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Site-Directed Mutagenesis of Glutamate 317 of Bovine α -1,3Galactosyltransferase and Its Effect on Enzyme Activity: Implications for Reaction Mechanism

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Abstract

Bovine α 1,3galactosyltransferase (α 1,3GalT) transfers galactose from UDP- α -galactose to terminal β -linked galactosyl residues with retention of configuration of the incoming galactose residue. The epitope synthesized has been shown to be critical for xenotransplantation. According to a proposed double-displacement reaction mechanism, glutamate-317 (E317) is thought to be the catalytic nucleophile. The proposed catalytic role of E317 involves an initial nucleophilic attack with inversion of configuration and formation of a covalent sugar-enzyme intermediate between E317 and galactose from the donor substrate, followed by a second nucleophilic attack performed by the acceptor substrate with a second inversion of configuration. To determine whether E317 of α 1,3GalT is critical for enzyme activity, site-directed mutagenesis was used to substitute alanine, aspartic acid, cysteine and histidine for E317. If the proposed reaction mechanism for the α 1,3GalT enzyme is correct, E317D and E317H would produce active enzymes since they can act as nucleophiles. The non-conservative mutation E317A and conservative mutation E317C are predicted to produce inactive or very low activity enzymes since the E317A mutant cannot engage in a nucleophilic attack, and the E317C mutant would trap the galactose residue. The results obtained demonstrate that E317D and E317H mutants retained activity, albeit significantly less than the wild-type enzyme. Additionally, both E317A and E317C mutant also retained enzyme activity, suggesting that E317 is not the catalytic nucleophile proposed in the double-displacement mechanism. Therefore, either a different amino acid may act as the catalytic nucleophile or the reaction must proceed by a different mechanism.

Keywords

site-directed mutagenesis; α -1; 3Galactosyltransferase; double-displacement reaction mechanism; modeling

1. Introduction

Cell surface oligosaccharides are expressed on membrane glycoproteins and glycosphingolipids, and function in multiple cellular processes, serve as receptors for certain

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viral or bacterial infectious agents, contribute to the structural stability and proper folding of membrane proteins, and confer immunological antigenicity such as blood group or xenotransplantation antigen specificities on cells and tissues. One such xenotransplantation antigen is the terminal α 1,3galactose epitope found at the cell surface of most mammals including most primates and New World monkeys, but not in humans, apes, and Old World monkeys [1]. Transfer of the α -galactose moiety onto terminal β -galactosyl residues of glycoconjugates is catalyzed by α 1,3galactosyltransferase (α 1,3GalT) [2,3]. An inactive form of the enzyme is found in species in which the DNA sequence encoding the enzyme contains frame shift mutations and deletions [4–6].

The gene for the α 1,3GalT enzyme has been cloned [7] and the X-ray crystal structure of the bovine enzyme has been solved [8–10]. The reaction mechanism for the α 1,3GalT enzyme involves retention of the anomeric configuration of the incoming galactose moiety. Clues to the mechanistic basis for retention of configuration have been obtained from analysis of the 3-dimensional structure of the enzyme active site [8–10]. One hypothesis has focused on the significance of E317 as a nucleophile that could potentially act as an initial acceptor for the incoming α -galactose residue. This would form an enzyme-glycosyl intermediate involving a transient inversion of configuration followed by transfer to the terminal β -galactosyl moiety in a concerted fashion with a second inversion to retain the original anomeric configuration [8]. Evidence supporting this type of mechanism has been found in studies with a different retaining alpha Galactosyltransferase, lipopolysaccharyl- α 1,4galactosyltransferase C from *Neisseria meningitidis*, in which an enzyme-glycosyl intermediate involving an Asp residue has been detected [11]. However, the enzyme-glycosyl intermediate formed occurred through an amino acid that had not been predicted to serve as the catalytic nucleophile.

