Sequential Interchange of Four Amino Acids from Blood Group B to Blood Group A Glycosyltransferase Boosts Catalytic Activity and Progressively Modifies Substrate Recognition in Human Recombinant Enzymes

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The human blood group A and B glycosyltransferase enzymes are highly homologous and the alteration of four critical amino acid residues (Arg-176 → Gly, Gly-235 → Ser, Leu-266 → Met, and Gly-268 → Ala) is sufficient to change the enzyme specificity from a blood group A to a blood group B glycosyltransferase. To carry out a systematic study, a synthetic gene strategy was employed to obtain their genes and to allow facile mutagenesis. Soluble forms of a recombinant glycosyltransferase A and a set of hybrid glycosyltransferase A and B mutants were expressed in Escherichia coli in high yields, which allowed them to be kinetically characterized extensively for the first time. A functional hybrid A/B mutant enzyme was able to catalyze both A and B reactions, with the \( k_{\text{cat}} \) being 5-fold higher for the A donor. Surprisingly, even a single amino acid replacement in glycosyltransferase A with the corresponding residue from glycosyltransferase B (Arg-176 → Gly) produced enzymes with glycosyltransferase A activity only, but with very large (11-fold) increases in the \( k_{\text{cat}} \) and increased specificity. The increases observed in \( k_{\text{cat}} \) are among the largest obtained for a single amino acid change and are advantageous for the preparative scale synthesis of blood group antigens.

Complex carbohydrates are becoming increasingly important for the key roles they play in cell signaling, molecular recognition, and many other biological processes (1). Specific glycosyltransferase enzymes are responsible for the synthesis of different disaccharide linkages in complex oligosaccharides by transferring a single monosaccharide unit from a nucleotide donor to the hydroxyl group of an acceptor saccharide. Cloned glycosyltransferase enzymes are powerful new tools in small to large scale synthesis of therapeutically significant oligosaccharides (2, 3). Glycosyltransferases are highly specific for the donor and acceptor substrates and have been grouped into families according to the type of sugar they transfer (4, 5). With a few exceptions, a different glycosyltransferase is required to synthesize each different glycosidic linkage, and it is estimated that there are over 100 different glycosyltransferases to account for all the documented oligosaccharides (6). Mammalian oligosaccharide chains are largely composed of nine monosaccharide units, which are transferred by glycosyltransferases to different acceptor structures, but there have been no extensive sequence similarities found among different glycosyltransferases. However, there are common structural features among mammalian transferases as they are all classified as type II integral membrane proteins, with a short amino-terminal cytoplasmic tail, a membrane-anchoring domain, a short proteolytically sensitive stem region, and a large catalytic domain that includes the carboxyl terminus (6).

The most homologous glycosyltransferase sequences are the A and B glycosyltransferases of the human ABO blood group system (7). The histo-blood group ABO(H) antigens are defined carbohydrate determinants found on the surface of red blood cells and are largely responsible for failure of mismatched blood transfusions. These ABO carbohydrate antigens occur on other cell types and are important in cell development, cell differentiation, and oncogenesis (8-10). Blood group A individuals express \( (1-3)\text{N-acetylgalactosaminyltransferase} \) (GTA), which catalyzes the transfer of GalNAc from the donor UDP-GalNAc to the \((\text{O})\text{H}-\text{precursor structure} \) Fuc\( \text{(1-2)Gal} \text{-} \text{OR} \) (7, 11) to give the A determinant Gal\( \text{N} \text{a} \text{c}(1-3)\text{Fuc}(1-2)\text{Gal}-\text{OR} \). Blood group B individuals express \( (1-3)\text{galactosyltransferase} \) (GTB), which uses the same \((\text{O})\text{H}-\text{structure} \) but catalyzes the transfer of Gal from UDP-Gal to make the B determinant Gal\( \text{a} \text{c}(1-3)\text{Fuc}(1-2)\text{Gal} \text{-} \text{OR} \) (7, 11) (Fig. 1). Blood group O individuals do not express either enzyme, and AB individuals express both (7).

