Structural Effects of Naturally Occurring Human Blood Group B Galactosyltransferase Mutations Adjacent to the DXD Motif*

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Mattias Persson‡, James A. Letts‡, Bahram Hosseini-Maaf§, Svetlana N. Borisova†, Monica M. Palcic‡, Stephen V. Evans‡,¶ and Martin L. Olsson‡

From the ‡Carlsberg Laboratory, Gamle Carlsberg Vej 10, 2500 Valby, Copenhagen, Denmark, the §Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada, and the ¶Department of Laboratory Medicine and Transfusion Medicine, Department of Laboratory Medicine, Lund University Hospital, Lund University, SE-22185 Lund, Sweden

Human blood group A and B antigens are produced by two closely related glycosyltransferase enzymes. An N-acetylgalactosaminyltransferase (GTA) utilizes UDP-GalNAc to extend H antigen acceptors (Fuc(1–2)Galβ-OR) producing A antigens, whereas a galactosyltransferase (GTB) utilizes UDP-Gal as a donor to extend H structures producing B antigens. GTA and GTB have a characteristic 211DVD213 motif that coordinates to a Mn2+ ion shown to be critical in donor binding and catalysis. Three GTB mutants, M214V, M214T, and M214R, with alterations adjacent to the 211DVD213 motif have been identified in blood banking laboratories. From serological phenotyping, individuals with the M214R mutation show the B variant expressing very low levels of B antigens, whereas those with M214T and M214V mutations give rise to AweakB phenotypes. The crystal structure of M214R showed that DVD motif coordination to Mn2+ was disrupted by Arg-214 causing displacement of the metal by a water molecule. Kinetic characterizations of the M214T and M214V mutants revealed they both had GTA and GTB activity consistent with the serology. The crystal structure of the M214T mutant showed no change in DVD coordination to Mn2+. Instead a critical residue, Met-266, which is responsible for determining donor specificity, had adopted alternate conformations. The conformation with the highest occupancy opens up the active site to accommodate the larger A-specific donor, UDP-GalNAc, accounting for the dual specificity.

The ABH histo-blood group antigens are inherited carbohydrate structures on erythrocytes and other cells. Individuals with blood group A carry at least one allele encoding a 3-α-N-acetylgalactosaminyltransferase (GTA), whereas those with blood group B have at least one allele giving rise to an 3-α-galactosyltransferase (GTB). GTA utilizes UDP-GalNAc as its donor substrate and the Fuc(1–2)Galβ-OR structure as an acceptor, whereas a galactosyltransferase (GTB) utilizes UDP-Gal as a donor to extend H structures producing B antigens, GTA and GTB are classified as metal-dependent, family 6 enzymes. GTA and GTB are highly homologous enzymes differing in only 4 of 354 amino acids, where GTA and GTB are highly homologous enzymes differing in only 4 of 354 amino acids, Arg/Gly-176, Gly/Ser-235, Leu/Met-266, and Gly/Ala-268. Therefore, they constitute a useful model system for glycosyltransferase structure-function studies.

Kinetic studies demonstrated that residue Leu/Met-266 is critical for differentiating between the UDP-GalNAc donor for GTA and the UDP-Gal donor of GTB. The crystal structure of the M214T mutant showed no change in DVD coordination to Mn2+. Instead a critical residue, Met-266, which is responsible for determining donor specificity, had adopted alternate conformations. The conformation with the highest occupancy opens up the active site to accommodate the larger A-specific donor, UDP-GalNAc, accounting for the dual specificity.
via the metal (4, 7, 9). In GTA and GTB the motif is defined by residues 211DVD213. The Asp residues coordinate a Mn$^{2+}$ ion, which in turn coordinates to the phosphates of the UDP-sugar donor. Both aspartate residues bind to the Mn$^{2+}$ including a bidentate coordinate bond by Asp-213 (4).

