Intermediate Trapping on a Mutant Retaining α-Galactosyltransferase Identifies an Unexpected Aspartate Residue

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Lipopolysaccharide-α,1,4-galactosyltransferase C (LgtC), a glycosyltransferase family 8 α,1,4-galactosyltransferase from Neisseria meningitidis, catalyzes the transfer of galactose from UDP galactose to terminal lactose-containing acceptor sugars with net retention of anomeric configuration. To investigate the potential role of discrete nucleophilic catalysis suggested by the double displacement mechanism generally proposed for retaining glycosyltransferases, the side chain amide of Gln-189, which is suitably positioned to act as the catalytic nucleophile of LgtC, was substituted with the more nucleophilic carboxylate of Asp-190. Electrospray mass spectrometric analysis revealed that a steady state reduced to 3% that of the wild type enzyme. Although the $K_m$ for UDP-galactose is not significantly altered, the $k_{cat}$ was reduced to 3% that of the wild type enzyme. Electro spray mass spectrometric analysis revealed that a steady state population of the Q189E variant contains a covalently bound galactosyl moiety. Liquid chromatographic/mass spectrometric analysis of fragmented proteolytic digests identified the site of labeling not as Glu-189 but, surprisingly, as the sequentially adjacent Asp-190. However, the side chain carboxylate of Asp-190 is located 8.9 Å away from the donor substrate in the available crystal structure. Kinetic analysis of a D190N mutant at this position revealed a $k_{cat}$ value 3000-fold lower than that of the wild type enzyme. A 2.6-Å crystal structure of the Q189E mutant with bound uridine 5'-diphospho-2-deoxy-2-fluoro-α-D-galactopyranose revealed no significant perturbation of the mode of donor sugar binding nor of active site configuration. This is the first trapping of an intermediate in the active site of a retaining glycosyltransferase and, although not conclusive, implicates Asp-190 as an alternative candidate catalytic nucleophile, thereby rekindling a longstanding mechanistic debate.

Oligosaccharides on glycoproteins and glycolipids distributed on cell surfaces and within extracellular matrices are known to play key roles in normal cell functions including cell growth and differentiation, recognition by the immune system, and cell-cell interactions (1–3). Changes in the composition of these glycoconjugates are often associated with disease states, including the metastasis of cancerous cells and autoimmune responses (4–7). They are also known to modulate interactions with viral and bacterial pathogens leading to infection and are involved in mechanisms to evade host immune responses (8–10). Glycosyltransferases, the anabolic enzymes responsible for the highly specific construction of these carbohydrate structures, therefore, not only represent an attractive class of therapeutic targets but also are important tools for the enzymatic synthesis of this synthetically challenging class of therapeutic agents. Of central importance to both of these applications is a detailed understanding of the mechanisms by which this class of enzyme catalyzes glycosyl group transfer.

On the basis of sequence similarities, glycosyltransferases are assigned to families in the same fashion as is done with the well characterized glycosidases (11, 12); the information is maintained on the Carbohydrate-active Enzymes Server at afnb.cnr-mrs.fr/~cazy/CAZY/index.html. Similarly, by comparison to the glycosidases, the relative anomic stereochemistries of the substrate and product classify the glycosyltransferase catalyst as either retaining or inverting. This analogy has led to the assumption that the two classes of enzymes use similar mechanisms to carry out their function (14, 15). The existing structural and kinetic evidence for both classes of inverting enzymes supports a direct displacement mechanism involving an $S_2$-like attack facilitated by an active site general base catalyst. Reactions catalyzed by retaining glycosidases are known to proceed via a double displacement mechanism involving the formation and subsequent breakdown of a covalent glycosyl-enzyme intermediate (16). Recent findings have, however, brought into question the applicability of this mechanism to retaining nucleoside diphosphate-utilizing glycosyltransferases since good candidates for the catalytic nucleophile are generally lacking, and in no case has an intermediate been trapped and characterized. The claimed observation of a galactosyl-enzyme intermediate on the retaining α,1,3-galactosyltransferase (EC 2.4.1.151) (17), despite low resolution and poor structure factors, is undermined by the more recent redetermination of that structure at a higher resolution (18). This new structure revealed that a large portion of the active site adopts a different conformation that is inconsistent with prior proposals.
The α-1,4-galactosyltransferase (LgtC<sup>1</sup>; EC 2.4.1.1) from *Neisseria meningitidis* is to date the only retaining glycosyltransferase for which the three-dimensional structure of a ternary complex with both intact donor and acceptor substrate analogues bound is available (19). Surprisingly, this structure revealed that the only active site side chain suitably positioned to act as the nucleophile in a double displacement mechanism is that of Gln-189. Although not generally considered a good candidate as a nucleophile, an amide can indeed play this role, as is seen with retaining hexosaminidases from families 18, 20, and 56, in which the acetamido substituent of the substrate functions as an intramolecular nucleophile with the formation of an oxazolinium ion intermediate (20, 21). Furthermore, a possible role of an amide as a nucleophile had been previously forwarded for the structurally similar glycogen phosphorylase on the basis of a very similar active site interaction (22). Subsequently another example has arisen in the glycosyltransferase family 20 trehalose-6-phosphate synthase OtsA (23).