We have prepared a series of site-directed mutants of bovine α 1,3GalT at the E317 residue to directly test if a transient enzyme-glycosyl intermediate is formed through this residue during catalysis. To test the double displacement hypothesis, we introduced both conservative and non-conservative mutations at the 317 position of the enzyme. We predicted that if E317 of α 1,3GalT were important for catalysis, the mutations introduced would result in proteins with low or no activity. In addition, we also predicted that if the proposed double-displacement mechanism were valid, the E317D and E317H would be active, whereas the E317A and the E317C mutants would produce inactive or very low activity proteins. The E317C mutant was prepared to covalently trap the galactose residue to the protein. Upon nucleophilic attack on the galactose by the Cys residue a covalent C-S bond would be formed with the Cys sulfhydryl group which would be stable towards a subsequent nucleophilic attack thought to be performed by the acceptor. This should create an inactive enzyme and result in an accumulation of the sugar-enzyme intermediate. The results demonstrated that all mutants retained detectable, although low activity and thus, these results have implications concerning the actual reaction mechanism for the enzyme.

2. Materials and methods

2.1. Materials

The pPROTA- α 1,3GalT construct that was used as a template was previously generated by Henion et al. [3]. Oligonucleotide primers used to introduce the E317A and E317D mutations were synthesized by Integrated DNA Technologies (IDT) at the Kansas State University Core Facility; whereas the primers used to introduce mutations E317C and E317H were obtained from Operon Technologies, Inc. (Alameda, CA). The QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Lipofectamine 2000 was from Invitrogen S.A. (Carlsbad, CA). COS-7 cells were obtained from ATCC (Manassas, VA). High glucose Dubelcco's Modified Eagle Medium (DMEM) and FCS were purchased from Hyclone Laboratories (Logan, UT). Penicillin-Streptomycin was obtained from the UCSF Cell Culture

Facility (San Francisco, CA). Kaleidoscope Prestained Standards, 4–20% Tris-HCl Ready Gels, and Trans-Blot Transfer Medium (0.45 μ m Pure Nitrocellulose Membrane) purchased from BioRad Laboratories (Hercules, CA). Goat IgG-reagent grade, rabbit IgG-Agarose (IgG beads), anti-goat IgG alkaline phosphatase conjugated, bovine serum albumin (BSA), *p*-nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Sigma Chemical Company. Sodium cacodylate, manganese chloride (MnCl₂), and Sep-Pak columns were obtained from Fisher Scientific (Fair Lawn, NY). Uridine 5'-diphosphogalactose disodium salt (UDP-Gal) was purchased from Sigma Chemical Company. Tritiated UDP-Gal (60 μ Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and Hydrofluor Complete Liquid Scintillation Counting Solution was from National Diagnostics (Manville, NJ). LacNAc-C₈ (C₈ = O(CH₂)₈CO₂CH₃) was generously provided by Dr. Ole Hindsgaul (Department of Chemistry, University of Alberta, Canada), whereas the LacNAc-sp-biotin was purchased from Glycotec Corporation (Gaithersburg, MD). All other chemicals were commercially obtained and were of the highest quality.

2.2. Quick-change site-directed mutagenesis of E317 of α 1,3GalT

The pPROTA- α 1,3GalT construct was used as a template in the QuickChange Site-Directed Mutagenesis procedure according to manufacturer's instructions. The pPROTA- α 1,3GalT construct consisted of truncated α 1,3GalT wild-type bovine sequence encoding amino acids E80 through V368, which was subcloned in the pPROTA vector. Forward mutagenic primers used to create the point mutations were: E317A 5'-CAA TGG CAT GAT **gca** AGC CAT CTA AAC-3', E317D 5'-CAA TGG CAT GAT **Gac** AGC CAT CTA AAC-3', E317C 5'-CC CAA TGG CAT GAT **tgC** AGC CAT CTA AAC-3', and E317H 5'-CAA TGG CAT GAT **cAc** AGC CAT CTA AAC-3'. Sequences shown in bolded lowercase represent mismatch bases introduced to obtain the desired mutation. Reverse mutagenic primers were of the same length and complementary to the reverse mutagenic primers. Following amplification, the reaction mixture was treated with *DpnI* to eliminate the template DNA. Each of the mutated amplification products were transformed in *E. coli* JM109 strain. The nucleotide sequence of each mutant construct was confirmed in two steps. Sequencing analysis was performed in two steps (Microchemical Core Facility at San Diego State University). First, it was confirmed that each of the desired mutations were incorporated and second, both strands of the entire coding region were analyzed to confirm that no unwanted mutations occurred in the rest of the enzyme's coding region. Sequences were analyzed using the Sequencher software by Gene Codes Corporation (Ann Arbor, MI).

