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Insights into role of the hydrogen bond networks in substrate recognition by UDP-GalNAc 4-epimerases

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Abstract

UDP-hexose 4-epimerases are critical in galactose metabolism and often important in lipopolysaccharide biosynthesis as well. Three groups of these enzymes have been reported based on their substrate specificity towards non-acetylated substrates (group 1), dual specificity towards *N*-acetylated and non-acetylated substrates (group 2) and specificity towards *N*-acetylated substrates (group 3). We recently reported the structure of a novel UDP-GalNAc 4-epimerase called WbgU and based on the structure proposed a model of specific substrate recognition by UDP-GalNAc 4-epimerases. In this work, we present an analysis of the proposed model of substrate recognition using site-directed mutagenesis of WbgU and crystal structure of the His305Ala mutant. This investigation reveals that the wild-type activity of WbgU is retained in most single-point mutants targeting the active site. However, a graded loss in activity is observed in double- and triple-point mutants with the quadruple-point mutant being completely inactive corroborating the proposed rationale of substrate recognition. Furthermore, crystal structure of the His305Ala mutant shows that the structure is significantly similar to the wild-type WbgU, albeit a loss in the critical hydrogen bond network seated at His305 and ensuing minor conformational changes. It is inferred that the specific and non-specific interactions throughout the active site confer it sufficient elasticity to sustain wild-type activity for several of the single-point mutations.

Keywords

Lipopolysaccharide; *N*-acetylglucosamine; Rossmann fold; 4-Epimerase; Galactose metabolism

1. Introduction

UDP-hexose 4-epimerases are essential in galactose metabolism [5–7] and often critical in lipopolysaccharide biosynthesis [8–12]. These enzymes have been classified into three groups based on whether they are specific towards non-acetylated substrates (group 1), *N*-

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Conflict of interest

The authors declare that they have no conflict of interest.

acetylated substrates (group 3) or both non-acetylated and *N*-acetylated substrates (group 2) [10]. We recently reported the structure of WbgU, a UDP-GalNAc 4-epimerase [1]. The investigation revealed that key interactions at Arg304 and His305 keep the substrate binding site in a conformation that renders specificity towards *N*-acetylated substrates such as UDP-GlcNAc and UDP-GalNAc. Arg304 forms a hydrogen bond with Thr232 and a salt bridge with Asp229. His305 forms a hydrogen bond network with Arg268 and Ser233. In addition, a variation from Thr144 in the groups 1 and 2 to Ser in the group 3 was also noted as potentially critical. Most of the remaining important interactions are provided by residues that are closer to the catalytic center such as Ser142, Ser143 and Asn195. In this work we present an elaborate analysis of this model of substrate recognition based on enzymatic characterization of several site-specific mutants of WbgU and crystal structure of the His305Ala mutant.

2. Materials and methods

2.1. Preparation of the site-directed mutants and protein expression

wbgU was inserted between *Xho*I and *Bam*HI restriction sites of pET-15b plasmid (Novagen) with an N-terminal (His)₆ fusion tag. This recombinant plasmid was used as a template in site-directed mutagenesis by QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, CA). Primer design and thermal cycling were performed as per the manufacturer's protocol. Briefly, primers having appropriate melting temperature were designed for the region flanking the target mutation for both strands of the plasmid DNA carrying wild-type *wbgU*. Thermal cycling was performed then by *Pfu*Ultra DNA polymerase. This was followed by *Dpn*I digestion of the parental plasmid DNA and transformation of the reaction mixture to the tet^R XL1-Blue Supercompetent cells. Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN, CA) and then transformed to BL21(DE3) (Novagen, NJ) for overexpression. Thus, several mutants were prepared namely, the single-point mutants Ser144Thr, Ser233Gly, Arg268Gly, Arg304Gly, His305Ala and Ser306Tyr; the double-point mutant Ser144Thr/His305Ala, the triple-point mutant Ser144Thr/Arg304Gly/His305Ala and the quadruple-point mutant Ser144Thr/Arg304Gly/His305Ala/ Ser306Tyr.