However, despite this precedent considerable doubt concerning a nucleophilic role of Gln-189 was raised by the finding that the Q189A variant of LgtC possesses relatively high residual activity, with a *k<sub>cat</sub>* value that is 3% that of the wild type enzyme (19). Doubt concerning a standard double displacement mechanism also derives from the lack of success in attempts to observe a covalently bound intermediate on LgtC, glycogen phosphorylase, and other glycosyltransferases despite exhaustive studies using techniques that have been successfully applied to observe the glycosyl-enzyme intermediates of retaining glycosidases (24). Consequently (and somewhat in desperation), an alternative front side S<sub>N</sub>2-like displacement mechanism termed S<sub>N</sub>2-like was proposed (19). The S<sub>N</sub>2-like mechanism has since been suggested for other retaining glycosyltransferases, including an α-1,4-N-acetyltrehalosaminyltransferase (EC 2.4.1.1) based on a recent crystal structure in which the only residue containing a side chain suitably positioned to act as the nucleophile is that of Arg-293 (25).

To investigate the degree of nucleophilic character contributed by Gln-189 during catalysis, a Q189E variant of LgtC was created, thereby replacing the side chain amide with a more nucleophilic carboxylate, and subjected to kinetic, mass spectrometric, and x-ray crystallographic analysis. Making the transference more "glycosidase-like" might result in intermediate accumulation or even perhaps a more efficient enzyme. The results provide the first direct observation of a glycosyl-enzyme intermediate covalently bound to the active site of a retaining glycosyltransferase but not in the anticipated mode, thereby implicating an alternative catalytic nucleophile.

**EXPERIMENTAL PROCEDURES**

**General Procedures**—All buffer chemicals and other reagents were obtained from Sigma-Aldrich unless otherwise stated. UDP-galactose was a generous gift from Neose Inc. Recombinant LgtC was overexpressed and purified from *Escherichia coli* (BL21) as described previously (26).

**Mutagenesis of LgtC**—The Q189E and Q189A mutants were generated using pCWltgC-25 (26) and a two-stage PCR mutagenesis protocol as described previously (19). The D190N mutant was created using the QuickChange<sup>®</sup> method. Coding (GATGGATATCATGAGGACTGAGAA- CATTTCGGAGCTGACATTGATCATTGACAT) primers containing a single-point mutation were used for PCR reaction using pCWltgC-25 C128S/C174S plasmid DNA (19) as template. All constructs were sequenced to verify the presence of the mutation of interest.

**Enzyme Kinetics**—A continuous coupled assay similar to that described previously (24, 27) in which the release of UDP is coupled to the oxidation of NADH (a = 340 nm, ε = 6.22 mm<sup>-1</sup> cm<sup>-1</sup>) was used to monitor the activity of all LgtC variants. Absorbance measurements were obtained using Cary 300 and Cary 4000 UV-visible spectrophotometers equipped with a circulating water bath and a Peltier temperature controller, respectively. GraFit version 4.0 (28) was used to calculate kinetic parameters by direct fit of initial rates to the respective equations for enzyme assays.

