Roles of active site tryptophans in substrate binding and catalysis by α-1,3 galactosyltransferase

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Aromatic amino acids are frequent components of the carbohydrate binding sites of lectins and enzymes. Previous structural studies have shown that in α-1,3 galactosyltransferase, the binding site for disaccharide acceptor substrates is encircled by four tryptophans, residues 249, 250, 314, and 356. To investigate their roles in enzyme specificity and catalysis, we expressed and characterized variants of the catalytic domain of α-1,3 galactosyltransferase with substitutions for each tryptophan. Substitution of glycine for tryptophan 249, whose indole ring interacts with the nonpolar B face of glucose or GlcNAc, greatly increases the $k_{\text{cat}}$ for the acceptor substrate. In contrast, the substitution of tyrosine for tryptophan 314, which interacts with the β-galactosyl moiety of the acceptor and UDP-galactose, decreases $k_{\text{cat}}$ for the galactosyltransferase reaction but does not affect the low UDP-galactose hydrolase activity. Thus, this highly conserved residue stabilizes the transition state for the galactose transfer to disaccharide but not to water. High-resolution crystallographic structures of the Trp249Gly mutant and the Trp314Tyr mutant indicate that the mutations do not affect the overall structure of the enzyme or its interactions with ligands. Substitutions for tryptophan 250 have only small effects on catalytic activity, but mutation of tryptophan 356 to threonine reduces catalytic activity for both transferase and hydrolase activities and reduces affinity for the acceptor substrate. This residue is adjacent to the flexible C-terminus that becomes ordered on binding UDP to assemble the acceptor binding site and influence catalysis. The results highlight the diverse roles of these tryptophans in enzyme action and the importance of $k_{\text{cat}}$ changes in modulating glycosyltransferase specificity.

Key words: catalysis/crystal structure/glycosyltransferase/mutation/substrate binding

Introduction

Major contributions to the recognition of specific carbohydrates by lectins and glycosidases are made by interactions of the protein with hydroxyl groups of monosaccharides, particularly through H-bonds with the side chains of polar amino acids and coordination bonds with protein-bound Ca$^{2+}$ ions (Sharon and Lis, 2001; Weiss and Drickamer, 1996). Aromatic amino acids are also found in a large proportion of carbohydrate binding sites, where they make hydrophobic interactions with the nonpolar faces of monosaccharide pyranose rings; the side chains of tryptophan and tyrosine also H-bond with monosaccharide hydroxyl groups. The importance of aromatic side chains in the specificity of lectins and glycosidases has been previously discussed (Muraki, 2002). Glycosyltransferases, enzymes that catalyze the transfer of a monosaccharide from an activated derivative into a defined linkage with a specific acceptor, determine the structures of glycan components of glycoconjugates produced by cells. The structural basis of their substrate specificities is therefore of fundamental significance in glycobiology, yet few structures have been determined for eukaryotic glycosyltransferases, and there is limited information available regarding the basis of their substrate specificity. Although it is likely that the interactions between glycosyltransferases and oligosaccharides will be similar to those in glycosidases and lectins, the multisubstrate character of glycosyltransfer reactions suggests that the determinants of substrate specificity in these enzymes are likely to be more complex.

UDP-galactose β galactosyl α1-3-galactosyltransferase (α3GT, E.C. 2.4.1.151) is a retaining GT that catalyzes the synthesis of α1-3-galactosyl β-OR structures in glycoconjugates (van den Eijnden et al., 1983) and is a component of trans-Golgi membranes. An inactive form of this enzyme is produced in humans and their closest relatives among the primates (Galili et al., 1988; Galili and Swansen, 1991). The absence of active α3GT allows the production of natural antibodies (1–3% of circulating IgG) to the product of its action, the α-Gal epitope, which provide defenses against pathogens (Avila et al., 1989) and viruses arising from mammalian hosts that have active α3GT (Takeuchi et al., 1996).

α3GT is a member of a family of homologous retaining galactosyl- and N-acetylglactosaminyl-transferases that form α-1,3 linkages to β-galactosyl and β-N-acetylglactosaminy residues in glycoconjugates but differ in specificity: the histo-blood group A and B glycosyltransferases (Yamamoto et al., 1990), Forssman glycolipid (Gb$_3$) synthase (Haslam and Baenziger, 1996), and isogloboside 3 (Gb$_3$) synthase (Keusch et al., 2000). These enzymes, like most
Eukaryotic glycosyltransferases, all type II membrane proteins with short cytosolic N-terminal domains, a membrane spanning region, a stem, and C-terminal catalytic domains (Paulson and Colley, 1989). Structures have been determined for recombinant truncated catalytic domains of bovine α3GT (Boix et al., 2001, 2002; Gastinel et al., 2001) and human histo-blood group A and B glycosyltransferases (Patenau et al., 2002).