Mutagenesis studies have shown that the DXD motifs in GT-A fold glycosyltransferases are not functionally equivalent. In some cases the DXD motif is important for enzyme activity but not for binding of the sugar nucleotide (10–12), whereas in other instances it is important for both nucleotide binding and activity (13, 14). The only exception reported to date is leukocyte type 2 6-N-acetylglucosaminyltransferase, which displays the GT-A fold type but lacks a DXD metal ion binding site. Instead, the role of the metal ion is served by two basic amino acids, Arg-378 and Lys-401 (15).

The subgroups of the ABO blood group system were originally characterized based on their serological behavior in red blood cells and plasma typing. Subsequently, individuals with anomalous blood grouping results were further categorized following DNA sequencing of their ABO genes (16–18). These subgroups include variants of blood group B, in which individuals with mutations adjacent to the 211DVD213 motif, M214V or M214T, have been classified as AweakB subgroups (19). The erythrocytes in these cases react weakly with some anti-A reagents as well as with all anti-B sera, suggesting that the blood group glycosyltransferase exhibits GalNAc as well as Gal transferase activity. A third mutation, M214R, has been classified as a B$_d$ subgroup (20). The amount of B determinant on these erythrocytes is very low, so that they do not agglutinate when incubated with monoclonal or polyclonal anti-B reagents. However, upon incubation with polyclonal blood grouping reagents, these cells will adsorb and subsequently elute measurable quantities of anti-B. Also, these individuals typically lack anti-B in their sera.

Here we report the expression, kinetic analysis, structure determination by single crystal x-ray diffraction, and structure-function correlations of variant GTB enzymes corresponding to the naturally occurring mutants, M214T, M214R, and M214V, which all have mutations adjacent to the DXD motif. Each of these constructs is based on the same synthetic gene that has been previously used for high level expression of soluble GTB in Escherichia coli (3, 21).

EXPERIMENTAL PROCEDURES

Materials—Oligodeoxynucleotides, UDP, UDP-Gal, and UDP-GalNAc were obtained from Sigma. The acceptor substrate (Fuc(1-2)Galβ(CH$_2$)$_2$CH$_3$) was a kind gift from Prof. Ole Hindsaul. Sep-Pak C$_{18}$ reverse-phase cartridges (Waters), UDP-[6-3$^3$H]GalNAc (50µCi/µmol) and UDP-[6-3$^3$H]Gal(250µCi/µmol) (American Radiolabeled Chemi-
Site-directed Mutagenesis—A QuikChange II XL site-directed mutagenesis kit (Stratagene) was used to generate mutants. The PCR step was performed in a thermocycler Peltier Thermal PTC-200 (Bio-Rad). The plasmid pCWΔlac vector harboring the T641G mutation was found in the immediate vicinity of the 211DVD213 motif were prepared. These enzymes were all cloned as soluble truncated forms of the catalytic domain (amino acids 63–354) and expressed in E. coli. The purification of the three constructs by ion-exchange chromatography and affinity chromatography on a UDP-hexanolamine resin was straightforward in all cases. The yields of final purified protein were 25 mg/liter for the M214T mutant, 53 mg/liter for the M214V mutant, and 25 mg/liter for the M214R enzyme.

Kinetics—Kinetic constants were determined for each enzyme at a high concentration of the alternate substrate (Table 1). For the M214R mutants, the $k_{cat}$ for both UDP-Gal (0.0042 s$^{-1}$) and UDP-GalNAc (0.00016 s$^{-1}$) was decreased substantially as compared with the wild type GTB, which exhibits a $k_{cat}$ of 5.1 s$^{-1}$ for UDP-Gal and 0.42 s$^{-1}$ for UDP-GalNAc (Table 1). The binding of UDP-Gal and acceptor was also affected for this mutant with an increase in $K_M$ for UDP-Gal from 27 μM for the wild type enzyme to 298 μM for the mutant. The acceptor $K_M$ had also been increased from 88 to 167 μM. Individuals with this mutation would generate low quantities of B antigen on their red blood cells, consistent with the results obtained in blood banking laboratories where the T641G mutation was found in the B allele of an individual who was phenotyped and categorized as Bel (20). This allele was originally designated *B105 by the authors and later Bel01 according to the Blood Group Antigen Gene Mutation Database (27). The M214T mutant has both glycosyltransferase A and glycosyltransferase B activity (Table 1). Kinetic analysis of the mutant using the B-donor UDP-Gal revealed that the $k_{cat}$ for UDP-Gal (5.4 s$^{-1}$) was unchanged, whereas the $k_{cat}$ for the A-donor UDP-GalNAc increased 3.8-fold compared with wild type GTB (Table 1). The mutation introduces a minor effect on the binding of the acceptor substrate reflected in an elevation in $K_M$ for Fuca1(1–2)Galβ(CH2)7CH3 acceptor (Table 1). This should result in the expression of normal levels of B antigen.