**Labeling of LgtC-25 Q189E**—LgtC-25 Q189E (25 µl, 1 mg/ml in 20 mM HEPES buffer, pH 7.5, 5 mM MnCl<sub>2</sub>, 5 mM 1,4-dithiothreitol) was incubated in the presence of 1 mM UDP-galactose for 2 min or 1 mM UDP-glucose for 2 h to obtain optimal amounts of labeled protein. All samples were quenched by the addition of an equal volume of 6 M urea to ensure complete gel filtration analysis and increase the relative proportion of labeled enzyme by "pulling" the equilibrium over, 15 units of pyruvate kinase and 5 mM phosphoenolpyruvate were added to the above incubations.

**Proteolysis**—LgtC (25 µl in 20 mM HEPES buffer, pH 7.5, native or labeled, 1 mg/ml) was incubated with trypsin (25 µl in 20 mM HEPES buffer, pH 7.5, 1 mg/ml) and incubated at room temperature until digestion was complete (30 min).

**Intermediate Turnover**—Excess UDP-galactose or UDP-glucose was removed from LgtC-25 Q189E labeled (as determined by MS analysis) with either Gal or Glu by exchanging the labeling buffer conditions (described above) with that of 20 mM HEPES buffer, pH 7.5, 5 mM MnCl<sub>2</sub>, and 5 mM 1,4-dithiothreitol using an Ultrafree<sup>®</sup> filter device (Millipore) following the manufacturer's instructions to obtain a final protein concentration of 2 mg/ml. When labeled with Gal, the peak corresponding to labeled enzyme had disappeared after solvent exchange (~30 min). When labeled with Glu, a significant peak corresponding to labeled enzyme was observed after solvent exchange. To this solution (10-µl aliquots) an equal volume of 20 mM HEPES buffer, pH 7.5, 5 mM MnCl<sub>2</sub>, 5 mM 1,4-dithiothreitol (control), or 20 mM HEPES buffer, pH 7.5, 5 mM MnCl<sub>2</sub>, 5 mM 1,4-dithiothreitol, 200 mM lactose was added. Aliquots were incubated for predetermined time periods before being quenched by the addition of 6 M urea and stored frozen before MS analysis. An observed first order rate constant for the turnover of LgtC-25 Q189E labeled with Glu was used to fit the data of the logarithm of the relative peak height of the labeled species versus time.

**Electrospray MS Conditions**—Mass spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an ion spray ion source. Whole proteins and peptides were separated by reverse-phase HPLC on an LC Packing UltiMate Micro HPLC system (Dionex, Sunnyvale, CA) that was directly interfaced with the mass spectrometer. In each of the MS experiments whole protein was loaded onto a C4 column (LC Packing, 300 Å, 1 × 150 mm) equilibrated with solvent A and then eluted with a step gradient of 60:40 A:B for 10 min, 40:60 A:B for 10 min, 20:80 A:B for 4 min followed by 100% B for 5 min (solvent A: 0.05% trifluoroacetic acid, 2% acetonitrile in water; solvent B: 0.045% trifluoroacetic acid, 80% acetonitrile in water). Solvents were pumped at a constant flow rate of 40 µl/min. Spectra were recorded in the single quadrupole scan mode (LC/MS) with the quadrupole mass analyzer scanned over a mass range of 500-2500 Da with a step of 0.5 Da and dwell time of 1 ms per step. The ion source voltage was set at 5.5 kV, and the orifice energy was set at 45 V. Protein masses were deconvoluted from multiply charged species using the Analyst 1.2 software package.

For each of the MS experiments the proteolytic digest was loaded onto a C18 column (LC Packing; 100 Å pepMap, 1 × 150 mm) equilibrated with solvent A and then eluted with a step gradient of 60:40 A:B for 10 min, 40:60 A:B for 20 min, 20:80 A:B for 4 min followed by 100% B for 5 min (solvent A: 0.05% trifluoroacetic acid, 2% acetonitrile in water; solvent B: 0.045% trifluoroacetic acid, 80% acetonitrile in water). Solvents were pumped at a constant flow rate of 50 µl/min. A post-column splitter was present in all experiments, splitting off 85% of the sample into a fraction collector and sending 15% into the mass spectrometer. Spectra were recorded in the single-quadrupole mode with the quadrupole mass analyzer scanned over an m/z range of 300–2200 Da with a step of 0.5 Da and a dwell time of 1.5 ms per step. The ion source voltage was set at 5.5 kV, and the orifice energy was set at 45 V. Native or labeled peptides were sequenced in the single quadrupole mode by increasing the orifice energy to 65 V.