The cDNA sequences of the GTA and GTB genes show that they are highly homologous and that alteration of only four critical amino acid residues (Arg-176 → Gly, Gly-235 → Ser, Leu-266 → Met, and Gly-268 → Ala) is sufficient to change the enzyme specificity from a blood group A to a blood group B enzyme (7, 12, 13). GTA and GTB transfer to the same \((\text{O})\text{H}-\text{structure} \) but catalyze the transfer of Gal from UDP-Gal to make the B determinant Gal\( \text{a} \text{c}(1-3)\text{Fuc}(1-2)\text{Gal} \text{-} \text{OR} \) (7, 11) (Fig. 1). Blood group O individuals do not express either enzyme, and AB individuals express both (7).

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** The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) Y11891.

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‡ The abbreviations used are: GTA, glycosyltransferase A encoded by the blood group A gene (EC 2.4.1.40); GTB, glycosyltransferase B encoded by the blood group B gene (EC 2.4.1.37).

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266 and 268 were crucial for the nucleotide donor specificity of the enzyme (13). However, the authors note that transient expression in HeLa cells may not be the true representation of in vivo conditions and, moreover, does not provide any mechanistic information about the enzymes. Therefore, detailed kinetic analyses of purified enzymes are necessary to determine the exact effects of the residue changes on substrate recognition and catalytic activity (14).

There is a need for economical production of oligosaccharides, especially for the purpose of studying their biochemical function and assessing their potential in therapeutics or as diagnostic tools. Enzymatic oligosaccharide synthesis using glycosyltransferases proceeds regio- and stereoselectively and is less laborious and less costly compared with chemical synthesis. The limitation to the use of glycosyltransferases as synthetic tools is their scarcity from natural sources. The expression of recombinant glycosyltransferases in bacterial systems makes milligram scale synthesis by the enzymatic approach more practical (15).

Previously, we described the expression in Escherichia coli of a soluble recombinant GTB and the kinetic characterization of its acceptor specificity using synthetic analogs of the H disaccharide acceptor (16). In this paper, we report the chemical synthesis and expression of DNA encoding GTA and a series of mutants. The sequence of the synthetic DNA was designed to contain codons preferred for optimal expression in E. coli (17). Gene design, extinction coefficients at 280 nm, and molecular weights of proteins were determined using the Wisconsin Sequence Analysis Software (Genetics Computer Group, Inc.). Fabello protease inhibitor (Boehringer Mannheim); UDP, UDP-GalNAc, and UDP-Gal (Sigma); UDP-[6-3H]GalNAc (10 Ci/mmol) and UDP-[6-3H]Gal (15 Ci/mmol) (American Radiolabeled Chemicals); Sep-Pak C18 reverse phase cartridges (Waters); EcoLite (+) liquid scintillation mixture (ICN); and Centriloc-10 protein concentrators (Amicon) were purchased commercially.

**Construction of the Synthetic Glycosyltransferase Wild-type A Gene and Mutants**—The ompA-GTA gene (1034 base pairs) was designed and synthesized as described previously for GTB (16). The mutant proteins were named based on their amino acid status at the four locations where GTA and GTB differ. The synthetic wild-type GTA (designated AAAA) and GTB (BBBB) genes were designed with unique restriction sites throughout the gene to facilitate mutagenesis. Glycosyltransferase mutants BAAA, BBAA, and BBBA were synthesized by digesting the GTB gene with KpnI/SphI and ligating in oligonucleotides to form the desired gene sequence. The DNA sequences of all four genes were confirmed on both strands.