RESULTS

Preparation of Mutant Enzymes—Three natural variants of GTB discovered in blood banking laboratories with mutations located at Met-214 in the immediate vicinity of the 211DVD213 motif were prepared. These enzymes were all cloned as soluble truncated forms of the catalytic domain (amino acids 63–354) and expressed in E. coli. The purification of the three constructs by ion-exchange chromatography and affinity chromatography on a UDP-hexanolamine resin was straightforward in all cases. The yields of final purified protein were 25 mg/liter for the M214T mutant, 53 mg/liter for the M214V mutant, and 25 mg/liter for the M214R enzyme.
TABLE 1
Kinetic constants and phenotype for wild type and mutant glycosyltransferases

| Enzyme | Phenotype | UDP-Gal | UDP-GalNAc |
|--------|-----------|---------|------------|
|        |           | $K_a$   | $K_b$      | $k_{cat}$  | $K_a$   | $K_b$  | $k_{cat}$  |
| Wild type | B | 88 | 27 | 5.1 | 180 | 138 | 0.42 |
| M214R | | 167 | 298 | 0.0042 | 156 | 135 | 0.00016 |
| M214T | $\Delta_a$weak | 148 | 39 | 5.4 | 290 | 174 | 1.6 |
| M214V | $\Delta_a$weak | 132 | 32 | 1.4 | 224 | 226 | 0.37 |

*Not determined; the specific activity was too low to measure accurately.

TABLE 2
Specific activity for wild type and mutant blood type B glycosyltransferases

| Enzyme | UDP-GalNAc 2 mM | UDP-GalNAc 0.13 mM | UDP-GalNAc 0.013 mM |
|--------|-----------------|--------------------|---------------------|
| Wild type | 1.2 | 0.19 | ND |
| M214V | 0.84 | 0.33 | 0.052 |
| M214T | 2.9 | 0.51 | 0.067 |
| M214R | 0.0011 | 0.00032 | ND |

TABLE 3
Data collection and refinement results for crystals of GTB mutants and complexes

| Enzyme | Resolution (Å) | Space group | Rwork (%) | Rfree (%) | Rmerge (%) | Completeness (%) | Unique reflections | PDB accession code |
|--------|----------------|-------------|-----------|-----------|------------|------------------|-------------------|-------------------|
| GTB_M214T | 20-1.71 | C2221 | 18.3 | 17.8 | 18.4 | 96.7 (99.4) | 33,405 | 2O1G |
| GTB_M214T-UDP | 20-1.67 | C2221 | 21.8 | 22.2 | 23.5 | 100.0 (100.0) | 36,659 | 2O1H |
| GTB_M214R | 20-1.99 | C2221 | 19.8 | 23.6 | 19.8 | 97.8 (99.7) | 21,496 | 2O1F |

DISCUSSION

The $^{211}$DVD$_{213}$ motif in the human blood group A and B glycosyltransferases coordinates a Mn$^{2+}$ ion, which in turn facilitates UDP-donor binding (4). It is therefore not surprising that the natural mutations observed in this important region of the enzyme can strongly affect catalysis, but it is significant that they also affect enzyme substrate specificity.

