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A Single Point Mutation Reverses the Donor Specificity of Human Blood Group B-synthesizing Galactosyltransferase*

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Blood group A and B antigens are carbohydrate structures that are synthesized by glycosyltransferase enzymes. The final step in B antigen synthesis is carried out by an α1–3 galactosyltransferase (GTB) that transfers galactose from UDP-Gal to type 1 or type 2, αFuc1→2βGal-R (H)-terminating acceptors. Similarly the A antigen is produced by an α1–3 *N*-acetylgalactosaminyltransferase that transfers *N*-acetylgalactosamine from UDP-GalNAc to H-acceptors. Human α1–3 *N*-acetylgalactosaminyltransferase and GTB are highly homologous enzymes differing in only four of 354 amino acids (R176G, G235S, L266M, and G268A). Single crystal x-ray diffraction studies have shown that the latter two of these amino acids are responsible for the difference in donor specificity, while the other residues have roles in acceptor binding and turnover. Recently a novel *cis-AB* allele was discovered that produced A and B cell surface structures. It had codons corresponding to GTB with a single point mutation that replaced the conserved amino acid proline 234 with serine. Active enzyme expressed from a synthetic gene corresponding to GTB with a P234S mutation shows a dramatic and complete reversal of donor specificity. Although this enzyme contains all four "critical" amino acids associated with the production of blood group B antigen, it preferentially utilizes the blood group A donor UDP-GalNAc and shows only marginal transfer of UDP-Gal. The crystal structure of the mutant reveals the basis for the shift in donor specificity.

Human blood group A and B antigens are produced by glycosyltransferase enzymes that catalyze the transfer of a monosaccharide from a nucleotide donor to Fucα1–2Galβ-R (H) acceptor substrates (1, 2). The A-synthesizing α1–3 *N*-acetylgalactosaminyltransferase (GTA,[1] EC 2.4.1.40, glycoprotein-fucosylgalactoside α-*N*-acetylgalactosaminyltransferase) transfers GalNAc from UDP-GalNAc to H-terminating acceptors producing the A antigen GalNAcα1–3[Fucα1–2]Galβ-R. The B-synthesizing α1–3 galactosyltransferase (GTB, EC 2.4.1.37, glycoprotein-fucosylgalactoside α-galactosyltransferase) utilizes UDP-Gal as its donor producing Galα1–3[Fucα1–2]Galβ-R. GTA and GTB are highly homologous enzymes, differing at only four amino acids of 354 (3). Changing these four amino acids, R176G, G235S, L266M, and G268A, alters the specificity of transfer from that of GTA to GTB. X-ray diffraction studies of crystals of GTA and GTB and their complexes with H-acceptor and UDP have revealed the basis for donor and acceptor specificity (4). Both residues 266 and 268 are involved in recognition of the donor monosaccharide with Leu/Met-266 being primarily responsible for the discrimination between GalNAc and Gal. Donor specificity is not absolute since small levels of crossover reactions have been observed where GTA is able to use UDP-Gal to synthesize B antigens at about 0.4% the rate of UDP-GalNAc transfer (5, 6). Similarly GTB can slowly synthesize the A antigen, and A antigen structures have been observed on normal group B red blood cells (7).

cis-AB enzymes are rare dual specificity hybrid enzymes capable of utilizing either donor. Several natural and recombinant *cis*-AB enzymes have been characterized with interchanges in the four amino acids such as AAAB (Arg-176, Gly-235, Leu-266, and Ala-268) (8), ABBA, AABA and BBBA, and BABA. Two other *cis*-AB enzymes result from point mutations of GTB: one at codon 266 (M266L) (9) and one at position 234 (P234S) (10). Another mutation at position 234 (P234A) has been reported to modify the specificity of the GTB to transfer not only Gal but also small amounts of GalNAc leading to a phenotype called B(A) (11). To elucidate the structural basis of functional modification induced by the P234S replacement, recombinant GTB P234S was characterized by kinetic and x-ray diffraction studies.