**Expression in E. coli**—Plasmids harboring the wild-type ompA-GTA, and the mutant BAAA, BBAA, and BBBA genes were used to transform E. coli TG-1 cells. To produce the recombinant glycosyltransferase proteins, E. coli strains containing the 5 different plasmids were grown at 30 °C in M-9 minimal medium supplemented with 0.4% casamino acids and 100 μg/ml ampicillin. After 18–24 h the cultures were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and made up to 1 × TB (12 g of trypthone, 24 g of yeast extract, and 4 ml of glycerol/liter of culture), a rich growth medium that maximizes cell density. The cultures were harvested 48–64 h later, and periplasmic extracts were prepared by a one-step osmotic shock. Cells from 2 liters of culture were resuspended thoroughly in 60 ml of ice-cold shock buffer (20 mM Tris-HCl, 1 mM EDTA (pH 7.2), 1 mM dithiothreitol, 0.5 mM NaCl) and dialyzed extensively against the same buffer. The extracts were made up to 2 mM MnCl2, spun in a microcentrifuge for 5 min to remove any insoluble material and loaded onto a 10-ml UDP-hexanolamine-Sepharose column at 0.4 ml/h with column buffer containing 2 mM MnCl2 and washed extensively until the A280 was at background levels. GTA was eluted with column buffer containing 20 mM MnCl2 and 2 mM UDP (18). Since 2 mM UDP has a strong

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**EXPERIMENTAL PROCEDURES**

**Materials and General Techniques**—Oligodeoxyribonucleotides were synthesized using model 380A and 394 DNA/RNA synthesizers (Applied Biosystems). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs and Life Technologies, Inc. All general molecular biology procedures were performed according to standard procedures (17). Gene design, extinction coefficients at 280 nm, and molecular weights of proteins were determined using the Wisconsin Sequence Analysis Software (Genetics Computer Group, Inc.). Fabello protease inhibitor (Boehringer Mannheim); UDP, UDP-GalNAc, and UDP-Gal (Sigma); UDP-[6-3H]GalNAc (10 Ci/mmol) and UDP-[6-3H]Gal (15 Ci/mmol) (American Radiolabeled Chemicals); Sep-Pak C18 reverse phase cartridges (Waters); EcoLite (+) liquid scintillation mixture (ICN); and Centriloc-10 protein concentrators (Amicon) were purchased commercially.

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**FIG. 1. Biosynthesis of the blood group A and B antigens from the O(H) precursor.** The human GTA enzyme catalyzes the transfer of GalNAc from the donor UDP-GalNAc (b) to the (O/H)-precursor structure Fuc(1–2)-Galβ-OR (a) to give the B antigen Fuc(1–2)(GalNAc1–3)Galβ-OR (c). The human GTB enzyme catalyzes the transfer of Gal from UDP-Gal (d) to the same (O/H)-precursor (a) to make the B antigen Fuc(1–2)(Galα1–3)Galβ-OR (e). R = carbohydrate residue attached to a glycoprotein or glycolipid.
absorbance at 380 nm, the purified protein peak in the column fractions was determined by taking 20 μl of each fraction and measuring the protein concentration using the Bio-Rad Micro Protein Assay. Pooled column fractions (3–4 ml) containing pure GTA enzyme were concentrated to 600–1000 μl volumes using Centriplus-10 units, dialyzed extensively against 20 mM sodium cacodylate (pH 6.8) containing 1 mM dithiothreitol before activity assays or use in enzymatic synthesis. The protein concentration of all the enzymes was determined using the Bradford method with bovine γ globulin as a standard (Bio-Rad).

Kinetic Properties of Transferase A and Transferase AB Mutants—For the kinetic analysis of GTA and GTA activity, Sep-Pak radiochemical reverse phase cartridges were used with the hydrophobic acceptor Fucose-1-2Galp-O-(CH2)7CH3, essentially as described previously (19–21). Reactions to measure glycosyltransferase A activity were incubated at 37 °C for 20–60 min and carried out in a 33-mM sodium cacodylate buffer (pH 7.0), 20 mM MnCl2, 0.2 mM UDP-[6-3H]GalNAc, and 0.0005–0.2 μl of purified GTA or mutant enzymes. Initial rate conditions were linear under these conditions, where no more than 10–15% of substrate was consumed. Six different concentrations of donor and acceptor were employed generally covering the range from about 0.3 up to 8 KM for the substrate. Data were analyzed for a general two-substrate system using Equation 1.