2.3. Expression of wild-type α 1,3GalT and E317 mutant constructs

Transient expression of wild-type and mutant DNA constructs into COS-7 cells was achieved using Lipofectamine 2000 (LF2000) according to the manufacturer's procedure. The expressed soluble protein A-tagged α 1,3GalT fusion (protein A is fused at the N-terminus of the proteins) enzymes were secreted into the media and subsequently purified using IgG-Agarose affinity chromatography as previously described [17].

2.4. Wild-type α 1,3GalT and mutant enzyme activity assays

The enzyme activity of the bead-bound wild-type and mutant enzymes was determined by quantifying the radioactive product formed when the enzyme transfers a radiolabeled galactose residue from the donor UDP-[³H]Gal to the acceptor Gal- β -1,4-GlcNAc-(CH₂)₈CO₂CH₃ (LacNAc-C₈) in the presence of Mn⁺². A 25 μ L reaction, containing 100 mM sodium cacodylate buffer pH 6.0, 30 mM MnCl₂, 1.5 mM UDP-Gal, 0.2 μ Ci UDP-[³H]Gal (60 μ Ci/mmol), 400 μ M LacNAc-C₈, and 5–10 μ L of bead-bound enzyme, was incubated for 1–3 hours at 37 °C. The hydrophobic property of the radioactive product formed [³H]Gal- α -1,3-Gal-

β -1,4-GlcNAc-C₈ was exploited to separate it from the reaction mixture using a Sep-Pak column as previously described [12].

The wild type and mutant proteins also were evaluated for activity in an assay using a glycolipid acceptor substrate (nLc₄Cer). The assay conditions were: 100 mM cacodylic buffer pH 6.0, 32 mM MnCl₂, 1.3 nmol/ul nLc₄-cer; 0.8% v/v Triton x 100, 2mM UDP-Gal; 5 ug/ul bovine serum albumin and 40nCi ¹⁴C-UDP-Gal. Sep-Pak chromatography was used to isolate the product glycolipid which was analyzed by thin layer chromatography using the solvent system, chloroform-methanol-water (60:35:8, v/vs). Radioactive product was detected using a STORM 840 phosphorimager system. Subsequently, glycolipids were visualized with 0.2g of orcinol dissolved in 100 ml of 1.5N H₂SO₄ followed by heating to 110° C for 5–10 minutes. Protein was estimated by densitometry of Coomassie stained gels. Known quantities of bovine serum albumin (BSA) were used for comparison with α 1,3GalT.

2.5. Analysis of wild-type α 1,3GalT and mutant enzymes by Western blot analysis

Wild-type α 1,3GalT and mutant proteins were separated using SDS-PAGE followed by transfer to a nitrocellulose membrane for colorimetric detection. In the SDS-PAGE procedure, 0.5–3.0 μ L of bead-bound protein was denatured with SDS, reduced with β -mercaptoethanol (BME), and heated to 100 °C for 5–10 minutes. Reduced and denatured experimental samples were loaded on a 4–20% Tris-HCl gel and run for 1 hour at 200 V. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane by electrophoretic transfer and the blot was blocked with 1% BSA, incubated with anti-goat IgG conjugated to alkaline phosphatase, and developed with a mixture of BCIP/NBT.