BL21 (DE3) transformants carrying the above mentioned recombinant plasmids were grown in LB-ampicillin medium to an OD₆₀₀ of 0.7 upon which the culture was induced with 0.15 mM IPTG at 30 °C for 5 h. The cells were harvested by centrifugation at 11,000*g* for 10 min and the pellet was resuspended in buffer A (20 mM Tris-HCl pH 9.0, 0.5 M NaCl) containing 10 mM imidazole. After sonication on ice, the lysate was clarified by centrifugation at 20,000*g* for 40 min at 4 °C and the supernatant was loaded onto a Ni-NTA agarose affinity column pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed first with buffer A with 60 mM imidazole added, and then with buffer A containing 110 mM imidazole. The sample was then eluted with elution buffer (20 mM Tris-HCl pH 9.0, 0.5 M NaCl, 0.5 M imidazole). The eluent was desalted by HiTrapTM Desalting HP column (GE Healthcare, NJ) to 50 mM Tris-HCl pH 9.0. Finally, the sample was concentrated with Amicon Ultra centrifugal filter unit of 10 kDa cutoff (Millipore, MA) and stored in buffer S (50 mM Tris-HCl pH 9.0, 20% glycerol) at -20 °C, till further use.

2.2. Characterization of the activity

The reactions were performed at 37 °C in a cocktail containing 20 mM Tris-HCl pH 8.0, 1 mM substrate and 100 µg/mL of the enzyme and quenched by boiling for 6 min. The precipitate was removed by centrifugation. The supernatant was analyzed by capillary electrophoresis (CE) using a 75 µm i.d. capillary (China) in a P/ACE MDQ system (Beckman Coulter) at 25 kV/80 µA. A 5 s vacuum injection of the sample was monitored at

262 nm and in the running buffer of 25 mM sodium tetraborate, pH 9.4. The ratios of UDP-GlcNAc:UDP-GalNAc for each reaction were measured at 1 h time-point for all the single-point mutants. For multi-point mutants, the respective ratios of UDP-GlcNAc:UDP-GalNAc were measured at 1 h time-point and 16 h time-point i.e., overnight reactions.

2.3. Crystallization of the His305mutant in complex with UDP-GlcNAc

The purified samples of all the mutants were subjected to screening for crystallization conditions using the previously established conditions as starting point [1]. The screening was performed in several home-made and commercially available crystallization screens (Hampton Research, CA) across three different kinds of setups namely, sitting drop vapor diffusion, hanging drop vapor diffusion and microbatch under oil. However, despite tedious search, crystals could be obtained only for the His305Ala mutant. These crystals, albeit difficult to reproduce, could be obtained both in microbatch under oil set ups and hanging drop vapor diffusion set ups. The best diffracting crystals could be obtained from a solution containing 25% PEG 3350, 0.2 M ammonium sulfate. The crystals were cryoprotected in the crystallization reagent containing 15–20% glycerol.

2.4. Structure determination of the His305Ala mutant

The diffraction data were collected at the beamline 19-ID-D of the Structural Biology Center (SBC), Argonne National Laboratory (IL). The intensities were indexed and integrated using HKL3000 [13,14] at SBC and scaled to structure factor amplitudes in the space group $P3_2$ using SCALEPACK [15]. The data processing statistics are listed in Table 1.

Diffraction data to 2.1 Å were used for structure determination of the His305Ala mutant. A monomer from the crystal structure of WbgU/UDP-GlcNAc complex [1] was used as a search model in molecular replacement using Phaser [16]. A unique solution could be obtained in the space group $P3_2$ with 4 monomers in the asymmetric unit. This solution had a final log-likelihood gain of 10148.8 and an R_{factor} of 0.39. It was used in PHENIX AutoBuild [17] for automated model building while forcing non-crystallographic symmetry (NCS) restraints during the model building and refinement. The ligands NAD(H) and UDP-GlcNAc were added to the model output by the refinement and refined in real space according to the difference electron density map (Fig. 2) while using the geometric constraints generated by PRODRG server [18]. The NCS restraints were then lifted from the subsequent refinement. Anisotropic displacement parameters were generated for this model using a TLS model [19,20] and it was then subjected to TLS and restrained refinement in REFMAC5 [19,21] while keeping the initial B factors fixed at 20 Å². All these refinement steps reduced $R_{\text{factor}}/R_{\text{free}}$ to 0.24/0.28. Water molecules were added to this model at the peaks above 1.2σ cutoff in the 2mF_o–DF_c electron density map using COOT [22]. Electron density peaks for two SO₄²⁻ ions were also identified that were appropriately modeled. The $R_{\text{factor}}/R_{\text{free}}$ for the final model is 0.22/0.27. The refinement statistics are listed in Table 1.