\[
v = \frac{v_{\text{max}} [A][B]}{K_{\text{M}}[A] + K_{\text{M}}[B] + [A][B]} \tag{Eq. 1}
\]

The kinetic parameters v_{\text{max}}, K_{\text{M}}, and v_{\text{max}}/K_{\text{M}} were derived from the best fit of the Michaelis-Menten equation using unweighted nonlinear regression with the SigmaPlot 4.1 program. Plots of the values of v_{\text{max}}, KM, and v_{\text{max}}/KM versus 1/[donor] of the six primary plots were all linear and gave K_{\text{M}} (the apparent Michaelis constant for the acceptor), v_{\text{max}} and K_{\text{M}} (the apparent Michaelis constant that is independent of the concentration of donor and reflects the effect that the binding of one substrate has on the binding of the other substrate). Plots of the values from the six primary plots of v_{\text{max}}, K_{\text{M}}, and v_{\text{max}}/KM versus 1/[donor] were all linear and gave K_{\text{M}} (the apparent Michaelis-Menten constant for donor) and v_{\text{max}}. It was noted that the extent to which donor affects the K_{\text{M}} for the acceptor is the same as the effect of acceptor on donor K_{\text{M}}, that is K_{\text{M}} = K_{\text{M}} (where K_{\text{M}} is the apparent Michaelis constant for donor that is independent of the concentration of acceptor).

The reactions to measure glycosyltransferase B activity were incubated at 37 °C for 30–120 min and carried out in 35 mM total volume with 50 mM sodium cacodylate buffer (pH 7.0), 20 mM MnCl2, 0.2 mM UDP-[6-3H]Gal, and 0.05–4.0 μl of purified GTA or mutant enzymes. Six different concentrations of donor and acceptor were employed and analysis carried out as described for the glycosyltransferase A activity.

One milliliter of activity is defined as the amount that catalyzes the conversion of 1 nmol of sugar transferred/min. The catalytic constant or turnover number, k_{cat}, is the maximum number of substrate molecules converted to product per active site per unit time, obtained from v_{\text{max}}/EI. The catalytic constant or turnover number, k_{cat}, is the maximum number of substrate molecules converted to product per active site per unit time, obtained from v_{\text{max}}/EI.

The Preparative Scale Enzymatic Synthesis of the Blood Group A and B Trisaccharides—The preparative scale enzymatic synthesis of 6.0 mg of blood group A trisaccharide was carried out in a reaction mixture, which contained 6.0 mg (13.7 μmol) of precursor H oligosaccharide Fucose-1-2Galp-O-(CH2)7CH3, 45 μM (22.4 μmol) of UDP-GalNAc, 100 mM sodium cacodylate (pH 7.0), 5 mM MnCl2, 1 mg/ml bovine serum albumin, and 50 milliliters of recombinant GTA enzyme in a total volume of 0.8–1.0 ml. The reaction was incubated overnight at 37 °C with GTA enzyme and UDP-GalNAc being added in two aliquots. The progress of the reaction was monitored by thin layer chromatography with the product purified upon completion using two Sep-Pak C18 reverse phase cartridges as described previously (15). BBBA was used in reactions with either UDP-GalNAc or UDP-Gal, for the preparative scale synthesis of both A and B trisaccharide products. The products were characterized by H NMR spectroscopy on a Varian Unity 500 spectrophotometer at 500 Mhz.