EXPERIMENTAL PROCEDURES

Cloning and Characterization of P234S—The original GTB and GTA gene sequences (amino acids 54–354) were described previously (12, 13). In this study, GTB (amino acids 63–354) and GTA (amino acids 63–354) are denoted as wild type and were constructed by PCR using the original GTA and GTB (amino acids 54–354) clones as templates. The forward primer MIN2 (5′-ATA TGA ATT CAT GGT TTC CCT GCC GCG TAT GGT TTA CCC GCA GCC GAA-3′) introduced an *Eco*RI site at the 5′ end, and the reverse primer PCR3B (5′-ATA ATT AAG CTTCTA TCA CGG GTT ACG AAC AGC CTG GTG GTT TTT-3′) introduced a *Hin*dIII site in the 3′ end of GTA and GTB genes. The amplified genes were cloned into the expression vector pCWΔlac (pCW was a gift of F. W. Dahlquist (14), and pCWΔlac was a gift of W. Wakarchuk),

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[1] The abbreviations used are: GTA, α1–3 *N*-acetylgalactosaminyltransferase; GTB, α1–3 galactosyltransferase; MOPS, 4-morpholinepropanesulfonic acid.

TABLE I
Effects of Pro-234 to Ser mutation on the catalytic properties of GTB
K_A for UDP-Gal was determined at 15 mM acceptor, and the K_B for acceptor was determined at 500 μM UDP-Gal donor.

Enzyme	UDP-Gal				UDP-GalNAc			
	K_A	K_B	K_{ia}	k_{cat}	K_A	K_B	K_{ia}	k_{cat}
	μM	μM	μM	s^{-1}	μM	μM	μM	s^{-1}
GTB	88	27	42	5.1	180	138	90	0.42
P234S	4500	106		0.24	3740	167	1100	14.4
GTA	67	3.2	78	0.088	9.9	8.7	3.4	17.5

TABLE II
Data collection and refinement statistics for P234S GTB mutant grown in the absence and presence of H-antigen

	P234S GTB	P234S GTB + HA[a]
Resolution (Å)	20–1.65	20–1.55
Space group	$C222_1$	$C222_1$
a (Å)	52.8	52.8
b (Å)	149.0	149.7
c (Å)	79.8	79.8
R_{merge} (%)[b,c]	5.0 (25.6)	4.6 (23.2)
Completeness (%)[b]	95.5 (72.0)	97.2 (78.8)
Unique reflections	38,353	46,470
Resolution (Å)	20–1.65	20–1.55
R_{work} (%)[b,d]	19.7	20.7
R_{free} (%)[b,e]	21.7	21.8
Overall B ($Å^2$)	24.2	25.9
r.m.s.[f] bond (Å)	0.005	0.005
r.m.s. angle (°)	1.3	1.3

[a] HA, H-antigen.
[b] Values in parentheses represent high resolution shell.
[c] $R_{merge} = \Sigma|I_{obs} - I_{ave}|/\Sigma I_{ave}$.
[d] $R_{work} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$.
[e] 10% of reflections were omitted in R_{free} calculations.
[f] r.m.s., root mean square.

transformed into *Escherichia coli* BL21 (Novagen), and characterized by DNA sequence analysis.

The P234S GTB mutant was constructed by directed mutagenesis using PCR (15) and GTB (residues 63–354) plasmid DNA as a template. Two fragments were amplified with *Pfx* DNA polymerase (Invitrogen) by using the forward primer MIN2 together with SM01 (5′-GTA GAA GCT gga GTG CAG GGT ACC GAA CAG CGG-3′) and the reverse primer PCR3B with SM02 (5′-CTG CAC tcc AGC TTC TAC GGT TCC TCC CGT GAA G-3′). SM01 and SM02 were designed so that the two fragments overlap with each other and have a single codon substitution (CCC to TCC) at codon 234 (lowercase letters). The two overlapping fragments were isolated, annealed by 3′ extension using PCR, and amplified using the outside primers MIN2 and PCR3B. The resulting fragment containing the desired mutation was digested with *Eco*RI and *Hin*dIII (underlined in the previous section) and inserted into the corresponding sites of pCWΔlac.

Both wild type and mutant enzymes were expressed and purified from *E. coli* as described previously (16) except 50 mM MOPS buffer, pH 7.0, containing 5 mM UDP, 0.5 M NaCl, and 1 mM dithiothreitol were used for elution from the UDP-hexanolamine affinity column, and the enzyme was concentrated in a Centriplus 30 filtration unit (Amicon). The P234S mutant had expression levels of 40 mg/liter of culture compared with wild type GTB and GTA with expression levels of 50–100 mg/liter. Protein concentrations were estimated with a Bio-Rad protein assay procedure that is based on the method of Bradford using bovine gamma globulin as a protein standard (17). SDS-PAGE was used to confirm homogeneity.