Two distinct structural states have been described for α3GT, a lower-resolution tetragonal form (Form I), in which the C-terminus of the enzyme is disordered (Gastinel et al., 2001), and a higher-resolution monoclinic form (Form II), which has a distinct and highly ordered conformation for the C-terminal region (Boix et al., 2001). The structured C-terminus was found by mutagenesis to be important for the catalytic activity of the enzyme (Boix et al., 2001). We have reported Form II structures for α3GT in complexes with Mn²⁺ and UDP at 1.53 Å resolution (Boix et al., 2001), Mn²⁺/UDP-galactose (UDP-Gal), Mn²⁺/UDP-glucose (UDP-Glc), and with both Mn²⁺/UDP and the acceptor substrates lactose and N-acetyllactosamine (Boix et al., 2002). The structures of these complexes identify four tryptophans, residues 249, 250, 314, and 356, in the active site of α3GT that interact with acceptor substrates (see Table I and Figure 1). Molecular modelling studies by Heissigerová et al. (2003) suggest that these specific tryptophans and the corresponding residues in other members of the α3GT family may be important in acceptor substrate selectivity.

To investigate the roles of the four tryptophans of α3GT in substrate specificity and catalysis, we introduced homology-based (Table II) and structurally conservative substitutions. Such mutations are expected to have minor effects on overall enzyme structure and to perturb (but not eliminate) catalytic activity. The quantitative effects of the mutations on individual kinetic parameters for two catalytic activities of α3GT, galactosyltransferase and UDP-Gal hydrolase activity, were then determined to evaluate the role of each residue in enzyme function (Zhang et al., 2001). Two mutants containing substitutions for Trp²⁴⁹ (Gly) and Trp³¹⁴ (Tyr), residues that make stacking interactions with the glucose and galactose components of the lactose acceptor, respectively (Boix et al., 2002), have major but distinct effects on the catalytic properties. Crystallographic structural analyses show that neither mutation significantly changes the overall structure of the enzyme or its active site. Nevertheless, the Trp²⁴⁹Gly substitution greatly reduces the affinity of the enzyme for lactose and the Trp³¹⁴Tyr mutation selectively perturbs $k_{cat}$ for galactosyl transfer to lactose without perturbing the rate of galactose transfer to water. Mutations of Trp²⁵⁰ have relatively subtle effects on activity, whereas the Trp³⁵⁶Thr mutation lowers

### Table I. Roles of residues selected for mutagenesis in α3GT

<table>
<thead>
<tr>
<th>Residue</th>
<th>Substrate</th>
<th>Enzyme atom</th>
<th>Substrate atom</th>
<th>Interactions with substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp²⁴⁹</td>
<td>Lac (gle)a</td>
<td>(&gt;10)⁹</td>
<td>ring</td>
<td>vdw⁸</td>
</tr>
<tr>
<td>Trp²⁵⁰</td>
<td>Lac (gle)a</td>
<td>NE1</td>
<td>O3'</td>
<td>Hb⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9)</td>
<td>ring</td>
<td>vdw</td>
</tr>
<tr>
<td>Trp³¹⁴</td>
<td>Gal component of UDP-Gal⁡</td>
<td>O</td>
<td>O3</td>
<td>Hb</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O4</td>
<td>Hb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>vdw</td>
<td></td>
</tr>
<tr>
<td>Trp³⁵⁶</td>
<td>Lac (gal)a</td>
<td>NG1</td>
<td>O6</td>
<td>Hb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>vdw</td>
<td></td>
</tr>
</tbody>
</table>

¹⁰Derived from structure of the α3GT-UDP-Lac complex.
¹¹(X)-number of contacts.
¹²vdw: van der Waal contact.
¹³Hb: hydrogen bond.
¹⁴Derived from structure of the α3GT-UDP-gal complex. The galactose is free as a result of cleavage of UDP-Gal.

**Fig. 1.** Location of tryptophans 249, 250, 314, and 356 in the acceptor substrate binding site of the α3GT-UDP-lactose complex. The Cα backbone of the enzyme is shown in gray, tryptophan side chains are pink, the UDP is yellow, the Mn²⁺ cofactor is blue, and the lactose is green with C atoms colored black and O and N atoms red.