**Catalytic, Data Collection, and Structure Refinement**—The C128S/Q189E mutant was crystallized under the same conditions (100 mM sodium acetate, pH 5.0, 5–20% polyethylene glycol 2000 monomethyl ether) as the wild type enzyme and in the presence of 5 mM MnCl<sub>2</sub>, 5 mM tris(2-carboxyethyl)phosphine hydrochloride, and 10 mM performic 5'-diphenyl-2-deoxy-2-fluoro-o-n-galactopyranose at 10/
Active Site Labeling of a Retaining Glycosyltransferase

Michaelis-Menten parameters for the transferase and hydrolase activities of wild type and various mutants of LgtC-25 with respect to UDP-galactose and UDP-glucose donor substrates measured at pH 7.5, 37 °C. The error range in the data is from 5% to 20%. ND, not determined.

| LgtC-25   | Activity   | UDP-galactose | UDP-glucose |
|-----------|------------|---------------|-------------|
|           |            | $k_{cat}$     | $K_m$       | $k_{cat}/K_m$ | $K_m$      |
| Wild type | Transferase | 24            | 20          | 1.2          | 0.001      |
|           | Hydrolase   | 0.02          | 4.5         | 0.004        | 0.0003     |
| Q189E     | Transferase | 68            | 23          | 0.030        | 0.002      |
|           | Hydrolase   | 0.003         | N/A         |              | N/A        |
| Q189A     | Transferase | 0.43          | 25          | 0.017        | ND         |
|           | Hydrolase   | 0.008         | N/A         |              | ND         |
| D190N     | Transferase | 0.010         | N/A         |              | ND         |

* Determined at saturating concentrations of lactose acceptor (100–240 mM).
* N/A, not applicable. Values could not be obtained because the high concentrations of enzyme required to obtain significant rates approached those of unsaturating donor substrate concentrations, in violation of Michaelis-Menten assumptions. The $k_{cat}$ values were obtained from rates observed at saturating donor substrate concentrations (100 µM).

ml. Data were collected at 100 K. Cryoprotectants contained 20% polyethylene glycol 400 plus mother liquor. Q189E crystals were of the same orthorhombic space group P2$_1$2$_1$2, and had similar unit cell dimensions as with the wild type, namely $a = 39.86$, $b = 75.94$, and $c = 86.91$ Å. A 2.6-Å data set was collected on a local Rigaku RU200 rotation anode equipped with OSMIC mirrors and a MAR image plate detector. Data were processed using DENZO and SCALEPACK (29). The wild type structure was used as the starting search model for molecular replacement with AMoRe (30) and also for refinement using CNS 1.1 (31). Glutamine at position 189 was first changed to alanine in the first round of refinement and, subsequently, to glutamate. Statistics for data collection and processing are available as supplemental information.

RESULTS

Kinetic Analysis—Expression and purification of the Q189E variant of LgtC-25 proceeded without difficulties, yielding protein that displayed the same purification behavior, yield, and CD spectrum as the wild type enzyme. Kinetic analysis indicates that although the $K_m$ for UDP-Gal is approximately unchanged, the measured $k_{cat}$ value of this mutant is ~3% that of the wild type enzyme (Table I). Interestingly, this is virtually identical to the residual activity of the Q189A mutant observed previously (Table I). It might be expected that substitution of the weakly nucleophilic side chain of glutamine with that of a carboxylate-containing side chain would increase the turnover rate of an enzyme that catalyzed a reaction via a double displacement mechanism if that residue was in fact acting as the catalytic nucleophile. The observed decrease in turnover rate, therefore, indicates either that this residue is not the catalytic nucleophile or that an uncharged nucleophile is required for optimal activity on a charged substrate. The recent finding that an active site tyrosine acts as the nucleophile in the double displacement mechanism of a trans-sialidase, which uses charged sialic acid-containing substrates, supports this latter interpretation (32). Alternatively, this finding is consistent with the notion that this enzyme uses a mechanism that differs from that involving the discrete nucleophilic catalysis of a double displacement mechanism.

