPS" and "R" were strictly conserved between CstII and human GD3-synthase, and "N", "S", and "R" are reported as functional sites of CstII [11]. Protein sequence comparison of all the human sialyltransferases suggested that the sequences, "NPS" and "R", were strictly conserved only in the human ST8Sia subfamily (Supplementary Material).

Expression of wild-type enzyme and mutants

Since endogenous GD3-synthase enzymatic activity is absent in CHO cells, a CHO cell expression system was developed to express wild-type human GD3-synthase and mutants. Using IL-2 signal peptide, a soluble form of GD3-synthase with a V5 tag and a $(His)_6$ tag fused at the C-terminus was secreted from CHO cells into the medium (Supplementary Material). Medium of the cells transfected with plasmids without a cDNA insert showed no endogenous GD3-synthase activity. The $(His)_6$ tag was added to the construct to aid in purification of the recombinant protein from the culture medium. Nonspecific-binding proteins are the major content trapped by immobilized metal ion affinity (data not shown). Anti-V5 antibody was used to monitor the expression level of the soluble form of native enzyme and mutants.

Plasmids containing insertions of native enzyme and mutants were transfected into CHO cells. The culture media were harvested and analyzed by SDS-PAGE, followed by Western blotting. The relative amount of recombinant proteins was determined by ImageJ (http://rsb.info.nih.gov/ij/). Similar amounts of wild-type and mutant enzymes were used for enzymatic assays (Fig. 2, lower panel). The relative amount of recombinant proteins was determined and used to normalize enzyme activities of native enzyme and mutants. The enzymatic activities of mutants N188D, P189A, S189A, and R272A are 42%, 91%, 33%, and 20% of that of wild-type enzyme (Fig. 2, upper panel), respectively. Mutant P189A retained 91% of enzyme activity, suggesting that Pro^{189} might not be critical in enzyme activity. The mutant R272K restored almost 98% of enzyme activity, indicating the positive charge on the Arg^{272} side chain plays an important role in the enzymatic activity. This conclusion was confirmed by mutant R272I which has a nonpolar side chain (isoleucine) substitution and retains only 19% of enzyme activity.

Kinetic parameters of wild-type human GD3-synthase and mutants

Kinetic parameters of wild-type human GD3-synthase and three mutants (N188D, S189A, and R272A) were determined, as described under Materials and methods. Results are summarized in Table 1. The apparent K_m values of wild-type enzyme for donor and acceptor are 88 μM and 83 μM, respectively, which are consistent with data from naturally occurring GD3-synthases [27]. The K_m values of N188 D and S189A for donor substrate increased to

1.25- to 2.5-fold of the native enzyme, while the K_m values of S189A and R272A for acceptor substrate increased in a range of 2- to 10-fold. This suggests that Asn^{188} is involved in donor substrate binding, Ser^{189} is donor and acceptor substrate binding-re lated, and Arg^{272} contributes to binding of the acceptor substrate. V_{max} of mutant R272A decreases 5-fold, suggesting that Arg^{272} may also participate in catalytic process.

Predicted 3D structure of human GD3-synthase and molecular docking

To understand the roles of the functional sites, the catalytic region of human GD3-synthase was modeled using CstII as a template. The modeled structure displays a typical α/β/α Rossmann fold [28], which consists of a central portion of parallel β-strands, flanked by several α-helices on each side (Supplementary Material). Invariant in the members of ST8Sia subfamily Asn^{188}, Pro^{189}, Ser^{190}, and Arg^{272} are located around the cleft-like active site, suggesting that these residues might participate in the enzymatic activity.

CMP-NeuAc is a common donor substrate for human GD3-synthase and CstII. According to the location of CstII for donor binding, CMP-NeuAc was docked into modeled human GD3-synthase (Fig. 3A). The hydrogen bonds between the enzyme and donor substrate were determined. The side chain on Asn^{188} has a strong hydrogen bond with the carboxyl group on the sialic acid group of the donor substrate (2.98 Å). Pro^{190} has no interaction with the donor. The distance between Ser^{190} and the donor substrate is 4.4 Å, suggesting that this residue might weakly interact with the donor. Arg^{272} is 8.8 Å away from the donor, suggesting that this residue has no function on donor substrate binding.