RESULTS

Gene Design and Synthesis of GTA and Mutants—The amino acid sequence of membrane-bound human GTA deduced from cDNA (7, 12) was used to redesign 1034-base pair synthetic GTA gene to contain E. coli preferred codons to maximize gene expression and unique restriction sites to facilitate mutagenesis (Fig. 2). The putative transmembrane domain (amino acids 1–53) was replaced with the bacterial ompA secretory signal to target GTA into the periplasm as a soluble protein. A DNA segment encoding the c-myc epitope and an affinity purification tail consisting of five histidine residues was added to the carboxyl terminus of the GTA gene in front of two termination codons (Fig. 2). GTA (AAAAA) and GTB (BBBB) differ by only four essential amino acids (residues 176, 235, 266, and 268), and the mutant proteins were named based on their amino acid status at the four locations. Only the unique restriction enzymes sites in GTA are shown in Fig. 2, but other restriction sites that differ between the GTA and GTB sequences are also present.

Expression and Purification—The soluble form of GTA and its mutants were purified from the periplasm to apparent homogeneity and characterized as described previously for GTB (16). The mutations in BAAA (Arg-176 → Gly) and BBBA (Arg-176 → Gly and Gly-235 → Ser) enhanced total expression levels of the enzymes compared with that of GTA and BBBA (Arg-176 → Gly, Gly-235 → Ser, and Leu-266 → Met), with a corresponding increased secretion of soluble recombinant protein into the periplasm (data not shown). In terms of enzymatic activity, the average yield of pure BAAA was 19 times higher than for GTA, due to being more highly expressed in E. coli and having increased catalytic activity.

Kinetic Characterization—Using a suitable range of six acceptor concentrations and six donor concentrations and then measuring the rate with all possible combinations of these substrate concentrations (i.e. 6 × 6 grid), the kinetic constants K_{M}, k_{cat}, k_{cat}/K_{M}, and k_{cat}/K_{M} were determined. In comparing wild-type glycosyltransferase A and B enzymes with each using their preferred donors, the k_{cat} (maximum rate possible when both acceptor and donor is saturated) is similar. However, glycosyltransferase A has a K_{M} for its preferred donor UDP-GalNAc that is 5.5 times lower than the K_{M} of the B enzyme for its preferred donor UDP-Gal, which results in a higher specificity constant (k_{cat}/K_{M}) for the A enzyme (Table 1).

Wild-type GTA and mutant BAAA and BBBA enzymes showed predominantly glycosyltransferase A activity (UDP-GalNAc) only. It was possible to kinetically characterize the small amounts of glycosyltransferase B activity (UDP-Gal) that was detectable in these glycosyltransferase A enzymes by using comparatively high concentrations of the recombinant enzymes in the assays for B activity. Kinetic analysis of the GTA enzyme using the B donor UDP-Gal shows that the wild-type A enzyme does not transfer the B donor (UDP-Gal) efficiently, largely because of a much lower k_{cat}. Similarly, the decreased k_{cat} also accounts for the lack of A activity (transferring of UDP-GalNAc) displayed by the B enzyme (Table 1). K_{M} and K_{P} for GTA was similar for both UDP-GalNAc and UDP-Gal. Thus, the difference in the donor specificity of the glycosyltransferase A and B enzymes is largely due to a difference in k_{cat} rather than K_{M} values.

The single mutant BAAA (Arg-176 → Gly) showed a very large 11-fold increase in k_{cat} and a 4-fold increase in the specificity constant k_{cat}/K_{p} compared to wild-type A enzyme (Table 1). Although the single amino acid change in BAAA was due to the substitution of residue Gly-176 from the B enzyme for Arg in the A enzyme, this enzyme did not show any additional ability to catalyze the B reaction, but instead showed an increased ability to catalyze the A reaction.

A further additional amino acid change in the double mutant BAAA (Arg-176 → Gly, Gly-235→ Ser) results in an enzyme with a 5-fold increase in k_{cat} compared with the wild-type A enzyme. In contrast to BAAA, the specificity constant k_{cat}/K_{P} is only increased slightly. Despite two amino acid substitutions with the corresponding residues in the B enzyme, BAAA is not able to catalyze either the A or B reaction with the same specificity as either the wild-type A or B enzyme.
BBBA (Arg-176 → Gly, Gly-235 → Ser, and Leu-266 → Met) has the ability to catalyze both the A and B reactions and is a functional hybrid A/B enzyme. With UDP-GalNAc, BBBA has a 2-fold increase in $k_{\text{cat}}$, but also a larger $K_A$ and $K_B$, which results in lower specificity constants compared with wild-type (Table I).