2.6. Estimation of the kinetic constants

Donor and acceptor saturation curves were obtained by varying the concentration of the one of the substrates while maintaining the concentration of the other substrate constant. Reactions were incubated for 1 hour for truncated wild-type enzymes, whereas mutant enzymes were incubated for 3–12 hours. After incubation, reactions were analyzed using the Sep-Pak method and the radiolabeled product formed was quantified using a liquid scintillation counter as previously described [12]. To obtain the apparent K_m for the donor and acceptor substrates, the experimental data were fit to the Michaelis-Menton equation using the curve-fitting program KaleidoGraph.

2.7. Molecular modeling

Energy minimizations were performed using Charmm [18], as implemented in Quanta 2000X (Accelrys, San Diego, USA). In order to speed up calculations, only residues within a 10 Å radius of the bound substrates and manganese ion were allowed to move freely during energy minimization while all other residues were fixed. The structure of α 1,3GalT bound to UDP-galactose and lactose as reported by Zhang et al. [14] (Protein Data Bank, [19], reference code 1O7O), was used for all modeling studies. The Gln317 mutation in this crystal structure was replaced with the original Glu317 side-chain. Additional mutations of this residue were made using the protein design module in Quanta 2000X. The conformation of bound substrate was taken from the crystal structure of galactosyltransferase LgtC from *Neisseria meningitidis* ([15], PDB entry 1G9R) and manually modified to change the fluorine atom of the inhibitor to a hydroxyl group. An extended conformation was also generated by manual manipulation of torsion angles in the substrate and both were subsequently minimized in the active site after superpositioning on UDP.

3. Results

3.1. Site-directed mutagenesis of E317

Point mutations resulting in both conservative and non-conservative amino acid replacements of E317 were made by PCR, site-directed mutagenesis of the wild-type bovine $\alpha 1,3\text{GalT}$ catalytic domain sequence (aa 80–368) cloned into the pPROTA expression vector [12]. These mutants were prepared to test the hypothesis that the catalytic mechanism of $\alpha 1,3\text{GalT}$ involves an intermediate in which the Gal residue forms a transient covalent attachment to the carboxyl group of E317. In these experiments, the E317D mutant represents a conservative change that retains a carboxylic acid group, but is shortened by a methylene unit compared to the wild-type amino acid. The E317H mutant contains a side chain that can act as a nucleophile similar to Glu. The E317A mutant produced a protein in which the amino acid at 317 (i.e. Ala) cannot serve as a nucleophile, and the E317C mutant is expected to trap the galactose residue and not allow transfer to occur to the acceptor. PCR site-directed mutagenesis to create each point mutation was conducted as described under “Materials and Methods” and the sequence of each expression construct was confirmed (results not shown). No other amino acid substitution in any construct was detected.

3.2. Expression of wild-type and mutant $\alpha 1,3\text{GalT}$

pPROTA constructs of the wild-type and mutant proteins were expressed in COS-7 cells and the secreted protein isolated on IgG-Agarose beads. The resultant beads, after washing, were subjected to Western blot analysis to detect and estimate the amount of expressed fusion proteins. As shown in Fig. 1, the wild-type enzyme and each mutant construct were expressed as fusion proteins migrating at approximately 70 kDa. The mutant proteins were expressed at approximately one half the level of the wild-type enzyme.

3.3. Activity of wild-type and mutant $\alpha 1,3\text{GalT}$ enzymes

Previous results have demonstrated that the apparent K_m values of wild-type $\alpha 1,3\text{GalT}$ are 710 μM and 190 μM for the sugar nucleotide donor UDP-Gal and acceptor LacNAc-C₈, respectively [13] and similar values were obtained in the present study (670 μM for UDP-Gal and 310 μM for LacNAc-C₈). Table I shows the comparative activity of wild-type and mutant $\alpha 1,3\text{GalT}$ enzymes using substrate concentrations approximately 2X apparent K_m . The activity of each of the mutant enzymes was substantially lower than that of the wild-type enzyme. The E317C and E317D mutants had the highest activity of the mutants under these reaction conditions, however both the E317A and E317H mutants also were active (i.e. activity was two-fold greater than that observed without acceptor substrate added to the reaction).