### Table II. Residues that correspond to α3GT acceptor substrate binding site tryptophans in homologous glycosyltransferases

<table>
<thead>
<tr>
<th>Bovine α3GT</th>
<th>Other α3GTs</th>
<th>iGb3 synthase</th>
<th>Forssman glycolipid synthase</th>
<th>Blood group A synthase</th>
<th>Blood group B synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp²⁴⁹</td>
<td>Trp</td>
<td>Trp</td>
<td>Gly</td>
<td>Gly</td>
<td>Ser</td>
</tr>
<tr>
<td>Trp²⁵⁰</td>
<td>Trp</td>
<td>His</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
</tr>
<tr>
<td>Trp³¹⁴</td>
<td>Trp</td>
<td>Trp</td>
<td>Trp</td>
<td>Trp</td>
<td>Trp</td>
</tr>
<tr>
<td>Trp³⁵⁶</td>
<td>Trp</td>
<td>Trp</td>
<td>Thr</td>
<td>Ala</td>
<td>Ala</td>
</tr>
</tbody>
</table>

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the catalytic activity for both reactions and weakens lactose binding. These different effects appear to reflect the proximity of the residues to the enzyme catalytic center and their roles in localized conformational changes during catalysis.

Results

Design and production of α3GT Trp mutants

The side chains of α3GTs 249, 250, 314, and 356 surround the binding site for lactose and LacNAc in their complexes with α3GT, UDP, and Mn2⁺ (Figure 1). The indole ring of Trp249 is nearly parallel with the reducing monosaccharide (Glc or GlcNAc) of the acceptor substrates and makes nonpolar van der Waals interactions with the hydrophobic B face of the pyranose ring (Table I). However, in the absence of acceptor substrate, the side chain adopts two alternative conformational states (Boix et al., 2002). As indicated in Table II, tryptophan is conserved at this site in other glycosyltransferases but not in Forssman glycolipid synthase or in histo-blood group A and B synthases (Haslam and Baenziger, 1996; Keusch et al., 2000; Yamamoto et al., 1990). The Gly mutation introduces the amino acid at the corresponding site in the histo-blood group A transferase that catalyzes GalNAc transfer to a similar acceptor in which fucose is α-linked to the 2-OH of lactose.

The side chain of Trp250 is located near the glucose or GlcNAc component of acceptor substrates in their complexes with enzyme (Boix et al., 2002). However, unlike Trp249, the indole ring is approximately perpendicular to the pyranose ring, lying at the edge of the entrance of lactose binding groove. Specific interactions of Trp250 with acceptor substrates are an H-bond between the indole ring N and the 3'-OH of both lactose and LacNAc and packing interactions of the indole ring with the 2'-acetamido group of LacNAc. There is no corresponding interaction with lactose, but the tryptophan makes about seven additional nonpolar contacts with both acceptors. Mutants were expressed with conservative substitutions of Tyr and Phe, corresponding to residues present in Forssman glycolipid synthase and the blood group enzymes (Table II).

Trp314 is conserved in all homologs of α3GT. In α3GT, the side chain has more than 10 hydrophobic interactions with the β-galactosyl moiety of the acceptor and the N of the indole ring is within H-bonding distance of O6 of the glucose moiety of lactose. Also, in the enzyme complexes with UDP-Gal and UDP-Glc (in which the substrate is cleaved) it has 10 and 8 hydrophobic contacts, respectively, with the monosaccharide ring. In the complex with UDP-Gal, the carbonyl OH H-bonds with the 2- and 3-OH groups of the galactose (Table I; Boix et al., 2002).

Trp356 is part of the acceptor binding site making six to seven nonpolar van der Waals contacts with acceptor substrates. It is adjacent to the C-terminal section of the polypeptide chain that undergoes a rearrangement between the Form I and Form II structures and is linked with UDP binding (Boix et al., 2001). This residue was mutated to Thr, the amino acid present at the corresponding site in the histo-blood group A and B transferases (Table II).

All of the α3GT mutants were expressed in soluble active form in good yield and showed similar solubility to the wild-type enzyme.

Effects of mutations on activity

Trp249 to Gly. Acceptance substrate (lactose) binding is so strongly perturbed by this mutation that the relationship between velocity and lactose concentration up to 100 mM at 1 mM UDP-Gal is linear. The limited solubility of lactose in water precludes the use of higher lactose concentrations in assays. Although \( k_{cat} \) and \( K_m \) with lactose as substrate cannot be determined for this mutant, the value of \( k_{cat}/K_m \) (apparent), determined from the slope of \( v \) versus [lactose] plot is 20-fold lower than that of the wild-type enzyme (Table III). The lack of curvature indicates that the \( K_m \) for lactose must exceed 200 mM. It was also not possible to achieve saturation with higher-affinity acceptor substrates including LacNAc and \( p\)-NO₂-phenyl-LacNAc because of their limited solubility in water. The apparent \( K_m \) for UDP-Gal, determined at a fixed nonsaturating concentration of lactose (25 mM), is similar to the wild-type enzyme (Table III), and the apparent \( k_{cat} \) determined at 25 mM lactose is 0.26 s⁻¹, a value that is much lower than the true \( k_{cat} \) because of the low acceptor concentration relative to the \( K_m \). The \( k_{cat} \) and \( K_m \) for UDP-Gal hydrolysis are essentially unchanged (Table III), and it is possible that \( k_{cat} \) for the transferase reaction is also unchanged, in which case the \( K_m \) for lactose would be about 1.5 I. Isothermal titration calorimetry studies (see Boix et al., 2002) indicate that the free energy and enthalpy of binding of UDP and UDP-Gal to the enzyme in the presence of Mn2⁺ were not significantly changed by the mutation. However, unlike wild-type α3GT, the binding of lactose in the presence of UDP and Mn2⁺ to the Trp249Gly mutant was not detected, suggesting that the negative enthalpy of binding and/or affinity of lactose for this inhibitory enzyme complex are reduced (data not shown).