Intermediate Trapping—To further investigate the contribution of nucleophilic catalysis in the mechanism of the Q189E mutant, the enzyme was incubated with various combinations and concentrations of donor and acceptor substrates to see if any covalently modified protein could be observed by ESMS. Interestingly, incubation with 1 mM UDP-galactose for 2 min followed by quenching in 3 M urea resulted in what appeared to be a steady state population (~10%) of protein for which the mass had increased by 163 mass units, corresponding to a covalently bound galactosyl moiety (Fig. 1B). In an attempt to increase the level of labeling, UDP-glucose was employed in place of UDP-galactose since it has a lower $K_m$ value and slower turnover. As hoped, UDP-glucose labeled the mutant to a slightly higher level (~30%) (Fig. 1C). Incubation in the presence of higher concentrations of donor substrate or in the presence of various concentrations of the incompetent acceptor analogue 4′-deoxylactose did not increase the relative amount of labeled protein. Surprisingly, quenching the reaction with acidic conditions (phosphate buffer, pH 1.6) resulted in the loss of any observable labeled protein. This finding is unusual in that glycosidase intermediates covalently bound by ester linkages are typically stable under acidic conditions. A covalently modified intermediate of the Q189A variant of LgtC-25 was not observed under similar conditions (data not shown).

Supporting the notion that the observed labeled portion of the mutant enzyme was in fact that of a catalytically competent steady state population rather than a “dead end”-inhibited population was the observation that removal of excess UDP-galactose from such mixtures by centrifugal dialysis resulted in complete loss of the labeled species as determined by ESMS. Presumably the galactosyl-enzyme intermediate was hydrolyzed in a similar fashion to that seen for the wild type enzyme, which exhibits a significant rate of background hydrolytic activity (Table I). However, because of the higher level of labeling and presumably a lower hydrolysis rate, removal of excess UDP-glucose from enzyme labeled with that nucleotide sugar allowed the isolation and observation of a labeled species that could be turned over in a time-dependent fashion by incubation.
in the presence of saturating amounts of lactose acceptor. Fitting the rate of decay of labeled enzyme (as determined by ESMS) to a first order rate equation allowed the calculation of a first order rate constant of 0.0016 s⁻¹, in close agreement with the observed $k_{\text{cat}}$ value for the UDP-glucose transferase activity of this mutant (Table I). Finally, in an attempt to “pull” the labeling equilibrium over, the Q189E mutant enzyme was incubated with UDP-galactose in the presence of pyruvate kinase and phosphoenolpyruvate to determine whether the relative proportion of labeled enzyme could be increased, consistent with the labeling event being a true equilibrium process (Scheme 1). Indeed, the introduction of this coupling system resulted in a dramatic increase in the relative proportion of the labeled enzyme species (Fig. 1D).

**Site of Labeling**—To determine whether the Q189E mutant was labeled at the anticipated position (the introduced carboxylate at position 189) after incubation with UDP-Gal, the mutant enzyme was digested with trypsin at pH 7.5, and the resulting peptide fragments were subsequently analyzed by reverse phase HPLC/ESMS. Peptide fragments from both labeled and native protein digests were subjected to comparative mapping analysis, allowing the identification of two peptides that eluted with the same retention time of 34.10 min having $m/z$ ratios of 964 and 1046, respectively. The difference in $m/z$ of 82 is consistent with these being doubly charged peptides carrying a label of mass 163 (the mass of a galactosyl moiety). From the trypsin cleavage sites contained within the LgtC-25 Q189E protein sequence, only one peptide with a mass of 1928 Da ($^{15}N^{15}C^{12}Q^{18}E^{18}A^{18}G^{18}N^{18}P^{18}L^{18}G^{18}C^{18}T^{18}$) is expected. The sequence of this peptide was subsequently confirmed by fragmentation of the isolated unlabeled species (data not shown).