For the M214V mutant there was almost no effect on the $k_{cat}$ for the A-donor UDP-GalNAc, whereas the $k_{cat}$ for UDP-Gal was reduced 3.6-fold. The $K_m$ values were affected in a similar way as the M214T enzyme (Table 1). The reduced activity is not because of instability of the M214V mutant. The mutant maintained 90% of its activity after incubation at 37 °C for 24 h, which is comparable with that of wild type GTB. The kinetic study predicted expression of B structures, but the low substrate turnover for the M214V mutant

on cell surfaces and low levels of A antigen, which is in agreement with the phenotyping. A T641C mutation resulting in M214T was found in the B allele (B(A)05 according to the data base (27)) of an individual phenotyped as AweakB (19), despite the absence of an A allele in trans, showing agglutination with antibodies for the A (weakly) and the B (strongly) antigen.

The very low substrate turnover for the M214R mutant can be understood upon examination of its crystal structure. In the wild type enzyme, the two aspartate residues coordinate a Mn$^{2+}$ ion; however, in the mutant enzyme Arg-214 joins with Asp-211 and Asp-213 to trap a water molecule in the Mn$^{2+}$ binding site, which would significantly hinder (if not preclude) donor binding (Fig. 2). The presence of a water
molecule rather than a low occupancy Mn\(^{2+}\) ion is indicated not only by the hydrogen bond to the functional group of the arginine residue but also by the longer distances observed between Asp-211 and Asp-213 and the water molecule (2.63 and 2.99 Å, respectively) than observed between these residues and the Mn\(^{2+}\) ion in the wild type enzyme (2.33 and 2.38 Å, respectively). Also a strong peak in the anomalous Fourier map would be expected for a Mn\(^{2+}\) ion; however, no peak was observed adjacent to the DVD motif. Interestingly, the observed length of the hydrogen bond from Arg-214 to the water molecule is somewhat shorter than usual at 2.18 Å. Short hydrogen bonds between charged groups are stabilized by polarization effects (28), and there is a clear charge gradient across the water molecule as it bridges between Arg-214 and the two aspartate residues of the \(^{211}\)DVD\(^{213}\) motif. Arg-214 did not approach either of the active site critical residues known to be involved in donor recognition (Met-266 and Ala-268); consequently there was no issue of the mutation affecting enzyme turnover by simply blocking the active site, as in the blood group O\(_2\) (O03) mutant enzyme (29). Several attempts to obtain a structure of M214R in complex with UDP were made under higher concentrations of Mn\(^{2+}\) (10 mM) and UDP (50 mM) than used for the wild type enzyme, but density for manganese or UDP was never observed (data not shown).

M214T showed both GTA and GTB activity (Table 1). The mutation of the bulky Met-214 to the smaller threonine in GTB resulted in the generation of a hydrophobic pocket adjacent to the active site. The side chain of Leu-324 moved, by nearly 1.7 Å, into this pocket. This, in turn, created a significant void in the active site surrounding the critical residue Met-266, with the result that the side chain of this residue can be seen to adopt at least three different conformations (Fig. 3), all of which conform to the Boltzmann-type distribution of side chain conformations in proteins (30). The major conformation maintains van der Waals contact with Leu-324 (Fig. 3, green); one of the minor conformations is similar to that observed for Met-266 in the wild type enzyme (yellow), and the other moves into the donor binding site (magenta). The anomalous Fourier map shows a small peak at 3.0 Å corresponding to the sulfur atom in the high occupancy conformation. The occupancies of these three conformations were adjusted to 0.50, 0.25, and 0.25, respectively, which yielded comparable temperature factors for each conformer.

The alternate conformation of Met-266 with highest occupancy shows the side chain maintaining van der Waals contact with Leu-324 and opening a large pocket in the active site (Fig. 4). Using the atomic coordinates of UDP from the M214T structure solved in complex with UDP, the likely position of UDP-Gal and UDP-GalNAc can be modeled into the binding site (Fig. 5). The modeled folded-back conformation for UDP-Gal is in accordance with the solution structure of bound donor determined by transferred NOE (nuclear Overhauser effect) experiments (31). This shows that the favored conformation of Met-266 provides space within the active site to accommodate the N-acetyl group of GalNAc in the donor binding site (Fig. 5), resulting in blood group A activity.
Crystals of the M214V mutant did not grow. However, it is possible to draw some structure-function correlations, because the change in side chain from the wild type methionine to valine is similar in size and shape to the M214T mutation described above. The smaller 214 side chain would again remove the stabilizing influence of Leu-324 away from Met-266 and create a more forgiving active site recognition pocket that would admit and subsequently transfer the larger A donor.