Steady-state kinetic studies were carried out on all enzymes using a Sep-Pak radiochemical assay using the hydrophobic acceptor Fucα1–2Galβ-O$(CH_2)_7CH_3$ (6, 18). Assays were carried out at 37 °C in a total volume of 12 μl containing substrates and enzyme in 50 mM MOPS buffer, pH 7.0, with 20 mM $MnCl_2$ and 1 mg/ml bovine serum albumin. Seven different concentrations of the donor and acceptor were used, and the amount of substrate consumed was less than 15% to ensure linear initial reaction rates. Data were analyzed as described previously (6, 13) for a general two-substrate reaction using Equation 1, .0

$$v = \frac{V_{max}[A][B]}{K_{ia}K_B + K_B[A] + K_A[B] + [A][B]} \quad \text{(Eq. 1)}$$

where [*A*] and [*B*] represent the concentration of acceptor and donor, respectively. K_A is the Michaelis constant for acceptor, K_B is the Michaelis constant for donor, and K_{ia} is the dissociation constant for acceptor (Table I). The high K_m values for the P234S mutant with UDP-Gal precluded complete two-substrate analysis therefore the K_m for UDP-Gal was determined at 15 mM acceptor and the K_m for acceptor was determined at 500 μM UDP-Gal donor.

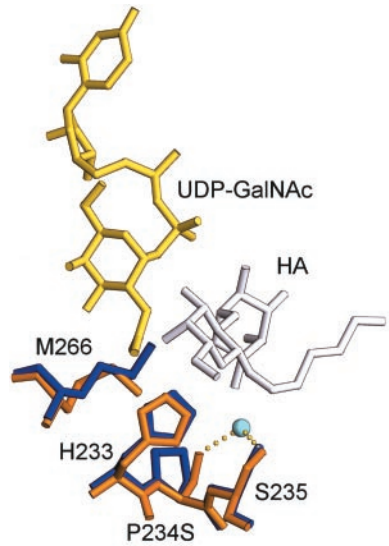

FIG. 1. **Superposition of the structures of native GTB (*blue*) and P234S (*orange*).** The substitution of P234S changes the conformation of Met-266, which allows the preferential binding of the blood group A donor UDP-GalNAc (modeled in *yellow*). A water molecule (*cyan*) observed to be hydrogen bonded to Ser-234 in the unliganded mutant structure must be displaced upon binding of the H-antigen acceptor (*white*), which accounts for the increased K_m for acceptor binding observed for the P234S mutant.

Crystallography—P234S was crystallized using conditions similar to the native GTB enzyme (4). Data were collected at beamline X8C at the National Synchrotron Light Source at Brookhaven National Laboratories under cryogenic conditions using a wavelength of 1.15 Å. Data sets for P234S both in the presence and absence of acceptor were solved using native GTB (Protein Data Bank accession code 1LZ7) as a starting model for rigid body refinement using the program CNS (19). Data collection and refinement statistics are presented in Table II.

RESULTS AND DISCUSSION

The reported structures of GTA and GTB are virtually identical except for the four critical amino acid residues (4). These enzymes represent a paradigm for structure-function relationships since changes in only four amino acids alter enzyme specificity. Of these residues, Arg/Gly-176 is far from the active site, Gly/Ser-235 appears in close vicinity to the acceptor binding site, and Leu/Met-266 and Gly/Ala-268 are both within the donor recognition pocket. The P234S mutation is striking in that this one mutation of a single residue in GTB results in the near abolishment of B donor (UDP-Gal) transfer and a large increase in A donor (UDP-GalNAc) transfer activity.