The activity of the wild-type enzyme and mutants was further evaluated using a glycolipid acceptor, nLc₄Cer. The product identified by thin layer chromatography is shown in Fig. 2. The product generated by the wild-type enzyme was observable by chemical detection (i.e., staining with orcinol), and detection of radioactive Gal incorporation into a glycolipid product that migrated with the expected product, IV³- α -Gal-nLc₄Cer. Three of the mutant proteins (E317A, E317D, E317H) also were analyzed for product formation with nLc₄Cer. Each produced a radioactive product that co-migrated with the product generated by the wild-type protein. Consistent with the results reported in Table I, the mutants produced far less product than the wild type, less than two percent in all cases under the reaction conditions employed.

3.4. Kinetic studies of wild-type and mutant $\alpha 1,3\text{GalT}$ enzymes

Initial kinetic experiments with the mutant enzymes using LacNAc-C₈ as the acceptor produced a straight line (velocity vs. [S]) when the LacNAc-C₈ concentration was varied up to the saturation limit of 2,400 μM , which is about 12-fold higher than the reported apparent K_m of

the wild-type enzyme [13], and 8-fold higher than the K_m obtained in this study. Similar results were obtained for all mutants. Thus, increasing enzyme activity was observed with increasing acceptor concentration, indicating that the enzymes were not saturating even at acceptor concentrations up to 2400 μM .

Since it was impossible to obtain accurate measures of the apparent K_m values for the mutants using LacNAc-C₈, a comparison of the velocity of product formation by the mutant enzymes at two LacNAc-C₈ concentrations, 400 μM and 2400 μM was done. Under these conditions, the highest enzyme activity was obtained for the E317C mutant followed by the E317D mutant. The 6-fold change in acceptor substrate concentration resulted in an approximate 4-fold and 3-fold increase in activity for E317C and E317A, respectively as shown in Fig. 3. Although the observed activities for the other two mutants were lower, the fold increases when the acceptor substrate was increased 6-fold, were higher, close to a linear increase. Therefore, even at the maximum concentration of LacNAc-C₈ none of the mutants were close to saturation.

A comparison of enzyme activity between wild-type enzyme and the various mutants was also done at two UDP-Gal concentrations, 1 mM and 3 mM. The activity in the presence of 3 mM UDP-galactose was substantially higher (2–2.5 fold) than at 1 mM UDP-Gal for three (E317A, E317C and E317D) of the four mutants, whereas there was only a modest (1.4-fold) increase for E317H (data not shown). These results indicate that although the apparent K_m for UDP-Gal cannot be determined for the mutant enzymes with LacNAc-C₈ as the acceptor substrate, it is substantially higher than that of the wild-type enzyme.

The substrate LacNAc-sp-biotin has a much higher solubility than LacNAc-C₈ and was used in substrate saturation studies of wild-type and the E317D mutant. The wild-type enzyme had an apparent K_m of 315 μM . A substrate saturation study to obtain an apparent K_m for UDP-Gal for E317D using LacNAc-sp-biotin as the acceptor substrate at a concentration of 8 mM was done and the apparent K_m for UDP-Gal was found to be 1.25 ± 0.25 mM, about 2-fold higher than the value for wild-type enzyme (Fig. 4 A). The E317D mutant was evaluated with varying concentrations of LacNAc-sp-biotin up to 7.2 mM as shown in Fig. 4 B. Under these conditions, an estimate of the K_m (3.8 ± 1.1 mM) for LacNAc-sp-biotin for E317D was obtained, which is more than 12 times higher than that for the wild-type enzyme. The substrate saturation studies of E317D clearly demonstrate that a minor change in the functional group at amino acid 317 of bovine $\alpha 1,3\text{GalT}$ results in a substantial increase in the apparent K_m for both substrates, and may reflect a change in the enzyme's affinity for the two substrates. The V_{max} apparent for the E317D was found to be about 6 percent (600 ± 22 pm/hr microgram) of wild-type enzyme.