Alterations of donor substrate specificity upon mutagenesis have been reported for other glycosyltransferases (32, 33). These include mutants of bovine 4-β-galactosyltransferase with increased glucosyltransferase activity with a single amino acid change, R228K (34), and increased UDP-GalNAc transferase activity with a Y289L mutation (35). The donor specificity of *Clostridium difficile* toxin B glucosyltransferase has been changed to UDP-GlcNAc by a double mutation based on comparison with a related toxin UDP-GlcNAc transferase and vice versa (36). A 3-β-glucuronosyltransferase was converted to an enzyme with increased UDP-Glc, -GlcNAc, and -Man transferase activity by a H308R mutation (37). A plant anthocyanin galactosyltransferase was converted to a glucosyltransferase with a Q382H mutation (38). It is unclear whether such sensitivity of donor specificity to a few mutations is generalized among glycosyltransferases and whether GTA and GTB are more sensitive than other glycosyltransferases to mutations.

In summary, mutations of residue 214 in the human blood group B enzyme were expected to affect catalysis, as they are adjacent to the 211DVD213 motif (4, 32), which is essential for donor binding. However, they have also been shown to be able to affect the size and shape of the active site donor recognition pocket. M214R shows very slow substrate turnover, as the arginine residue significantly impacts on the ability of the 211DVD213 motif to bind Mn^{2+} while providing no steric barrier to donor sugar recognition. M214T and M214V enzymes exhibit both glycosyltransferase A and B activity, which can be rationalized by the fact the M214T and probably the M214V mutation lead to a rearrangement of the active site residues to permit the binding and subsequent transfer of the bulky A donor. In the case of M214T and M214R, the serological results are in high agreement with the kinetics obtained. More than a century after its discovery, the ABO blood group system is still one of the most dynamic and relevant model systems for studies of glycobiochemical phenotype-genotype correlations as well as understanding the structure-function relationships of glycosyltransferases.
REFERENCES