Sialyllactose is an acceptor sugar analog, to which CstII can catalyze the transfer of a sialic acid residue with an α2,8-linkage. Because of program limitations, only the oligosaccharide group (sialyllactose) of the acceptor GM3 was introduced into the complex structure of human GD3-synthase and CMP-NeuAc (Fig. 3B). Compared to the donor substrate, the docking model of sialyllactose indicates that the acceptor oligosaccharide is more exposed to the solvent. This suggests that the non-carbohydrate portion of the acceptor substrate (ceramide) may have a weak contact with the active site of the enzyme. Asn^{188} and Pro^{189} have no contact with the receptor substrate. Ser^{190} interacts with the galactose of the acceptor substrate at a distance of 2.22 Å. The positive side chain of Arg^{272} formed a solid hydrogen bond with the O8 of the sialic acid group.

Molecular docking of human GD3-synthase with donor and acceptor substrates suggests that Asn^{188} may act as an anchor for donor substrate binding, Pro^{189} may not play a role in the activity site, Ser^{190} has a slight interaction with donor and stronger interaction with acceptor substrate, and Arg^{272} has a strong contact with the acceptor substrate with a hydrogen bond. The results of modeling of donor and acceptor substrates into the enzyme are in a good agreement with the data of kinetic analysis.

Discussion

Many functional sites in sialylmotifs are reported to act as an anchor of the donor substrate [5,6], which can explain why mutant N188D loses only part of binding ability for the donor, although Asn188 has a strong interaction with the donor substrate.

In our protein model, Ser190 protrudes the binding pocket and locks the acceptor with a contact to the galactose group of acceptor substrate, suggesting that Ser190 may function to block other oligosaccharide group except sialyllactose. But, the mutational analysis does not suggest that a dramatic change of kinetic parameters occurred when Ser190 was replaced by alanine, while the corresponding Ser53 in CstII significantly enhances the α2,8-sialyltransferase specificity [11]. A possible explanation is that there may be some other

residues working in concert with Ser190 in human GD3-synthase for the selection of acceptor substrate.

The conserved arginine plays a key role in anchoring acceptor substrate in CstII, which is also invariant in CstI [11]. The docking model of CstII with different types of acceptor substrates (α2,3- or α2,8-linkage) suggests that this conserved arginine is a critical residue to anchor the acceptor substrate and does not contribute to the acceptor specificity [10].

In conclusion, we identified several novel functional sites in human GD3-synthase, using a strategy combined with structural information and sequence analysis. We also showed that protein modeling can be used to explain the roles of functional sites in vertebrate sialyltransferases, even when the sequence similarity is low between the target and the template. However, the protein model can only adopt the backbone of the template when sequence identity is low. Except the well-conserved region, the detailed structural information of side chain is uncertain [29]. A good example is that the flexible loops in human α2,3-sialyltransferases are unable to be modeled due to the huge sequence variance between the flexible loops of the model and the template [12]. At the present, CstI and CstII are the only templates available for molecular modeling of vertebrate sialyltransferases. The sequence similarities between vertebrate sialyltransferases and CstI or CstII are only 15–20% [12], suggesting that the structural information of side chains in current models of vertebrate sialyltransferases might be inaccurate. In the current research, however, Asn188, Pro189, Ser190, and Arg272 are highly conserved in human GD3-synthase and CstII, the structural information of their side chains in the protein model should be reliable. We suggest that current protein modeling approach can be applied to structure–functional relationship study only for those regions which are highly conserved between vertebrate sialyltransferases and CstI/CstII.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by an NIH Grant (NS11853). We thank Dr. B.C. Wang, University of Georgia, Athens, for valuable suggestions during the course of this work. Acknowledgment is due to Dr. Rhea Markowitz for editorial assistance.