The results of kinetic analysis with UDP-Gal and UDP-GalNAc for wild type GTB, P234S GTB, and wild type GTA are presented in Table I. For the P234S GTB mutant, the k_{cat} for UDP-Gal has decreased from the 5.1 s^{-1} of wild type GTB to 0.24 s^{-1}. This is comparable to 0.088 s^{-1}, the k_{cat} of the wild

type GTA cross-reaction utilizing UDP-Gal as a donor. While the binding of UDP-Gal has been affected with an increase in the K_m for UDP-Gal from 27 μM of wild type GTB to 106 μM for the mutant, a much larger effect is seen on acceptor binding. There is a 50-fold increase in acceptor K_m compared with that of wild type GTB. For P234S, k_{cat} for UDP-GalNAc is 14.4 s^{-1}, comparable to the k_{cat} of wild type GTA (17.5 s^{-1}). The binding of UDP-GalNAc to the mutant has been affected somewhat with a K_m of 167 μM and K_{ib} of 49 μM. Acceptor binding to P234S has been dramatically affected with over a 300-fold increase in both K_m and K_{ia}.

The crystal structure of the mutant enzyme provides a structural basis for the reversal in enzyme donor preference and weakened acceptor binding. Fig. 1 shows the position of residue 234 in P234S and wild type GTB relative to two of the residues that normally serve to differentiate between the donor sugars. It is clear that in the mutant enzyme the replacement of proline by serine creates a void at the proline C-γ position. This atom normally makes van der Waals contact with Met-266 in wild type GTB, and its removal enables Met-266 to alter its side chain orientation. This conformational change provides sufficient space to accommodate the *N*-acetyl group of UDP-GalNAc and also creates an unfavorable void and lack of enzyme-substrate complementarity in the donor-binding pocket for UDP-Gal, analogous to the void observed in GTA recognition of UDP-Gal (4).

Acceptor binding is also affected in P234S. The movement of the Met-266 side chain results in the loss of its van der Waals contacts with the acceptor moiety. Also the mutation of P234S creates a new van der Waals contact between the serine hydroxyl group and the C-6 methyl group of the fucose ring of the acceptor. Significantly the mutation to serine causes the ordering of a water molecule about Ser-234, which must be displaced in order for acceptor to bind. The effect of these changes is to weaken the binding of acceptor for reaction with either UDP-Gal or UDP-GalNAc donors. In addition, the point mutation at residue 234 occurs in the immediate vicinity of the critical residue Ser-235 that is thought to act on the acceptor by forcing the aliphatic tail to adopt different conformations between GTA and GTB. We have previously speculated that this residue may be responsible for selecting the glycoconjugate structures possessing the H-antigen acceptor (4). It is therefore plausible that changes introduced at the neighboring residue 234 would influence acceptor binding and ultimately the behavior of the mutant. Further, in the production of GTA/GTB hybrid enzymes the placement of a Ser residue (as in GTB) instead of a Gly residue (as in GTA) at position 235 results in weaker acceptor binding (13). The presence of tandem serine residues may further destabilize acceptor binding via steric and local charge distribution effects.

There are two examples reported of glycosyltransferases with altered donor specificity for a single amino acid replacement. In β1–4-galactosyltransferase, mutation of tyrosine 289 to leucine gave an enzyme that is capable of transferring GalNAc at a rate comparable to Gal (20). The x-ray structure of the mutant showed that there was sufficient room for the enzyme to accommodate the *N*-acetyl group of the alternate donor. For β1–3-glucuronosyltransferase, mutation of histidine 308 to arginine produces an enzyme that can efficiently utilize UDP-Glc, UDP-Man, and UDP-GlcNAc as well as UDP-GlcA donor (21). However, unlike these examples of broadened donor specificity, the P234S mutation in glycosyltransferase B results in a complete reversal of donor saccharide recognition. The reason for the difference in behavior of cloned P234S, which produces predominantly blood group A structures, and the phenotype of red blood cells of individuals with this mutation that react with both anti-A and anti-B reagents is not clear. The cloned enzyme is a truncated, soluble form, whereas full-length membrane-associated enzyme biosynthesizes cell surface structures. Cell surface blood group structures are also found on glycoproteins, whereas a lipid-like acceptor was used to characterize the cloned enzyme.

This study highlights the dramatic effect of a single amino change on the catalytic reaction of a glycosyltransferase enzyme that ultimately affects blood group structure. We have shown that residues adjacent to the four conserved amino acids in GTA and GTB can dramatically influence the specificity of A and B transferases by inducing conformational changes that alter the specificity and kinetics of these enzymes. The enzymology and structure of other point mutations at Pro-234 of both GTA and GTB enzymes are currently under investigation.

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