Enzymatic Synthesis of Blood Group A and B Trisaccharides—GTA and BAAA were used in preparative scale synthesis of the blood group A trisaccharide. Recombinant GTA (50 milliunits) was used in the preparative scale synthesis of 6 mg of the blood group A trisaccharide from the H disaccharide acceptor Fuc$_a$(1–2)Gal$_b$-$O$-(CH$_2$)$_7$CH$_3$. The reaction was judged to be 50% complete after heating at 37 °C. The reaction was left for another 16–20 h, which resulted in 100% conversion to the A trisaccharide product. Aliquots of the reaction mixture were removed at different times, and the enzyme activity was found to be relatively stable at 37 °C for 2 days. Under the preparative scale synthesis conditions, the mutant BAAA has the advantage of having a higher specific activity, $k_{\text{cat}}$, and $k_{\text{cat}}/K_m$ compared to wild-type GTA. The use of the glycosyltransferase AB mutant BBBA with either UDP-GalNAc or UDP-Gal donors in enzymatic synthesis also resulted in 100% conversion to the blood group A or B trisaccharide determinants.

The structures of the synthetic A (Fuc$_a$(1–2)[GalNAc$_a$(1–3)]Gal$_a$(1–3)Gal$_b$-$O$-(CH$_2$)$_7$CH$_3$) and B trisaccharide products (Fuc$_a$(1–2)[Gal$_a$(1–3)Gal$_b$-$O$-(CH$_2$)$_7$CH$_3$]) were confirmed by 1H NMR. In the A trisaccharide product, the terminal GalNAc is linked to Gal in an $\alpha 1\rightarrow 3$ linkage. The resonance of the $\alpha$-anomeric H of GalNAc was at $d_5.32$ ppm ($J_{1,2}$ 3.8 Hz) and that of the $\alpha$-anomeric H of Fuc was at $d_5.17$ ppm ($J_{1,2}$ 3.8 Hz). For the B trisaccharide product, the $\alpha$-anomeric H of the terminal Gal linked to the inner Gal in an $\alpha 1\rightarrow 3$ linkage results in the H-1 resonance at $d_5.30$ ppm ($J_{1,2}$ 3.8 Hz) and the $\alpha$-anomeric H of Fuc at $d_5.24$ ppm ($J_{1,2}$ 2.8 Hz) (22) (data not shown).

**DISCUSSION**

In this paper, we describe the chemical synthesis and expression of functional human GTA genes and their mutants using the synthetic gene strategy. Purified recombinant GTA and its mutant BAAA from 1 liter of E. coli had enzymatic activity equivalent to that recoverable from 0.2 and 32 million liters of human blood group A sera (19–21, 23–25), without the need for laborious multistep chromatographic purification (23, 26). It is interesting to note that the improved yield of active blood group A and B glycosyltransferases are boxed. The nucleotide numbers are on the right-hand side of the figure. Only unique restriction enzyme sites are shown.

**FIG. 2.** Amino acid and nucleotide sequence of synthetic DNA used to encode the human glycosyltransferase A. The residue numbers appear on top of the amino acid sequence and are numbered according to the membrane-anchored form. The amino acid sequence begins with the ompA secretion leader (bold italics) fused to the catalytic domain of GTA, which starts at residue 54. A-c-myc peptide (italic) and a His affinity tail are added to the carboxyl end. The four essential amino acids (residues 176, 235, 266, and 288) that differ between the blood group A and B glycosyltransferases are boxed. The nucleotide numbers are on the right-hand side of the figure. Only unique restriction enzyme sites are shown.
A enzyme was accomplished without significantly altering the \( K_a \) for the acceptor, and, surprisingly, mutant BAAA showed 11-fold higher A activity resulting from a single amino acid change of Arg-176 \( \rightarrow \) Gly. The availability of large quantities of the recombinant enzymes has made possible the first systematic kinetic characterization of the human blood group A and B enzymes. This allowed for an exact comparison of steady-state kinetic parameters of wild-type A enzyme, the mutants, and the B enzyme for both UDP-GalNAc and UDP-Gal donors.