Trp250 to Tyr or Phe. The activity levels of the two mutants under standard assays conditions are similar to those of the wild-type protein. A detailed analysis indicates that for galactose transfer to lactose, \( k_{cat} \) is slightly higher than in the wild type for both mutants while \( K_m \), the \( K_m \) for UDP-Gal is increased 6–10-fold. Both mutants also have slightly increased \( K_m \) and \( k_{cat} \) values for UDP-Gal hydrolysis (Table III). In the ordered sequential mechanism for α3GT:

\[
E \overset{k_1[A]}{\rightleftharpoons} EA \overset{k_2}{{\rightleftharpoons}} EAPQ \overset{k_3[P]}{\rightleftharpoons} EQ \overset{k_4[Q]}{\rightleftharpoons} E
\]

where \( A \) is the donor substrate, UDP-Gal, \( B \) is the acceptor substrate, and \( P \) and \( Q \) are expected to be the trisaccharide product and UDP, respectively. \( K_a = k_{cat}/[A]k_5(k_5 + k_7) \) and \( k_{cat} = k_{cat}[A]k_5(k_5 + k_7) \) so that \( k_{cat}/K_a = k_1 \), the on-rate for UDP-Gal binding (Purich and Allison, 2000).

Both of these mutations therefore increase the overall rate of product-release steps and lower the on-rate of UDP-Gal binding three- to fourfold. This interpretation is consistent with the slightly increased \( k_{cat} \) for UDP-Gal hydrolysis and may reflect a local structural change resulting from the mutation.
The $K_m$ of $\alpha$3GT for LacNAc is about 12-fold lower than the $K_m$ for lactose, which could reflect contributions to the free energy of binding arising from interactions of the LacNAc 2-acetamido group with Trp250. The substitution of Tyr or Phe, residues with smaller side chains than Trp, may perturb these interactions and affect the binding of LacNAc but not lactose. To test this hypothesis, apparent $K_m$ and $k_{cat}$ were determined with LacNAc for the wild-type enzyme and the two mutants (at 1 mM UDP-Gal). The values of these parameters were: wild type: $K_m$ = 2.67 ± 0.27 mM, $k_{cat}$ = 4.59 ± 0.18 s$^{-1}$; Trp250Phe: $K_m$ = 1.85 ± 0.25 mM, $k_{cat}$ = 5.97 ± 0.26 s$^{-1}$; Trp250Tyr: $K_m$ = 2.76 ± 0.24 mM; $k_{cat}$ = 6.19 ± 0.22 s$^{-1}$. The apparent kinetic parameters for the wild-type enzyme are similar to true values determined in experiments in which the concentrations of both UDP-Gal and LacNAc were varied: $K_m$ = 1.6 ± 0.14 mM; $k_{cat}$ = 5.6 ± 0.2 s$^{-1}$. These results indicate that the substitutions for Trp250 do not selectively disfavor the binding of LacNAc relative to lactose.

**Trp$^{314}$ to Tyr**

Galactosyltransferase activity is greatly reduced in the Trp$^{314}$Tyr mutant but UDP-Gal hydrolase activity is essentially unchanged relative to the wild-type enzyme (Table III). As discussed previously for a different mutant of $\alpha$3GT (Zhang et al., 2003), the high proportion of hydrolase activity makes it difficult to determine kinetic parameters with high precision using the radiochemical assay, and we determined apparent kinetic parameters for the transferase reaction. These indicate that the main effect of the mutation is to reduce the $k_{cat}$ for the galactosyltransferase reaction 30-fold but the kinetic parameters for UDP-Gal hydrolase activity are insignificantly affected. These effects are closely similar to those in the previously described Gln$^{247}$Glu mutant (Zhang et al., 2003).

**Trp$^{356}$ to Thr**

This mutant has a reduced level of overall catalytic activity. The loss in hydrolase activity reflects a 15-fold reduction in $k_{cat}$ but, for the galactosyltransferase reaction, a reduction in $k_{cat}$ and an increase in the $K_m$ for lactose (determined at 1 mM UDP-Gal) contribute to a greater reduction in activity. There is some uncertainty in the precise values of the parameters for the transferase reaction because the highest substrate concentration in the assay (100 mM) is less than the calculated value of $K_m$ (126 mM), but it is clear from the results that $k_{cat}$ is reduced 10–15-fold for both reactions and acceptor substrate affinity is about 5-fold lower than for the wild-type enzyme (Table III).