1. Yamamoto, F., Clausen, H., White, T., Marken, J., and Hakomori, S. (1990) Nature 345, 279–283
2. Yamamoto, F., and McNeil, P. D. (1996) J. Biol. Chem. 271, 10515–10520
3. Seto, N. O. L., Compton, C. A., Evans, S. V., Bundle, D. R., Narang, S. A., and Palcic, M. J. (1999) Eur. J. Biochem. 259, 770–775
4. Patenaude, S. I., Seto, N. O. L., Borisova, S. N., Szpacenko, A., Marcus, S. L., Palcic, M. M., and Evans, S. V. (2002) Nat. Struct. Biol. 9, 685–690
5. Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003) Curr. Opin. Struct. Biol. 13, 303–317
6. Davies, G. J., Gloster, T. M., and Henrissat, B. (2005) Curr. Opin. Struct. Biol. 15, 638–649
7. Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., and Imberty, A. (2006) Glycobiology 16, 29R–37R
8. Hu, Y., and Walker, S. (2002) Chem. Biol. 9, 1287–1296
9. Gastinel, L. N., Bignon, C., Misra, A. K., Hindsgaul, O., Shaper, J. H., and Joziasse, D. H. (2001) EMBO J. 20, 638–649
10. Munro, S., and Freeman, M. (2000) Curr. Biol. 10, 813–820
11. Cotton, C., Muller, S., Schöttler, M., Schen, S., Prante, C., Brinkmann, T., Kuhn, J., and Kleesiek, K. (2004) J. Biol. Chem. 279, 42566–42573
12. Li, J., Rancour, D. M., Allende, M. L., Worth, C. A., Darling, D. S., Gilbert, J. B., Menon, A. K., and Young, W. W., Jr. (2001) Glycobiology 11, 217–229
13. Busch, C., Hofmann, F., Selzer, I., Munro, S., Jeckel, D., and Aktories, K. (1998) J. Biol. Chem. 273, 19566–19572
14. Gulberti, S., Fourmel-Gigleux, S., Mulliert, G., Aubry, A., Netter, P., Magdalou, J., and Ouzine, M. (2003) J. Biol. Chem. 278, 32219–32226
15. Pak, J. E., Arnoux, P., Zhou, S., Sivarajah, P., Satkunarajah, M., Xing, X., and Rini, J. M. (2006) J. Biol. Chem. 281, 26693–26701
16. Olsson, M. L., Ishaida, N. M., Hosseini-Mafa, B., Hellberg, Å., Moulds, M. K., Saraneva, H., and Chester, M. A. (2001) Blood 98, 1585–1593
17. Seltsam, A., Hallensleben, M., Kollmann, A., and Blassczyk, R. (2003) Blood 102, 3035–3042
18. Roubinet, F., Janvier, D., and Blancher, A. (2002) Transfusion 42, 239–246
19. Deng, Z. H., Yu, Q., Wu, G. G., Lian, Y. L., Su, Y. Q., Li, D. C., Wang, D. M., and Zhang, G. S. (2005) Vox Sang. 89, 251–256
20. Ogasawara, K., Yabe, R., Uchikawa, M., Saitou, N., Bannai, M., Nakata, K., Takenaka, M., Fujisawa, K., Ishikawa, Y., Juji, T., and Tokunaga, T. (1996) Blood 88, 2732–2737
21. Marcus, S. L., Polakowski, R., Seto, N. O. L., Leinala, E., Borisova, S., Blanche, R., Roubinet, F., Evans, S. V., and Palcic, M. M. (2003) J. Biol. Chem. 278, 12403–12405
22. Palcic, M. M., Heerze, L. D., Pierce, M., and Hindsgaul, O. (1988) Glycoconj. J. 5, 49–63
23. Pfugrath, J. W. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 1718–1725
24. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
25. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
26. Collaborative Computational Project (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
27. Blumenfeld, O. O., and Patnaik, S. K. (2004) Hum. Mutat. 23, 8–16
28. Rajagopal, S., and Vishveshwara, S. (2005) FEBS J. 272, 1819–1832
29. Lee, H. L., Barry, C. H., Borisova, S. N., Seto, N. O. L., Zheng, R. B., Blancher, A., Evans, S. V., and Palcic, M. M. (2005) J. Biol. Chem. 280, 525–529
30. Butterfoss, G. L., and Hermans, I. (2003) Protein Sci. 12, 2719–2731
31. Angulo, J., Langpap, B., Blume, A., Biet, T., Meyer, B., Krishna, N. R., Peters, H., Palcic, M. M., and Peters, T. (2006) J. Am. Chem. Soc. 128, 13529–13538
32. Qasba, P. K., Ramakrishnan, B., and Boeggeman, E. (2005) Trends Biochem. Sci. 30, 53–62
33. Hancock, S. M., Vaughan, M. D., and Withers, S. G. (2006) Curr. Opin. Chem. Biol. 10, 509–519
34. Ramakrishnan, B., Boeggeman, E., and Qasba, P. K. (2005) Biochemistry 44, 3202–3211
35. Ramakrishnan, B., and Qasba, P. K. (2002) J. Biol. Chem. 277, 20833–20839
36. Jank, T., Reinert, D. J., Giesemann, T., Schulz, G. E., and Aktories, K. (2005) J. Biol. Chem. 280, 37833–37838
37. Ouzine, M., Gulberti, S., Levoïn, N., Netter, P., Magdalou, J., and Fourmel-Gigleux, S. (2002) J. Biol. Chem. 277, 25439–25445
38. Kubo, A., Arai, Y., Nagashima, S., and Yoshikawa, T. (2004) Arch. Biochem. Biophys. 429, 198–203