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

Received for publication, November 25, 2002, and in revised form, January 10, 2003
Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M21202200

Sandra L. Marcus‡, Robert Polakowski‡, Nina O. L. Seto§, Eeva Leinala¶, Svetlana Borisova§, Antoine Blancher §, Francis Roubinet**, Stephen V. Evans†, and Monica M. Palcic‡ ‡‡

From the ‡Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2E2, Canada, §Institute for Biological Sciences, National Research Council of Canada, Ottawa K1A 0R6, Canada, †Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, K1H 8M5, Canada, ¶Laboratoire d’Immunogénétique Moléculaire, Université Paul Sabatier, Hôpital de Rangueil, 1 Avenue Jean Poulhes, 31059 Toulouse Cedex 9, France, and **Laboratoire d’immunohématologie, Etablissement Français du Sang, Avenue de Grande Bretagne, BP 3210, 31027 Toulouse, France

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 Fuc1–2Galβ-R (H) acceptor substrates (1, 2). The A-synthesizing α1–3 N-acetylgalactosaminyltransferase (GTA)1 EC 2.4.1.40, glycoprotein-fucosylgalactoside α-galactosyltransferase) utilizes UDP-GalNAc as its 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 GTA 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 AAAβ (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 65–354) 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 GGC TAT GGT TTA CCC GCA GCA GAA-3′) introduced an EcoRI site at the 5′ end, and the reverse primer PCRB3B (5′-ATA ATT AAG CT- TCTA TCG GGT AGC AAG AGC CTG GGT GTT TTT-3′) introduced a HindIII site in the 3′ end of GTA and GTB genes. The amplified genes were cloned into the expression vector pCWlac (pCW was a gift of F. W. Dahlquist (14), and pCWlac was a gift of W. Wakarchuk).
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'-GTG CAG GTG ACC GAA CAG CCG-3') and the reverse primer PCR3B with SM02 (5'-CGT CAC AGC TTC TCC GTC TCC TTC GAA CAG 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 EcoRI and *Hind*III (underlined in the previous section) and inserted into the corresponding sites of pCW3lac.

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 mM NaCl, and 1 mM diethiothreitol 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 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 type GTB and GTA with expression levels of 50 μM UDP-Gal donor.

**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 ([9, 10]) and the reverse primer PCR3B with SM02 (5'-CGT CAC AGC TTC TCC GTC TCC TTC GAA CAG 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 EcoRI and *Hind*III (underlined in the previous section) and inserted into the corresponding sites of pCW3lac.

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**Table I**

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

| Enzyme    | Resolution (Å) | Space group | a (Å) | b (Å) | c (Å) | Completeness (%)<sup>a</sup> | Re, %<sup>b</sup> | R<sub>work</sub> (%)<sup>b</sup> | R<sub>free</sub> (%)<sup>b</sup> | Overall B (Å<sup>2</sup>) | r.m.s./bond (Å<sup>2</sup>) | r.m.s. angle (°) |
|-----------|----------------|-------------|-------|-------|-------|-----------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| P234S     | 20–1.65        | C222<sub>1</sub> | 52.8  | 149.0 | 79.8  | 95.5 (72.0)                | 19.7          | 21.7          | 2.4            | 24.2           | 0.005          | 1.3            |
| GTB       | 20–1.55        | C222<sub>1</sub> | 52.8  | 149.7 | 79.8  | 97.2 (78.8)                | 20.7          | 21.8          | 2.5            | 25.9           | 0.005          | 1.3            |

<sup>a</sup> HA, H-antigen.

<sup>b</sup> Values in parentheses represent high resolution shell.

<sup>c</sup> R<sub>merge</sub> = Σ[I<sub>obs</sub> − I<sub>calc</sub>] / [I<sub>obs</sub>].

<sup>d</sup> R<sub>work</sub> = Σ[I<sub>obs</sub> − I<sub>calc</sub>] / [ΣI<sub>obs</sub>].

<sup>e</sup> 10% of reflections were omitted in R<sub>free</sub> calculations.

<sup>f</sup> r.m.s., root mean square.

**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<sub>m</sub> for acceptor binding observed for the P234S mutant.

Michaels constant for donor, and K<sub>m</sub> is the dissociation constant for acceptor (Table I). The high K<sub>m</sub> values for the P234S mutant with UDP-Gal precluded complete two-substrate analysis therefore the K<sub>m</sub> for UDP-Gal was determined at 15 mM acceptor and the K<sub>m</sub> for acceptor was determined at 500 μM UDP-Gal donor.

Crystallography—P234S was crystalized 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<sub>cat</sub> for UDP-Gal has decreased from the 0.24 s<sup>−1</sup> of wild type GTB to 0.24 s<sup>−1</sup>. This is comparable to 0.088 s<sup>−1</sup>, the k<sub>cat</sub> of the wild...
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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 234 in P234S and wild type GTB relative to two of the residues that normally serve to differentiate between the donor sugars.

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 $\mu$m of wild type GTB to 106 $\mu$m 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, $K_m$ 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 $\mu$m and $k_{cat}$ of 49 $\mu$m. Acceptor binding to P234S has been dramatically affected with over a 300-fold increase in both $K_m$ and $k_{cat}$.

The crystal structure of the mutant enzyme provides a structural basis for the reversal in enzyme donor preference and weakened acceptor binding. It is clear that in the mutant enzyme the replacement of proline 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 234 in P234S and wild type GTB relative to two of the residues that normally serve to differentiate between the donor sugars.

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Acknowledgments—Assistance with DNA sequencing was provided by the Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta. We thank F. W. Dahlquist for the pcW vector, W. Wakarchuk for the pCW Lac vector, and O. Hindsgaul for the Fuc1-2Galβ1-3Galβ1-4Glcα1 acceptor.