**Structures of the Trp$^{249}$Gly and Trp$^{314}$Tyr mutants**

The Form II structure of the complex of Trp$^{249}$Gly with UDP and Mn$^{2+}$ was determined at 2.07 Å resolution (Table IV). The mutation has no significant effect on the overall structure of the enzyme and the active site. The root mean square deviation from the structure of the wild-type enzyme is 0.11 Å, and there are only minor structural changes in the vicinity of the mutation that are shown by superimposing the structures of the mutant and wild-type enzymes (Figure 2). Two specific structural changes can be directly attributed to the mutation; first, the loss of the Trp side chain disrupts an interaction between Trp$^{249}$ and Asp$^{340}$ and, second, a new structured water molecule is

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Table III. Kinetic parameters of recombinant $\alpha$1,3-GT and its variants for the galactosyltransferase reaction

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>WT</th>
<th>W$^{249}$G</th>
<th>W$^{250}$Y</th>
<th>W$^{250}$F</th>
<th>W$^{314}$Y</th>
<th>W$^{356}$T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosyltransferase reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>6.4 ± 0.7</td>
<td>&gt;2.3</td>
<td>8.2 ± 0.6</td>
<td>16.5 ± 0.9</td>
<td>0.22 ± 0.006$^{b}$</td>
<td>0.52 ± 0.02$^{b}$</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.43 ± 0.07</td>
<td>0.84 ± 0.17$^{a}$</td>
<td>2.24 ± 0.27</td>
<td>4.00 ± 0.33</td>
<td>0.72 ± 0.07$^{a}$</td>
<td>0.85 ± 0.03$^{a}$</td>
</tr>
<tr>
<td>$K_0$ (mM)</td>
<td>19.9 ± 3.4</td>
<td>&gt;200$^{c}$</td>
<td>9.8 ± 1.2</td>
<td>21.5 ± 1.8</td>
<td>9.7 ± 0.8$^{b}$</td>
<td>126 ± 8$^{b}$</td>
</tr>
<tr>
<td>$K_{cat}/K_m$ × $K_0$ (×10$^6$ sec$^{-1}$M$^{-2}$)</td>
<td>2.3 ± 0.7</td>
<td>&lt;0.019</td>
<td>5.5 ± 4.6</td>
<td>5.1 ± 3.6</td>
<td>0.16 ± 0.02</td>
<td>0.017</td>
</tr>
<tr>
<td>Hydrolyase reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$) × 10$^3$</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
<td>29 ± 1</td>
<td>23 ± 0.2</td>
<td>18 ± 0.3</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>$K_a$ (mM)</td>
<td>0.10 ± 0.00</td>
<td>0.21 ± 0.04</td>
<td>0.23 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>$k_{cat}/K_a$ (×10$^3$ s$^{-1}$M$^{-1}$)</td>
<td>0.16</td>
<td>0.076</td>
<td>0.13</td>
<td>0.15</td>
<td>0.16</td>
<td>0.0044</td>
</tr>
<tr>
<td>$k_{cat}$ hydrolase</td>
<td>400</td>
<td>&gt;140</td>
<td>283</td>
<td>717</td>
<td>12</td>
<td>473</td>
</tr>
</tbody>
</table>

These values were determined at 37°C as described in Materials and methods.

$^{a}$The values for $K_m$ (apparent) for UDP-Gal ($K_a$) were determined by varying [UDP-Gal] at a fixed lactose concentration of 25 mM.

$^{b}$The apparent $k_{cat}$ and $K_a$ for lactose ($K_m$) were determined by varying [lactose] at a fixed UDP-Gal concentration of 1.0 mM. The apparent $k_{cat}$ was corrected for the wild-type enzyme.

$^{c}$Determined by varying [lactose] at 1 mM UDP-Gal.

$^{d}$These are $K_m$ values for UDP-Gal hydrolysis (see text for discussion).
present in the mutant enzyme located in the space produced by removal of the Trp side chain (Figure 2).

Structures were determined for the Trp 314Tyr mutant in complexes with UDP and lactose and UDP and LacNAc at 1.7 and 2.1 Å, respectively (Table IV). The change in the amino acid side chain is clearly visible in the structure, but otherwise, the superimposed mutant and wild-type structures show essentially no change that can be attributed to the mutation (Figures 3A and 3B). Also, the bound ligands superimpose closely with those in the wild-type α3GT complexes. Although the mutation reduces the number of contacts between the side chain of Tyr 314 and acceptor substrate, it does not produce any change in the orientation of the substrate in this complex.