Abbreviations

CMP-NeuAc	cytidine 5′-monophospho-*N*-acetylneuraminic acid
ST3Gal	ST3 β-galactoside α2,3-sialyltransferase
ST6Gal	ST6 β-galactoside α2,6-sialyltransferase
ST6GalNAc	ST6 (α-*N*-acetylneuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide α2,6-sialyltransferase
ST8Sia	ST8 α2,8-sialyltransferase

References

[1]. Wen DX, Livingston BD, Medzihradszky KF, Kelm S, Burlingame AL, Paulson JC. Primary structure of Gal beta 1,3(4)GlcNAc alpha 2,3-sialyltransferase determined by mass spectrometry sequence analysis and molecular cloning. Evidence for a protein motif in the sialyltransferase gene family. J. Biol. Chem. 1992; 267:21011–21019. [PubMed: 1400416]

[2]. Kapitonov D, Yu RK. Conserved domains of glycosyltransferases. Glycobiology. 1999; 9:961–978. [PubMed: 10521532]

[3]. Jeanneau C, Chazalet V, Augé C, Soumpasis DM, Harduin-Lepers A, Delannoy P, Imberty A, Breton C. Structure–function analysis of the human sialyltransferase ST3Gal I: role of *N*-glycosylation and a novel conserved sialylmotif. J. Biol. Chem. 2004; 279:13461–13468. [PubMed: 14722111]

[4]. Geremia RA, Harduin-Lepers A, Delannoy P. Identification of two novel conserved amino acid residues in eukaryotic sialyltransferases: implications for their mechanism of action. Glycobiology. 1997; 7:v–vii. [PubMed: 9134419]

[5]. Datta AK, Paulson JC. The sialyltransferase "sialylmotif" participates in binding the donor substrate CMP-NeuAc. J. Biol. Chem. 1995; 270:1497–1500. [PubMed: 7829476]

[6]. Datta AK, Sinha A, Paulson JC. Mutation of the sialyltransferase S-sialylmotif alters the kinetics of the donor and acceptor substrates. J. Biol. Chem. 1998; 273:9608–9614. [PubMed: 9545292]

[7]. Datta AK, Chammas R, Paulson JC. Conserved cysteines in the sialyltransferase sialylmotifs form an essential disulfide bond. J. Biol. Chem. 2001; 276:15200–15207. [PubMed: 11278697]

[8]. Kitazume-Kawaguchi S, Kabata S, Arita M. Differential biosynthesis of polysialic or disialic acid Structure by ST8Sia II and ST8Sia IV. J. Biol. Chem. 2001; 276:15696–15703. [PubMed: 11278664]

[9]. Chiu CP, Lairson LL, Gilbert M, Wakarchuk WW, Withers SG, Strynadka NC. Structural analysis of the alpha-2,3-sialyltransferase Cst-I from *Campylobacter jejuni* in apo and substrate-analogue bound forms. Biochemistry. 2007; 46:7196–7204. [PubMed: 17518445]

[10]. Chiu CP, Watts AG, Lairson LL, Gilbert M, Lim D, Wakarchuk WW, Withers SG, Strynadka NC. Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. Nat. Struct. Mol. Biol. 2004; 11:163–170. [PubMed: 14730352]

[11]. Gilbert M, Karwaski MF, Bernatchez S, Young NM, Taboada E, Michniewicz J, Cunningham AM, Wakarchuk WW. The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J. Biol. Chem. 2002; 277:327–337. [PubMed: 11689567]

[12]. Sujatha MS, Balaji PV. Fold-recognition and comparative modeling of human alpha2,3-sialyltransferases reveal their sequence and structural similarities to CstII from *Campylobacter jejuni*. BMC Struct. Biol. 2006; 6:9–25. [PubMed: 16620397]

[13]. Patel RY, Balaji PV. Fold-recognition and comparative modeling of human beta3GalT I, II, IV, V and VI and beta3GalNAcT I: prediction of residues conferring acceptor substrate specificity. J. Mol. Graph. Model. 2007; 26:255–268. [PubMed: 17212986]

[14]. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389–3402. [PubMed: 9254694]

[15]. Jones DT. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 1999; 292:195–202. [PubMed: 10493868]

[16]. Shi J, Blundell TL, Mizuguchi K. FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J. Mol. Biol. 2001; 310:243–257. [PubMed: 11419950]

[17]. Söding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 2005; 33:W244–W248. [PubMed: 15980461]

[18]. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. Nucleic Acids Res. 2007; 35:D21–D25. [PubMed: 17202161]