2.5. Structural analysis

Structural alignment of the mutant WbgU structure was computed relative to the previously published crystal structure of WbgU [1] by generating a fit for the C^α atoms of the corresponding N-terminal domains (residue range 5–194 and 239–264) by the program LSQKAB [23]. Surface areas and volumes of the active site were calculated for both of the crystal structures using CASTp server [24] after removing all the ligands and the solvent. Subsequent approximations for these values were made to nearest 50, as suggested by Novotny et al. [25]. Molecular graphics were drawn using UCSF Chimera [26] and POV-Ray [27].

3. Results

3.1. Activity

Capillary electrophoresis (CE) was used to characterize the enzymatic activities of wild-type WbgU and WbgU mutants. The reaction products were identified by comparison to standard compounds. The epimers could be baseline-resolved with ~11.0 and ~11.7 min retention times for UDP-GlcNAc and UDP-GalNAc, respectively. For wild-type WbgU, the epimerization reaction between UDP-GlcNAc and UDP-GalNAc, when complete resulted in a 72:28 ratio of UDP-GlcNAc:UDP-GalNAc, irrespective of the initial substrate (data not shown). Five of the reactions involving single-point mutants namely, Ser144Thr, Ser233Gly, Arg268Gly, Ar304Gly and His305Ala were found to have a similar UDP-GlcNAc:UDP-GalNAc ratio as that of wild-type WbgU at the 1 h time-point (Fig. 1A). However, the reaction for Ser306Tyr mutant did not yield any product at all (data not shown). The reaction of the double-point mutant (Ser144Thr/His305Ala) resulted in a UDP-GlcNAc:UDP-GalNAc ratio of 85:15 at 1 h time-point and 75:25 for the overnight reaction (Fig. 1B). This ratio was observed to be 96:4 at 1 h time point and 89:11 for the overnight reaction in case of the triple-point mutant (Fig 1B). The analysis of the reaction of quadruple-point mutant Ser144Thr/Ar304Gly/His305Ala/ Ser306Tyr revealed that it has no activity (data not shown).

3.2. Crystal Structure of the His305Ala mutant

The final model of WbgU:His305Ala mutant contains four molecules each of WbgU, UDP-GlcNAc, NAD(H), two SO_4^{2-} ions and a total of 355 solvent molecules and is refined to 2.1 Å resolution with the value of $R_{\text{factor}}/R_{\text{free}}$ being 0.22/0.27 (Table 1). The electron density was found to be well defined for most part, notable exception being a loop region extending from His287–Ile293. The electron density for the cofactor NAD(H) as well as the substrate UDP-GlcNAc is also very well defined (Fig. 2). The overall structure of WbgU comprises of N- and C-terminal domains. The cofactor NAD(H) binding site sits on and parallel to the carboxy edge of the β -sheet of the C-terminal domain, which is a modified Rossmann fold. The substrate binding site is mostly contributed by the much smaller C-terminal domain (Fig. 3). The comparative analysis between the crystal structures of wild-type WbgU and the His305Ala mutant revealed that overall tertiary fold is very similar albeit a small increase in surface area and volume of the active site in the His305Ala mutant (Fig. 3 and Table 2).

4. Discussion

UDP-hexose 4-epimerases are classified into three groups based on whether they are specific towards non-acetylated substrates (group 1); both non-acetylated and *N*-acetylated substrates (group 2) or *N*-acetylated substrates (group 3) [10]. Although the mechanism of catalysis carried out by these enzymes has been well-studied [6,28–31], substrate discrimination by the various groups is not very well understood. In the recently published crystal structure of WbgU, we proposed a model to rationalize the substrate specificity of all the three groups on a common platform and deduced that the group 1 and the group 2 epimerases share a very similar active-site architecture, which is significantly different from the group 3 epimerases [1]. Briefly, the key interactions at Arg304 and His305 in the substrate-binding region called ‘297–308’ belt were primarily implicated in conferring the observed specificity. In this work, we delve further into understanding substrate specificity of the group 3 epimerases towards *N*-acetylated substrates. To accomplish this, enzymatic activity of several single- and multipoint mutants and crystal structure of the His305Ala were analyzed.