Discussion

The structures of complexes of enzymes with substrates and inhibitors provide important information regarding binding site locations and the nature of enzyme–ligand interactions. However, the complexes whose structures can be determined in this way are generally not on the catalytic pathway of the enzyme. This is the case for α3GT, where structures have been determined at high resolution for enzyme complexes (1) containing one UDP ligand (Boix et al., 2001), (2) two UDPs (Zhang et al., 2003), (3) the products of

Table IV. Statistics for data collection and refinement

<table>
<thead>
<tr>
<th>Data set</th>
<th>Trp249Gly</th>
<th>Trp314Tyr-Lactose complex</th>
<th>Trp314Tyr-LacNAc complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocrystallization conditions</td>
<td>10 mM UDP, 0.1 mM MnCl₂</td>
<td>10 mM UDP, 0.1 mM MnCl₂</td>
<td>10 mM UDP, 0.1 mM MnCl₂</td>
</tr>
<tr>
<td>Soaking conditions</td>
<td>None</td>
<td>100 mM lactose for 3 weeks</td>
<td>100 mM LacNAc for 3 weeks</td>
</tr>
<tr>
<td>Wavelength used for data collection (Å) and Synchrotron station</td>
<td>1.4 (PX 14.1-SRS-UK)</td>
<td>0.87 (PX 9.6-SRS-UK)</td>
<td>1.4 (PX 14.1-SRS-UK)</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50.0–2.07</td>
<td>50.0–1.7</td>
<td>50.0–2.1</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁ (2 mol/a.u.)</td>
<td>P2₁ (2 mol/a.u.)</td>
<td>P2₁ (2 mol/a.u.)</td>
</tr>
<tr>
<td>Cell dimensions (Å)</td>
<td>(a = 93.6, b = 94.2, c = 94.5)</td>
<td>(a = 94.8, b = 94.4, c = 94.5)</td>
<td>(a = 94.8, b = 94.4, c = 94.5)</td>
</tr>
<tr>
<td>Number of observations</td>
<td>544,531</td>
<td>234,582</td>
<td>131,633</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>52,584</td>
<td>81,958</td>
<td>44,358</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>92.2 (83.6)</td>
<td>97.6 (87.7)</td>
<td>98.1 (89.5)</td>
</tr>
<tr>
<td>I/σ (I)</td>
<td>11.2 (4.2)</td>
<td>18.8 (3.7)</td>
<td>27.7 (17.4)</td>
</tr>
<tr>
<td>(R_{\text{symm}}) (%)(^d)</td>
<td>11.0 (24.7)</td>
<td>5.7 (22.0)</td>
<td>4.0 (5.8)</td>
</tr>
<tr>
<td>(R_{\text{cryst}}) / (R_{\text{free}}) (%)(^f)</td>
<td>19.3/22.0</td>
<td>15.4/20.2</td>
<td>16.4/19.9</td>
</tr>
<tr>
<td>Deviations from ideality</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.006</td>
<td>0.008</td>
<td>0.006</td>
</tr>
<tr>
<td>Bond angles(deg)</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Ligand molecules</td>
<td>2 UDPs (one/monomer); 1 Mn²⁺ per monomer</td>
<td>2 UDPs (one/monomer); 1 Mn²⁺ per monomer; 1 Lac in B-chain</td>
<td>2 UDPs (one/monomer); 1 LacNAc in B-chain and 1 glycerol</td>
</tr>
</tbody>
</table>

\(^a\)Outermost shell is 2.07–2.0 Å.

\(^b\)Outermost shell is 1.76–1.7 Å.

\(^c\)Outermost shell is 2.19–2.11 Å.

\(^d\)\(R_{\text{symm}} = \frac{\sum_h (|I(h)| - <I(h)>)}{\sum|h|I(h)}\) where \(I(h)\) is the \(i\)th measurement and \(<I(h)>\) is the weighted mean of all measurements of \(I(h)\).

\(^e\)\(R_{\text{cryst}} = \frac{\sum_h |F_o - F_c|}{\sum_h F_o}\) where \(F_o\) and \(F_c\) are the observed and calculated structure factor amplitudes of reflection \(h\).

\(^f\)\(R_{\text{free}}\) is equal to \(R_{\text{cryst}}\) for a randomly selected 5–10% subset of reflections, not used in refinement.

![Fig. 2. Structure of the active site of the UDP complex of the Trp249Gly mutant of α3GT superimposed with the wild-type enzyme. The mutant enzyme is gray and the wild-type enzyme pink. The UDP is yellow and the Mn²⁺ cofactor blue. The location of a water molecule that replaces the tryptophan side chain is displayed as a gray ball.](https://example.com/fig2.png)
UDP-galactose hydrolysis, (4) products of UDP-glucose hydrolysis, (5) a dead-end inhibitory complex containing UDP and lactose, and (6) the corresponding complex with UDP and LacNAc (Boix et al., 2003). These structures all contain the Form II conformer of α3GT. Lower-resolution structures have been reported for the Form I conformer, in which the C-terminal region is disordered, in complexes with UMP and UDP-Gal (Gastinel et al., 2001), but we have been unable to identify a UDP ligand in our Form I structure despite extensive soaking in 20 mM UDP (Zhang et al., 2003). Although these structures do not establish the roles of specific enzyme–ligand interactions in specificity or catalysis, they do provide an information base of potential interactions that can be further investigated by mutational studies of structure–activity relationships.