Although GTA and GTB differ by only four amino acids and use the same acceptor, the alteration of these amino acids affects the binding of both the UDP-sugar donor and the acceptor. Kinetic characterization revealed that alteration of even one of the four amino acids that differ between these two enzymes may affect the \( K_a \) of both the acceptor and the donor substrates. The \( K_a \) for the acceptor also differed depending on whether UDP-GalNAc or UDP-Gal was used as the donor (Table I). GTB has a \( K_a \) for the donor UDP-Gal higher than the \( K_a \) that GTA has for the donor UDP-GalNAc, which suggests that the binding of GTB to UDP-Gal is weaker than the binding of GTA to UDP-GalNAc, with the corresponding \( k_{cat} \) values being similar.

The major difference in the utilization of the alternate donors by the glycosyltransferase A and B enzymes is largely due to a difference in \( k_{cat} \) rather than \( K_a \). For GTA the \( k_{cat} \) for UDP-Gal is only 0.4% that of UDP-GalNAc. GTA had an apparent \( K_m \) for UDP-Gal similar to the \( K_m \) for UDP-GalNAc, indicating that the enzyme was able to bind both donors, but the significantly decreased \( k_{cat} \) for UDP-Gal showed that it was not able to readily catalyze the transfer. Similarly, the lack of GTB activity observed for BAAA (Arg-176 \( \rightarrow \) Gly) and BAAA (Arg-176 \( \rightarrow \) Gly, Gly-235 \( \rightarrow \) Ser) was not necessarily due to an increased \( K_m \), but greatly increased in BAAA for both the acceptor and the donor. Generally, irrespective of the mechanism, the major factor governing specificity is the stability of the enzyme-bound transition state, which exists during the conversion of the enzyme-bound substrate to product. It is possible that the removal of the Arg side chain at position 176 in mutant BAAA stabilizes the transition state with the donor UDP-GalNAc more effectively, resulting in the higher specificity observed.

Our results suggest that alteration of the four critical amino acids that differ between GTA and GTB affected the kinetic constants of both the acceptor and the donor, possibly due to topologically close binding of the donor and acceptor. Mutations at residue 176 in BAAA appear to have little effect on the binding of the acceptor, but do affect the enzyme turnover, as this mutant showed a very large increase in \( k_{cat} \). Mutations at residues 176 and 235 in BAAA showed a 5-fold increase in \( k_{cat} \) and weaker acceptor binding, and the binding of the donor was not greatly affected. These results suggest a role in enzyme turnover for residue 176. Residue 235 could affect the binding of the acceptor, and segments around residues 266 and 268 could be most critical for binding of the nucleotide sugar donor.

Recombinant GTA and BAAA enzymes were used in the preparative scale synthesis of the blood group A tri saccharide, with BAAA having the advantage of having a 11-fold higher \( k_{cat} \) compared with wild-type GTA. A further systematic mutagenesis of GTA and GTB may lead to glycosyltransferase enzymes with even more significant increases in catalytic activity.

Very little is known about structure/function relationships in glycosyltransferases. With a few exceptions, there is very little nucleotide or amino acid sequence similarity among glyco syltransferases even if they use the same acceptor or nucleotide donors. There are no x-ray crystal structures of mammalian glycosyltransferases known, and the large scale fermentation of *E. coli* to produce unglycosylated glycosyltransferases may produce enough protein for crystallization and subsequent three-dimensional structure determination. The availability of recombinant GTA, GTB, and their mutants can form the framework for further genetic engineering of new glycosyltransferases, allowing investigation into structure and function re-
relationships to produce new glycosyltransferase enzymes as synthetic tools and diagnostic reagents.

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