To determine which residue within the identified peptide contained a covalently bound galactosyl moiety, isolated native and labeled peptides were individually injected and fragmented by increasing the orifice voltage to 65 V. For the labeled peptide species, in addition to the fragmentation pattern found for the unlabeled peptide, peaks were observed having $m/z$ values of 1617, 1600, 1436, 1327, 1309, and 1065, corresponding to peptide fragments containing a covalently bound galactosyl moiety (Fig. 2). Most significantly, the peak at $m/z = 1065$ can only correspond to the peptide fragment $^{13}N^{13}D^{13}I^{13}N^{13}L^{13}N^{13}L^{13}L^{13}F^{13}K^{13}$ lacking ammonia ($m/z$ 902) and containing the galactose label ($m/z$ 163). To our considerable surprise, therefore, the site of labeling has to be Asp-190. Substitution of Glu-189, suitably positioned in the ground state crystal structure to play the role of a catalytic nucleophile, with a more nucleophilic Glu residue, therefore, results in the accumulation of a steady state population of covalently modified protein. However, the site of labeling is not the mutated side chain, as was anticipated but, rather, a sequentially adjacent aspartate residue.

**D190N Analysis**—To probe the mechanistic importance of Asp-190, the D190N mutant of LgtC-25 was generated. Initial attempts to generate this variant using the wild type enzyme entry 1GA8) failed, as the D190N variant was found to be 3000-fold less than that of the wild type enzyme (Table I).

Crystallographic Analysis of LgtC-25 Q189E—An x-ray crystal structure of the Q189E mutant of LgtC-25 was obtained at a 2.6-Å resolution with a molecule of uridine 5'-diphospho-2-deoxy-β-fluoro-α-D-galactopyranose bound in the active site. The Q189E structure can be overlaid with the wild type structure with a root mean square deviation of 0.25 Å for 269 Ca atoms with near identical relative positioning of the carboxylate oxygen of Asp-190 and the anomic carbon of the donor (Fig. 3).

**DISCUSSION**

Several interpretations of this surprising result are possible. The first possibility is that labeling does in fact occur at Glu-189, but a subsequent migration results in the label being observed at the alternate Asp-190 site. However, the relative positioning of both of these residues within the active site (both on the β face of the galactose ring) would prevent migration from occurring in the intact enzyme (Fig. 3). Additionally, it is hard to envision a chemically sound mode of migration on the denatured or digested protein or even during gas-phase fragmentation in the mass spectrometer. It must, therefore, be considered possible that Asp-190 is in fact the catalytic nucleophile of LgtC and that retaining glycosyltransferases catalyze reactions via a double displacement mechanism involving discrete nucleophilic catalysis. The Q189E mutation may lead to a slowing down of the deglycosylation step relative to the glycosylation step, allowing the accumulation of an observable, steady state population of labeled enzyme. Indeed, formation of the glycosyl-enzyme intermediate would neutralize the negative charge of Asp-190, which is in close proximity to the introduced carboxylate of Glu-189, eliminating charge-charge repulsion, thereby altering the relative rates of intermediate formation and breakdown. This interpretation is supported by the observed pH-dependent stability of the labeled species, because at low pH the introduced carboxylate would be protonated, preventing unfavorable charge interaction with Asp-190. This argument is further supported by the lack of labeling observed with the Q189A mutant, in which mutation does not introduce a negatively charged species.

It is worth noting that previous suggestions regarding the mechanism of LgtC and retaining glycosyltransferases, in general, have been based primarily on the proximity of candidate nucleophiles to the anomic carbon of a donor sugar within a crystal structure. Indeed, on this basis Asp-190 was discounted as a potential catalytic nucleophile due to the distance (8.9 Å) separating the carboxylate oxygen and the reaction center in the crystal structure of LgtC with bound donor analogue (PDB entry 1GA8). If Asp-190 were in fact the catalytic nucleophile, a conformational change would need to occur during catalysis that would bring it into an appropriate position and orientation with respect to the donor sugar substrate. The need for a significant conformational change during catalysis is also obligated by the observed crystal structure of the self-glucosylating
enzyme glycogenin (EC 2.4.1.186), the other glycosyltransferase family 8 enzyme for which a structure with a bound intact donor sugar substrate exists (33). This enzyme transfers a glucosyl moiety from UDP-glucose to the hydroxyl of Tyr-195 on the surface of another monomer of glycogenin. The carboxylate oxygen of the proposed nucleophile of this enzyme (Asp-163) was found to be 6.1 Å from the anomeric carbon of the donor sugar, whereas that of Asp-160 (structurally equivalent to Asp-190 of LgtC) was found to be 6.9 Å away. Equally significant is the observed distance of 1101 Å, separating the donor substrate from the hydroxyl oxygen of the Tyr-195 acceptor.