The present results indicate that although tryptophans 249, 250, 314, and 356 contribute to the binding sites for acceptors in these structures, they make very distinct contributions to the catalytic properties of the enzyme. The Trp^{249}Gly mutation specifically perturbs the $K_m$ for acceptor but appears to have little effect on $k_{cat}$, indicating that mutation of Trp^{249} has similar effects on acceptor binding in the Michaelis complex and transition state. This is consistent with the relatively distant location of this residue from the catalytic center of the enzyme, its role in interacting with the Glc or GlcNAc component of the acceptor, and the absence of any significant change in overall structure arising from the mutation. It is interesting that the corresponding residue in human blood group A and B glycosyltransferases is one of the four sites at which these closely related enzymes differ in sequence (Gly and Ser, see Table II). This residue also influences acceptor substrate affinity in these enzymes because substitution of Ser for Gly in the truncated catalytic domain of the blood group A glycosyltransferases produces a threefold increase in the $K_m$ for acceptor substrate but insignificant changes in $k_{cat}$ or donor substrate specificity (Seto et al., 1999).

Substitutions of Tyr or Phe for Trp^{250} produce small changes in catalytic activity, specifically, a small increase in $k_{cat}$ for both the hydrolase and transferase activities and an increased $K_m$ for UDP-Gal for the transferase activity. These changes may reflect a small change in active site accessibility that produces increased rates of product release and reduces the on-rate for UDP-Gal binding.

The enzyme mutant with a Trp^{314} to Tyr substitution has properties that are similar to those of a previously described Glu^{247} to Glu variant (Zhang et al., 2003). The $k_{cat}$ for the transferase reaction is reduced, but hydrolase activity is essentially unchanged; the mutation also produces a small reduction in the $K_m$ for acceptor. Both the Trp^{314} to Tyr and Glu^{247} to Glu mutations reduce the ratio of the transferase to hydrolase activity ~40-fold (Table III). Therefore, the Trp^{314}Tyr mutation selectively destabilizes the transition state for the transferase reaction, as shown by its effect on catalytic efficiency for this reaction (Table III), but does not affect acceptor binding in the Michaelis complex or any aspect of UDP-Gal hydrolysis. Trp^{314} interacts with both acceptor substrates in their UDP complexes and with the monosaccharides in the enzyme complexes with UDP-Gal and UDP-Glc (Boix et al., 2002). The mutation does not alter the structure of the enzyme or the binding of UDP, lactose or LacNAc. These results suggest that Trp^{314} has a role in stabilizing the bisubstrate transition state for the transfer of galactose to a disaccharide substrate but not for transfer to water.

The effects of the mutation of Trp^{356} to Thr differs from the others investigated here in reducing the $k_{cat}$ for the both transferase and hydrolase reactions. This effect appears to be linked to its location immediately adjacent to the C-terminal region of α3GT (residues 358–365) that undergoes a conformational change between the Form I and II structures (Boix et al., 2001; Gastinel et al., 2001).
A previously described α3GT mutant with a substitution of Lys for Arg365, a residue that interacts with O2 of the α-P of UDP, showed large reductions in $k_{cat}$ and catalytic efficiency for the transferase reaction (Zhang et al., 2003) and a similar reduction in UDP-Gal hydrolyase activity to that seen in the Trp356Thr mutant (Table III). This suggests that the conformational change is linked to the ability of α3GT to catalyze galactose transfer to a carbohydrate acceptor or to water, possibly through the enclosure of the catalytic site and bound UDP-Gal by the transconformation and formation of an appropriate environment for catalysis (Zhang et al., 2003). However, Trp356 also interacts with the acceptor substrate, and the Thr mutation also increases the $K_m$ for lactose. Thus mutations of different tryptophans influence substrate specificity in this glycosyltransferase through their effects on substrate affinity (Trp 249), transition state stabilization (Trp 314), or both (Trp 356).

**Materials and methods**

**Enzyme expression, mutagenesis, and activity measurements**

Mutants of the catalytic domain of bovine α3GT (residues 80–368) were constructed, expressed in *Escherichia coli* BL21(DE3), and purified as described previously (Zhang et al., 2001). The sequences of mutants were checked by automated DNA sequencing of the expression vector.