3.5. Modeling Studies of wild-type and mutant $\alpha 1,3\text{GalT}$ enzymes

Minimization of E317 mutations in $\alpha 1,3\text{GalT}$ showed little disturbance of the local environment around the newly introduced amino acids, with the exception of the bulkier His (Fig. 5). The side chains of the newly introduced amino acids Asp, Ala and Cys at position 317 do not require significant reordering in the active site. As shown in Fig. 5, only the bulkier imidazole group of the His side chain displayed steric hinderance with the backbone carbonyl of the nearby Tyr279, causing a small shift in its position. Zhang and coworkers [14] observed a perturbation of the orientation of the lactose acceptor as a result of introducing the E317Q mutation. The observed perturbation is small, however, with atom displacements less than 1 Å, and such movements are within the error of energy minimization. As the mutations reported here are not expected to cause large structural rearrangements in the active site, it is likely that their effects on catalytic activity are due to their chemical nature. With E317 potentially stabilizing a positively charged oxo-carbenium intermediate [10] it is expected that a smaller residue will provide less direct stabilization of the intermediate. Indeed, all mutations of E317 to residues with smaller side chains reduce enzyme activity significantly. The requirement for

an electronegative environment to stabilize reaction intermediates is perhaps born out in the higher specific activities of the Asp and Cys mutants, versus those introducing the neutral Ala, or the potentially positively charged His side chains.

4. Discussion

Gastinel and co-workers published the first X-ray crystal structure of the catalytic domain (amino acids 80–368) of bovine α 1,3GalT at the 2.5Å level [8]. The structure (Form I) was obtained from crystals formed in the absence of substrates, and analyzed both in absence and presence of UDP-Gal and Mn^{2+} after soaking the crystals in this substrate. Both structures were of globular shape with folding of an α/β protein having central β -sheet and α -helical elements. The electron density of the side chain carboxyl group of E317 was found to be extended in the substrate-bound enzyme, and this observation led Gastinel and co-workers to propose that galactose was covalently bound to E317. Thus, they posited that the reaction mechanism occurred by a double-displacement process.

A subsequent study on the bovine α 1,3GalT construct by Boix and co-workers further refined the structure to a level of 1.53Å [9]. At this higher resolution, a clearer picture of the structure of the catalytic site of the enzyme was obtained. In the substrate bound form (Form II), the C-terminal segment was found to adopt a highly structured conformation that was part of the catalytic site, presumably due to a donor substrate induced conformational change upon binding.

In another study by this group the structural and mechanistic properties of this enzyme [10] were further clarified. Crystal structures of α 1,3GalT complexed with donor UDP-Gal or UDP-Glc and acceptor lactose or N-acetyllactosamine substrates at resolutions up to 1.46Å were obtained [10]. Structural and calorimetric binding studies were consistent with an ordered binding mechanism as previously described by Zhang and co-workers [15] in which the donor substrate binds first causing a conformational change followed by acceptor binding with the direct participation of UDP. The results demonstrated that catalysis proceeded by cleavage of the galactose-UDP bond producing non-covalent complexes containing buried galactose.

The location of the buried monosaccharide and results from molecular modeling studies also suggested that E317 was an important residue in UDP-Gal binding, but for a different reason than acting as the catalytic nucleophile. Interestingly, modeling studies identified a steric clash between the galactose ring and the E317 side chain, and with the acceptor substrate when UDP-Gal was docked in a normal extended conformation [10]. Modeling using a different conformation of UDP-Gal in which the UDP component is extended, but the pyranose ring is bent back under the phosphates such that it is almost parallel to the diphosphate [10,16] demonstrated that UDP-Gal in this distorted conformation could readily be docked to the enzyme without steric clashes. Thus, it is possible that the mechanistic significance of E317 is to induce this distorted conformation of UDP-Gal to properly place it in the enzyme catalytic site. In contrast to the covalent intermediate double-displacement mechanism proposed by Gastinel and co-workers [8], this mechanism is more consistent with an S_Ni mechanism involving nucleophilic attack by the acceptor simultaneously with UDP release on the same side of the galactose ring [16].