It is also possible that the observed site of labeling is simply an artifact arising from an altered active site configuration that differs significantly from that of the wild type enzyme in which Asp-190 becomes suitably positioned to attack the UDP-galactose donor as a result of the introduced mutation. Indeed, substitution of a neutral Gln residue with a charged Glu might introduce significant charge repulsion, leading to an altered active site conformation. To determine whether this latter interpretation was the source of the unexpected site of labeling, a crystal structure of the Q189E variant was obtained at 2.6 Å with uridine 5’-diphospho-2-deoxy-2-fluoro-β-D-galactopyranose bound in the active site and compared with the equivalent structure of the wild type enzyme. The nearly identical observed active site configurations of these two structures render it unlikely that the unexpected site of labeling was the result of an altered active site conformation or mode of donor sugar binding.

Mutation of the catalytic nucleophiles of retaining β-glycosidases typically results in decreased observed $k_{cat}$ values by factors of $10^5$-10$^8$-fold (34). However, equivalent mutations in α-retaining enzymes have been shown to result in less dramatic effects. For example, mutation of the catalytic nucleophile (Asp-229) of the glycosyltransferase family 13 cyclodextrin glucanotransferase (EC 2.4.1.19), the enzyme responsible for the conversion of starch and other α-1,4-linked glucopiranosyl chains into cyclic oligosaccharides, to asparagine led to observed decreases in activity on the order of 2000-fold (35, 36). The kinetic analysis of the D190N variant may, therefore, be interpreted as consistent with the role of this residue as the catalytic nucleophile in a double-displacement mechanism. Recently, it was found that mutation of the proposed nucleophile of β-galactosyl-α-1,3-galactosyltransferase (Glu-317, structurally equivalent to Gln-189 of LgtC) to glutamine reduced the observed $k_{cat}$ for galactosyltransferase activity 2400-fold (37). However, a detailed study of the binding affinities of wild type and E317Q enzyme for donor and acceptor substrates led the authors to propose that this residue is required for proper acceptor substrate orientation and does not play the role of a catalytic nucleophile (37).

An exact $K_{m}$ value could not be obtained for the D190N mutant of LgtC-25 because the enzyme appeared to be saturated by substrate concentrations (5 μM), approaching that of
results serve as a warning to other investigators, indicating the need to determine the site of labeling when a covalently modified protein is observed by mass spectroscopy.