The substrate binding pocket of WbgU is present in the C-terminal domain and is composed of residues from different regions of the protein. The key region interacting with UDP-GlcNAc is contributed by the residues Ser142-Ser144, Phe194-Asn195, Ala209-Trp214, Tyr225-Arg234 and Glu297-Ala308 [1]. Of these, the loop between Glu297-Ala308, a region referred to as '297–308' belt, and the residues Ser143 and Ser144 are primarily responsible for interacting with the *N*-acetyl moiety of the UDP-GlcNAc. The conformation of the 297–308 belt, in turn, rests mainly upon two sets of interactions: the first set pivoted at Arg304 as a salt bridge between Arg304 and Asp229 and a hydrogen bond between Arg304 and Thr232, and the second set pivoted at His305 in a hydrogen bond network formed with Ser233 and Arg268. Additionally, it is noted that Asp302 also forms a hydrogen bond with Arg234. This could be another potentially critical interaction, especially since Arg234 interacts with several regions of the active site including the substrate itself.

Enzymatic analysis of WbgU mutants following the aforesaid rationale revealed several details. Firstly, the single-point mutant Ser306Tyr was found to lose its activity (data not shown) as predicted previously [1], since a Tyr at this position would pose severe steric clashes with substrate binding site of the protein and very likely disrupt the catalytic site itself. This result is also in agreement with the previous experimental work on the group 3 epimerases [10]. The analysis of the five remaining single-point mutants, namely, Ser144Thr, Ser233Gly, Arg268Gly, Arg304Gly, His305Ala revealed that their activity is essentially identical to the wild-type WbgU, resulting in a UDP-GlcNAc:UDP-GalNAc ratio of ~72:28 at 1 h time-point. The crystal structure of His305Ala mutant shows that both of the hydrogen bond partners of His305 in the wild-type protein, i.e. Ser233 and Arg268, are in significantly different conformations in the mutant, due to loss in hydrogen bonding (Fig. 3). This loss in hydrogen bonding does 'loosen up' the substrate binding site very slightly, an effect clearly visible in the mutant structure in the regions Arg304–Gln307 and Ser143–Asn161 and also reflected in slightly increased active site volume and surface area in case of the His305Ala mutant (Table 2). Despite this, overall conformation of the active site of the His305Ala mutant is very similar to the wild-type protein, and so are the relative positions occupied by the substrate (Fig. 3). These observations indicate that the substrate binding site has sufficient elasticity to compensate for loss of one of the aforesaid critical interactions at a time, even if this leads to loss of one of the 'critical' hydrogen bond networks.

More interestingly, the results obtained for the double-and the triple-point mutants showed a graded loss of activity. The double-point mutant Ser144Thr/His305Ala exhibited a shift in the ratio of UDP-GlcNAc:UDP-GalNAc from the ratio of 72:28 noted for the wild-type WbgU to the ratio of 85:15 at 1 h time-point and to 75:25 in the overnight reaction. This ratio changed further to 96:4 for the triple-point mutant Ser144Thr/Arg304Gly/His305Ala at 1 h time-point and to 89:11 for the overnight reaction. The cumulative trend in the form of gradual increase in the ratio of UDP-GlcNAc:UDP-GalNAc clearly supports the proposed model of substrate recognition which pivots on the conformation of the 297–308 belt and the key interactions at Arg304 and His305. Although the double-and the triple-point mutants could not be crystallized, it is very likely that the loss of interactions as a consequence of these mutants adversely affects the conformation of the substrate binding site by loosening it, which in turn, reduces the number of favorable interactions with the substrate. The slight increase in the active site volume and surface area of the His305Ala mutant when compared to those of the wild-type WbgU support this. The quadruple mutant Ser144Thr/Arg304Gly/His305Ala/Ser306Tyr showed no activity at all for either UDP-GlcNAc or even UDP-Glc (data not shown). This is likely due to continued presence of the steric clashes produced as a consequence of Ser306Tyr mutation. In other words, this indicates that a loss of both sets of the primary interactions that are deemed responsible for the recognition of *N*-acetylated substrates, i.e. at Arg304 and His305, is still unable to accommodate a Ser306Tyr mutation.