[19]. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 2003; 31:3497–3500. [PubMed: 12824352]

[20]. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 1993; 234:779–815. [PubMed: 8254673]

[21]. Eisenberg D, Lüthy R, Bowie JU. VERIFY3D: assessment of protein models with three-dimensional profiles. Methods Enzymol. 1997; 277:396–404. [PubMed: 9379925]

[22]. Holm L, Sander C. Evaluation of protein models by atomic solvation preference. J. Mol. Biol. 1992; 225:93–105. [PubMed: 1583696]

[23]. Melo F, Feytmans E. Assessing protein structures with a non-local atomic interaction energy. J. Mol. Biol. 1998; 277:1141–1152. [PubMed: 9571028]

[24]. Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. Automated docking using a lamarckian genetic algorithm and empirical binding free energy function. J. Comput. Chem. 1998; 19:1639–1662.

[25]. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951; 193:265–275. [PubMed: 14907713]

[26]. Dasgupta S, Chien JL, Hogan EL. Biosynthesis of GM1b and similar neolactoseries gangliosides by a partially purified chicken skeletal muscle sialyltransferase. Effect of sphingomyelin and acetylcholine. Biochim. Biophys. Acta. 1990; 1036:11–17. [PubMed: 2223821]

[27]. Higashi H, Basu M, Basu S. Biosynthesis in vitro of disialosylneolactotetraosylceramide by a solubilized sialyltransferase from embryonic chicken brain. J. Biol. Chem. 1985; 260:824–828. [PubMed: 3838172]

[28]. Rao S, Rossmann M. Comparison of super-secondary structures in proteins. J. Mol. Biol. 1973; 76:241–256. [PubMed: 4737475]

[29]. Dunbrack RL Jr. Sequence comparison and protein structure prediction. Curr. Opin. Struct. Biol. 2006; 16:374–384. [PubMed: 16713709]

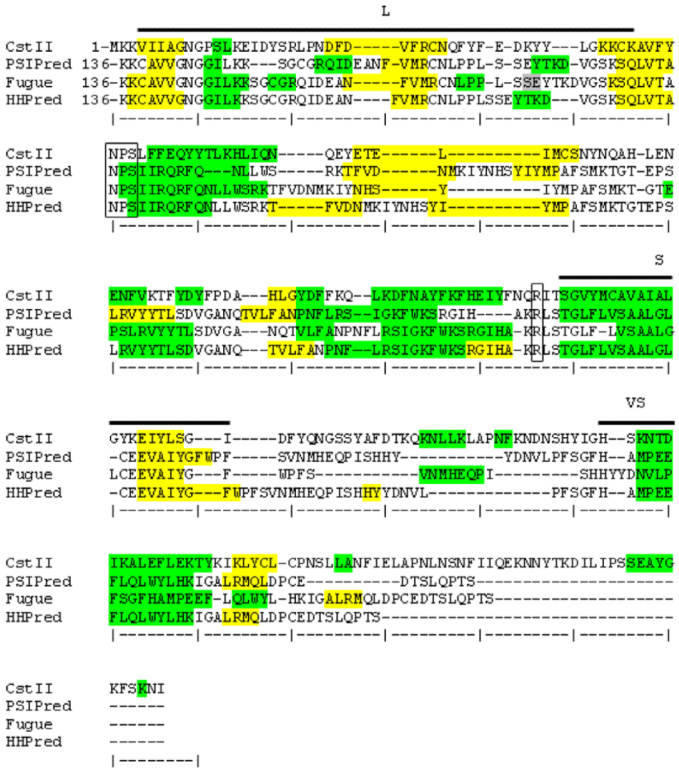

Fig. 1.

Secondary structure alignment of hSTSia I with CstII. Secondary structure alignment is performed by PsiPred, Fugue, and HHpred servers, with partial amino-acid sequence containing sialylmotif of human GD3-synthase (hSTII, residues 136–356) and full-length sequence of CstII. In the alignment, the first line is the sequence of CstII, others are human GD3-synthase whose secondary structure is predicted by different prediction servers; the possible activity sites, “NPS” and “R”, are circled. Protein secondary structure is indicated by background colors, blank: coil; green: α-helix; yellow: β-strand.