Steady-state kinetic studies with wild-type and mutant enzymes were performed with a radiochemical assay as previously described (Zhang et al., 2001). For characterizing the transferase activity of most mutants, enzyme activity was measured in the presence of 10 mM MnCl$_2$ at a series of UDP-Gal concentrations (generally 0.1–2.0 mM) and different lactose concentrations. The data were analyzed by fitting to the rate equation for a symmetrical sequential initial velocity pattern (Zhang et al., 2001) to give values for $k_{cat}$, $K_m$ (for UDP-Gal), $K_{in}$ (for UDP-Gal from E.UDP-Gal complex) and $K_a$ ($K_m$ for acceptor) (Zhang et al., 2001, 2003). As discussed in the text, in some cases the concentration of UDP-Gal was varied at a single fixed concentration of lactose (generally 25 mM) and lactose was varied at a single fixed concentration of UDP-Gal, generally 1.0 or 1.2 mM. Analysis of these data by fitting to the standard single-substrate Michaelis-Menten equation provided apparent values for $K_m$ for each substrate and two $V_m$ values. The apparent $V_m$ value, obtained by varying [lactose] at fixed [UDP-Gal] was adjusted by multiplying by (1 + [UDP-Gal]/$K_a$), where $K_a$ is the apparent $K_m$ for UDP-Gal to get an improved estimate of the true $V_m$ and $k_{cat}$. This method was used in the case of mutants with relatively high UDP-Gal hydrolase activities, as discussed next.

An enzyme concentration 2–5 μg/ml was used for galactosyltransferase assays and UDP-Gal hydrolase activities were determined at 10–20-fold higher concentrations (Zhang et al., 2003). The transferase and hydrolase activities of the less active Trp356Thr mutant were determined at enzyme concentrations and incubation times of 1.6 μM/15 min and 6.4 μM/60 min.

**X-ray crystallography**

Crystals of the Trp 249Gly and Trp 314Tyr mutants were grown at 16°C by the vapor diffusion hanging drop method as described elsewhere (Boix et al., 2001) by mixing 2 μl of the protein at 5 mg/ml in 20 mM 2-(N-morpholino)ethanesulfonic acid-NaOH buffer, pH 6.0, 10% glycerol, containing 10 mM UDP and 0.1 mM MnCl$_2$, with an equal volume of a reservoir solution containing 5% PEG 6000 and 0.1 M Tris–HCl, pH 8.0. Single crystals appeared after 3–4 days. Crystals of Trp314Tyr complexes with lactose and LacNAc were obtained by soaking in 100 mM solution of lactose and LacNAc, respectively. Before data collection, all crystals were flash-cooled at 100 K in a cryoprotectant containing 10% PEG 6000, 0.1 M Tris-HCl, pH 8.0, 25% glycerol, and appropriate ligands. High-resolution data sets (space group $P_{2_1}$ with 2 molecules in the asymmetric unit) were collected at the Synchrotron Radiation Source (Daresbury, UK) (stations PX9.6 and PX14.1 using a CCD-ADSC detector system). Raw data images were indexed and scaled using the DENZO and SCALEPACK modules of the HKL suite (Otwinowski and Minor, 1997), see Table IV.

The cell dimensions for all the data sets were isomorphous with the previously reported Form II α3GTUDP structure (Boix et al., 2001), PDB entry 1K4V (Table IV). Therefore these coordinates were used as a starting model. Crystallographic refinement was performed with the CNS program package (Brünger et al., 1998). Multiple rounds of bulk solvent correction, energy minimization, individual isotropic B-factor refinement, simulated annealing, and model building using the O program (Jones et al., 1991) were carried until the $R_{free}$ value was optimized. Appropriate ligands (UDP, lactose, LacNAc, and Mn$^{2+}$ ion) were incorporated into each model after careful observation of the respective electron density map. Water molecules were gradually included into the model at positions corresponding to peaks in the $\text{Fo} - \text{Fc}$ electron density map with heights greater than 3 sigma and at H-bond distance from appropriate atoms. The final refinement statistics are given in Table IV.

For the Trp 314Tyr-lactose complex structure at 1.7 Å resolution, further refinement was carried out using SHELX-97 (Sheldrick and Schneider, 1997). Initial conjugate gradient least squares refinement in SHELXL showed that all residues lie in the allowed regions for the electron density. Final analysis of the Ramachandran ($\varphi$, $\psi$) plot showed that all residues lie in the allowed regions for all three structures. The final refinement statistics are included in Table IV. The atomic coordinates for α3GT Trp 249Gly mutant, Trp 314Tyr mutant–lactose complex, and Trp 314Tyr mutant-N-acetyl-lactosamine.
complex (codes 1VZT, 1VZU, and 1VZX, respectively) have been deposited in the RCSB Protein Data Bank. All figures were generated using MOLSCRIPT (Kraulis, 1991).

Acknowledgments

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References