Unfortunately, the double-, triple- and quadruple-point mutants could not be crystallized, rendering it difficult to draw a more precise conclusion in this regard.

To summarize, this investigation provides evidence for the importance of Ser144, Arg304 and His305 in maintaining the active site conformation required for the specific recognition of the *N*-acetylated substrates in the group 3 UDP-hexose 4-epimerases. It also suggests that the active site architecture in these enzymes is fairly resilient to changes brought upon by single-point mutations, even if they lead to loss in one of the key hydrogen bond networks.

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Fig. 1.

The ratio of UDP-GlcNAc:UDP-GalNAc at specific time-points in reactions for different mutants. *X*-axis represents the retention time in min and the *Y*-axis is AU. (A) The ratio of UDP-GlcNAc:UDP-GalNAc at 1 h time-point for all the single-point mutants except Ser306Tyr is ~72:28; (B) The ratio of UDP-GlcNAc:UDP-GalNAc at 1 h time-point and overnight reaction for the double-point mutant Ser144Thr/His305Ala and the triple-point mutant Ser144Thr/Arg304Gly/His305Ala, depicted at the top of each trace.

Fig. 2. $F_o - F_c$ electron density map in the region of UDP-GlcNAc and NAD(H) depicted at an absolute electron density of $0.19 \text{ e}/\text{\AA}^3$ corresponding to 3.0σ cutoff.

Fig. 3.

The structural alignment of wild-type WbgU (grey) with the His503Ala mutant (yellow) viewed along the β -sheet (left) and rotated by $\sim 40^\circ$ (right). The substrate (UDP-GlcNAc) is bound in the C-terminal domain and the cofactor NAD(H) is bound on the carboxy edge of the β -sheet in the N-terminal domain. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Data collection and refinement statistics of WbgU:His305Ala mutant.

Data collection statistics	
Source	19 ID-D APS
Space group	P3 ₂
Unit cell parameters	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.3, 78.3, 231.8
<i>α</i> , <i>β</i> , <i>γ</i> (deg)	90.0, 90.0, 120.0
Temperature (K)	103
Wavelength (Å)	0.98
Resolution (Å)	50.0–2.10 (2.14–2.10)
<i>R</i> _{merge} ^a	0.12 (0.46)
Completeness (%)	99.8 (100)
(<i>I</i> /σ(<i>I</i>))	18.3 (2.9)
Number of unique reflections	92338
Average redundancy	3.7 (3.6)
B factor from Wilson plot (Å ²)	29.3
PDB entry	3ruc
Refinement statistics	
Resolution (Å)	50.0–2.10
Number of reflections	87697
<i>R</i> _{factor} ^b	0.22
<i>R</i> _{free} ^b	0.27
Number of protein atoms	10708
Number of water atoms	355
Number of ligand atoms	328
Rms deviation from ideal values for bond distances (Å) ^c	0.02
Rms deviation from ideal values for bond angles (deg) ^c	1.9
Average B-factors	
Main chain (Å ²)	31.0
Side chain and waters (Å ²)	32.4
All atoms (Å ²)	31.7
Ramachandran plot ^d	
Favored (%)	97.1
Disallowed (%)	0.6

The numbers in parentheses refer to the highest resolution shell.

$$^a R_{\text{merge}} = \frac{\sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl} \rangle|}{\sum_{hkl} \langle I_{hkl} \rangle}$$

$$^b R_{\text{factor}} = \frac{\sum |F_{\text{obs}} - F_{\text{calc}}|}{\sum F_{\text{obs}}}, \text{ where } R_{\text{free}} \text{ refers to the } R_{\text{factor}} \text{ for 5\% of the data that were excluded from the refinement.}$$

^c Ideal values used from Engh and Huber [2].

^dValidation by Molprobity [3,4].

Table 2

Active site dimensions of wild-type WbgU and the His305Ala mutant.

Protein	Vol (Å ³)	SA (Å ²)	RMSD (Å)	
			N-term	C-term
Wild-type WbgU/UD-GlcNAc [1]	2500	1800		
WbgU-H305A/UDP-GlcNAc	2600	1850	0.16	0.25