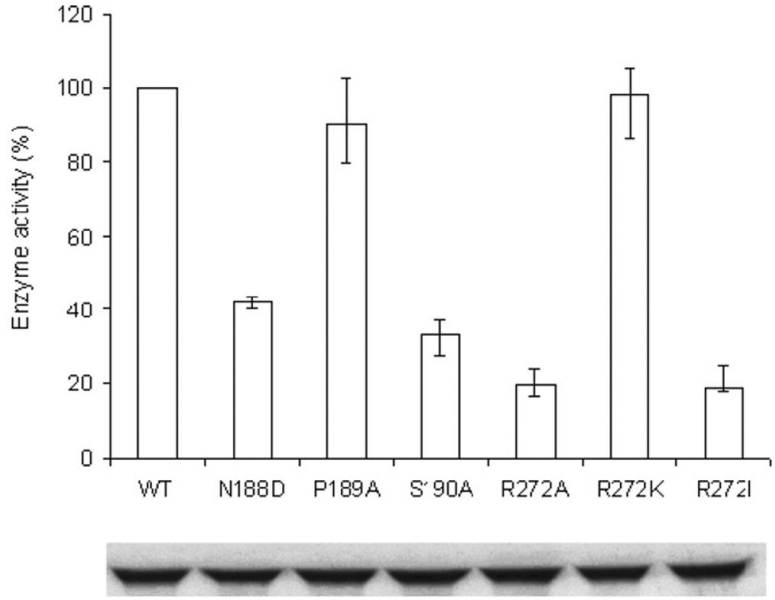

Fig. 2.

Activities of wild-type human GD3-synthase and mutants. The amounts of enzymes were compared by Western blot analysis as described under Materials and methods. Enzyme amounts of proteins were adjusted to the similar amount of wild-type enzyme (lower panel). The relative amounts of mutants to that of wild-type enzyme were determined by ImageJ. Enzyme assays were performed as described under Materials and methods and values are the average of triplicate experiments. Normalized by the relative amount of proteins, enzyme activities of mutants were expressed relative to that of wild-type enzyme (upper panel).

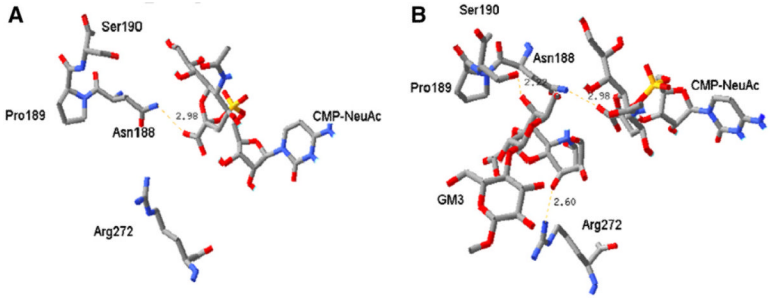

Fig. 3.

Stereoscopic representation of the hydrogen bonds between substrates and functional sites. (A) Same representation of the hydrogen bonds between CMP-NeuAc and functional sites. (B) Same representation of the hydrogen bonds between CMP-NeuAc with GM3 oligosaccharide group and functional site. The hydrogen bonds are computed by the same software. The distances between atoms are marked with Arabic numerals and the unit of distance is Å. This figure was prepared with Swiss-Pdb Viewer 3.7 (http://www.expasy.org/spdbv/).

Table 1

Kinetic parameters of wild-type GD3-synthase and mutants[a]

GD3-synthase	CMP-NeuAc			GM3		
	K_m (μm)	V_{max} (pmol/h)	V_{max}/K_m	K_m (μm)	V_{max} (pmol/h)	V_{max}/K_m
Wild-type	88(±7)	167(±11)	1.90	83(±8)	165(±12)	1.98
N188D	220(±32)	74(±4)	0.34	87(±10)	74(±5)	0.85
S190A	110(±13)	55(±6)	0.50	184(±20)	57(±4)	0.31
R272A	81(±7)	37(±2)	0.46	979(±133)	37(±5)	0.04

[a] Kinetic constants were determined for the wild-type enzyme and mutants, as described under Materials and methods.