The series of site-directed mutagenesis studies conducted in this paper were designed to directly address these differing mechanistic hypotheses. Under a double-displacement mechanism, site-directed mutagenesis of E317 to Asp would be expected to retain perhaps most enzyme activity, however changes to either Ala or Cys would be expected to result in inactive proteins or ones with very low activity. The results obtained demonstrated that changes of E317 to either Asp, His, Ala, or Cys resulted in mutant enzymes that had substantially reduced, but measurable

activity compared to the wild-type enzyme under standard reaction conditions. Increasing the concentration of both substrates resulted in a significant increase in the transferase activity of each mutant.

Initial attempts to define optimal assay conditions or experimentally determine apparent K_m values for the mutant enzymes using LacNAc-C₈ was impossible since none of the mutant proteins could be saturated with this acceptor substrate up to the limits of its solubility. However, it was clear from the preliminary analyses using LacNAc-C₈ that the apparent K_m for both acceptor and donor are substantially higher than that for the wild-type enzyme. Substituting an acceptor substrate (LacNAc-sp-biotin) that has a greater aqueous solubility than LacNAc-C₈ produced substrate saturation curves that yielded apparent K_m values for the E317D mutant. The apparent K_m values for E317D for both substrates were higher, especially the apparent K_m for the acceptor which was about 10 times higher than that observed for the wild-type enzyme. Even under conditions in which substrate concentrations are substantially greater than the apparent K_m values, the E317D mutant only has less than ten percent of wild type enzyme activity, suggesting that conservative mutation of this residue also alters the catalytic process as well as substrate affinity.

Zhang et al. [14] also reported on mutants (E317Q and E317D) of E317. When these mutants were initially analyzed they appeared to be inactive, but assays with higher protein concentrations and extended times demonstrated that they had low activity. Calorimetric studies demonstrated that the E317D had a reduced affinity for UDP, whereas the E317Q affinity was unchanged compared to wild-type enzyme. Substrate saturation studies were carried out with the E317Q mutant and showed that the protein had an increased K_m for the acceptor lactose compared to wild-type enzyme. The apparent K_m value obtained was ten times higher than the highest concentration (25 mM) of lactose tested, and therefore is tentative. Interestingly, the K_m for UDP-Gal of the E317Q mutant was reported to be similar to that of the wild type enzyme.

Taken together, the results presented indicate that either a residue in the active site other than E317 is involved in a nucleophilic double-displacement mechanism or, perhaps, that the reaction proceeds via a reaction mechanism not requiring a covalent intermediate such as an S_Ni mechanism.

As described earlier, Boix et al. [10] have previously reported that in a modeled complex of α 1,3GalT with UDP-Gal, the bound nucleotide sugar is in a conformation in which the galactose ring is bent back under the two phosphates and lies nearly parallel to the diphosphate plane. They reported that an extended conformation of UDP-Gal could not be accommodated in the active site due to steric clashes between the galactose ring and the side chain of E317, and with the acceptor substrate when it was included in the structure. In our hands, it appears that extensive steric clashes with the acceptor substrate are the main cause for the poor fit of the extended UDP-Gal conformation in the active site, whereas UDP-Gal itself fits into the active site in the absence of acceptor substrate with only minimal relaxation of the surrounding residues (see Fig. 6A). This suggests that the role of Glu317 is not strictly that of a steric barrier to force UDP-Gal into the folded, high-energy conformation (Fig. 6B) as proposed by Boix et al. [10]. Analysis of the environment of the galactose in the extended and folded conformations suggests, that in the latter case, the galactose residue is more tightly bound to the enzyme due to more complete shielding from solvent and a larger number of hydrogen bonding opportunities to the enzyme (cf. Fig. 6B). Since E317 is located near the site of catalysis for both conformations of UDP-Gal, mutations of this residue cannot be used to distinguish between these two possible binding modes.