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**REFERENCES**

1. Varzi, A. (1993) *Glyobiology* 3, 97–130
2. Kobata, A. (1993) *Acc. Chem. Res.* 26, 319–324
3. Deek, R. A. (1996) *Chem. Rev.* 96, 683–720
4. Dennis, J. W., Granovsky, M., and Warren, C. E. (1999) *BioEssays* 21, 412–421
5. Dennis, J. W., Granovsky, M., and Warren, C. E. (1999) *Biochim. Biophys. Acta* 1473, 21–34
6. Biochem. Biophys. Acta 1473, 67–85
7. Fukuda, M. (1996) *Cancer Res.* 56, 2257–2244
8. Jack, D. L., Dodds, A. W., Anwar, N., Isom, C. A., Law, A., Frosch, M., Turner, M. W., and Klein, N. J. (1998) *J. Immunol.* 160, 1546–1553
9. Ljungdahl, L., Scolari, A., and Wadstrom, T. (1996) *FEBS Lett.* 392, 171–176
10. Moran, A. P., Prendergast, M. M., and Appelmelk, B. J. (1996) *FEBS Lett.* 392, 171–176
11. Campbell, J. A., Davies, G. J., and Hendrischt, B. (1997) *Biochem. J.* 326, 929–939
12. Castinno, P. M. and Deleury, E., Davies, G. J., and Hendrischt, B. (2003) *J. Mol. Biol.* 328, 307–317
13. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* 277, 505–524
14. Sinnott, M. L. (1996) *Chem. Biol.* 3, 1171–1202
15. Davies, G., Withers, S. G., and Sinnott, M. L. (1997) *In Biochemical Biological Catalysis* (Sinnott, M. L., ed) pp 119–208, Academic Press, Ltd., London
16. Zeche, D. L., and Withers, S. G. (2000) *Acc. Chem. Res.* 33, 11–18
17. Gastinel, L. N., Bignon, C., Misra, A. K., Hindsgaau, O., Shaper, J. H., and Jezzai, H. J. (2000) *EMBO J.* 20, 638–649
18. Boix, E., Swaminathan, G. J., Zhang, Y. N., Natesh, R., Brew, K., and Ackhara, K. R. (2001) *J. Biol. Chem.* 276, 49609–49614
19. Persson, K., Ly, H. D., Dieckelmann, M., Wakarchuk, W. W., Withers, S. G., and Strynadka, N. J. C. (2001) *Nat. Struct. Biol.* 8, 166–175
20. Mark, B. L., Voozdal, D. J., Knapp, S., Triggs-Raine, B. L., Withers, S. G., and James, M. N. G. (2001) *J. Biol. Chem.* 276, 10330–10337
21. Mark, B. L., and James, M. N. G. (2002) *Can. J. Chem.* 80, 1064–1074
22. Mitchell, E. P., Withers, S. G., Ernert, P., Vasella, A. T., Garmon, E. F., Oikonomou, M. N., and Johnson, L. N. (1996) *Biochemistry* 35, 7341–7355
23. Gibson, R. F., Turkenburg, J. P., Charnock, S. J., Lloyd, R., and Davies, G. J. (2002) *Chem. Biol.* 9, 1337–1346
24. Ly, H. D., Lougheed, B., Wakarchuk, W. W., and Withers, S. G. (2002) *Biochemistry* 41, 6572–6572
25. Pedersen, L. C., Dong, J., Tanigashi, F., Kitagawa, H., Krahm, J. M., Pedersen, L. G., Sugahara, K., and Neigish, M. (2003) *J. Biol. Chem.* 278, 14429–14429
26. Wakarchuk, W. W., Cunningham, A., Watson, D. C., and Young, N. M. (1998) *Protein Eng.* 11, 295–302
27. Gosselin, S., Allhusmani, M., Streiff, M. B., Takabayashi, K., and Palic, M. M. (1994) *Annu. Biochem.* 220, 92–97
28. Leatherbarrow, K. R. (1999) *Grofit*, Version 4.0, Erithacus Software Ltd., Staines, UK
29. Owinowski, Z., and Minor, W. (1997) *Methods Enzymol.* 276, 307–326
30. Navaza, J. (1994) *Acta Crystallogr.* Sect. A 50, 157–163
31. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nogu, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr.* Sect. D 54, 905–921
32. Watts, A. G., Damaker, I. Amaya, M. L., Buchschain, A., Alzari, P., Frach, A. C., and Withers, S. G. (2003) *J. Am. Chem. Soc.* 125, 7532–7533
33. Gibbons, B. J., Roach, P. J., and Hurley, T. D. (2002) *J. Mol. Biol.* 319, 463–477
34. Ly, H. D., and Withers, S. G. (1999) *Annu. Rev. Biochem.* 68, 487–522
35. Knecht, R. M. K., Stryskopytov, P., Penninga, D., Faber, O. G., Rabeboom, H. J., Kalk, K. H., Dijkhuizen, L., and Dijkz, B. W., and Withers, S. G. (1999) *Biochemistry* 38, 8976–8984
36. Withers, S. G. (1999) *Biochemistry* 38, 8976–8984
37. Zhang, Y. N., Swaminathan, G. J., Deshpande, A., Boix, E., Natesh, K., and Klein, N. J. (1998) *J. Immunol.* 160, 1546–1553
38. Kraulis, P. (1991) *J. Appl. Crystallogr.* 24, 946–950

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**FIG. 3.** Superimposition of the wild type and Q189E mutant active sites of LgtC-25. Side chains for the active site residues are labeled and depicted in Corey-Pauling-Koltun coloring with carbon atoms in gray (wild type) or yellow (mutant), nitrogen atoms in blue, and oxygen atoms in red. Uracil 5'-diphosphate-2-deoxy-2-fluoro-o-galactopyranose is shown with carbon atoms in cyan (wild type complex) or magenta (mutant complex), nitrogen atoms in blue, oxygen atoms in red, and fluorine atoms in light gray. The Mn²⁺ ion is shown as a violet sphere and the water molecule as blue. Dashed lines indicate hydrogen bonding. The figure was prepared with MOLSCRIPT (38), rendered with Raster3D (13).