Confirming the existence of the “folded” binding mode of UDP-Gal would comprise a first step towards determining the reaction mechanism. Since E317 is located near the site of catalysis for both conformations of UDP-Gal, mutations of this residue cannot be used to distinguish between either possible binding mode. Similarly, mutations to the left of E317 may also interfere with binding of the acceptor rather than only UDP-Gal in either binding mode. Residues located in the galactose binding pocket, but distant from UDP, as shown in Fig. 6B, however, are unique for the binding mode involving a high-energy UDP-Gal conformation as proposed by Boix et al. [10]. Therefore, mutation of residues Ser199, Ala281, His315 and Asp316 to larger amino acids could provide evidence for the existence of the folded UDP-Gal conformation and strengthen the argument for the proposed S_Ni mechanism [10].

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The abbreviations used are

α1,3GalT	alpha 1,3-galactosyltransferase
LacNAc-C₈	Gal β 1,4GlcNAc-O-(CH ₂) ₈ COOCH ₃
LacNAc-sp-biotin	Gal β 1,4GlcNAc-O(CH ₂) ₃ NHCO(CH ₂) ₅ NH-biotin
nLc₄Cer	neolactotetraosylceramide or Gal β 1,4GlcNAc β 1,3Gal β 1,4Glc β 1,1Cer
PCR	polymerase chain reaction
UDP-Gal	UDP- α -galactose
UDP-Glc	UDP-glucose

Figure 1. Western blot analysis of the expressed protein α 1,3GalT fusion proteins

A single major band corresponding to the expected size of the protein A- α 1,3GalT fusion protein (~70 kDa) was observed for the wild-type and each site-directed mutant enzyme tested, as indicated. Conditions for Western blotting were as described under “Materials and Methods”. Positions of molecular weight markers are shown on the right and the numbers correspond to the mass of each marker in kD.

Figure 2. Thin layer chromatographic analysis of products formed from nLc₄Cer
(A) Orcinol staining of the glycolipid substrate, nLc₄Cer (upper band) and products formed (lower band, indicated by an *). (B) Radioactive product detected. Products formed from reactions run with: wild type α 1,3GalT (lane 1), E317A (lane 2), E317D (lane 3), E317H (lane 4). Lane 5A shows the position of the acceptor substrate nLc₄Cer. Lane 5 in B shows the results from a control reaction without glycolipid acceptor substrate and wild type enzyme.

Figure 3. Velocity of product formation of mutant enzymes at two concentrations of LacNAc-C8 acceptor
Specific activity was measured with acceptor substrate concentrations of 400 μM (gray shaded bars) and 2400 μM (open bars) LacNAc-C8.

Figure 4. Substrate saturation curves for E317D using LacNAc-sp-biotin and UDP-Gal
(**A**) E317D, V_o vs. donor concentration (acceptor concentration was 8.0 mM); (**B**) E317D, V_o vs. acceptor concentration (donor concentration was 1.0 mM).

Figure 5. The effect of the E317H mutation on its local environment in the active site
 The introduction of the bulkier Histidine side chain for Glu317 causes a small movement of the backbone carbonyl of Tyr279. The original crystal structure is depicted with green carbons, the energy minimized model in white. LacNAc = Gal β 1,4GlcNAc

Figure 6. Molecular modeling of $\alpha 1,3$ GalT

(A) Model of UDP-Gal bound in an extended conformation. The side chain of Glu317 is indicated in white to the left. (B) Model of UDP-Gal bound in the folded conformation. Residues making up the galactose binding pocket, but distant from UDP are indicated.

Table I
Activities of Wild-Type and Mutant Bovine α 1,3GalT

Enzyme	Activity Compared to wild-type α 1,3GalT (%)
Wild-type	100
E317A	0.1
E317D	0.4
E317C	0.8
E317H	0.1

Substrate concentrations 2X K_m for the wild-type enzyme, with LacNAc-C8 as the acceptor. The wild-type enzyme had a specific activity of 10–20 nmol/hr/ μ g.