GlmU (*N*-acetylglucosamine-1-phosphate uridylyltransferase) bound to three magnesium ions and ATP at the active site

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N-Acetylglucosamine-1-phosphate uridylyltransferase (GlmU), a bifunctional enzyme exclusive to prokaryotes, belongs to the family of sugar nucleotidyltransferases (SNTs). The enzyme binds GlcNAc-1-P and UTP, and catalyzes a uridylyltransfer reaction to synthesize UDP-GlcNAc, an important precursor for cell-wall biosynthesis. As many SNTs are known to utilize a broad range of substrates, substrate specificity in GlmU was probed using biochemical and structural studies. The enzymatic assays reported here demonstrate that GlmU is specific for its natural substrates UTP and GlcNAc-1-P. The crystal structure of GlmU bound to ATP and GlcNAc-1-P provides molecular details for the inability of the enzyme to utilize ATP for the nucleotidyltransfer reaction. ATP binding results in an inactive pre-catalytic enzyme–substrate complex, where it adopts an unusual conformation such that the reaction cannot be catalyzed; here, ATP is shown to be bound together with three Mg^{2+} ions. Overall, this structure represents the binding of an inhibitory molecule at the active site and can potentially be used to develop new inhibitors of the enzyme. Further, similar to DNA/RNA polymerases, GlmU was recently recognized to utilize two metal ions, Mg_A^{2+} and Mg_B^{2+} , to catalyze the uridylyltransfer reaction. Interestingly, displacement of Mg_B^{2+} from its usual catalytically competent position, as noted in the crystal structure of RNA polymerase in an inactive state, was considered to be a key factor inhibiting the reaction. Surprisingly, in the current structure of GlmU Mg_B^{2+} is similarly displaced; this raises the possibility that an analogous inhibitory mechanism may be operative in GlmU.

1. Introduction

The family of sugar nucleotidyltransferases (SNTs), also referred to as sugar nucleotide pyrophosphorylases, catalyze the transfer of the NMP (nucleoside monophosphate) moiety of NTP (nucleotide triphosphate) to the phosphate group of a sugar-phosphate molecule to produce sugar nucleotides. A general depiction of the reaction catalyzed is

The product NDP-sugar is then utilized by glycosyltransferases as the activated form of the sugar. In many essential cellular processes such as antigen synthesis, post-translational glycosylation of proteins, cell-wall synthesis and energy metabolism, activated sugars act as donors and are transferred to acceptor molecules such as proteins, lipids, carbohydrates or small organic molecules (Caputto *et al.*, 1950; Frey, 1996; Tetteroo *et al.*, 1987). This explains the prevalence of SNTs in biological systems. These molecules are also precursors of many therapeutically important molecules such as antigens and antibiotics (Luzhetskyy *et al.*, 2008). Given this important role of SNTs, several studies have been designed to understand their substrate specificities for different nucleotides and sugar phosphates (Mizanur *et al.*, 2004; Gronwald *et al.*, 2008; Kotake *et al.*, 2004; Moretti & Thorson, 2007; Dickmanns *et al.*, 2011). This is because SNTs with a broad specificity allow the utilization of different nucleotides and sugar phosphates and such SNTs can easily be employed industrially for the synthesis of diverse sugar nucleotides. Attempts have also been made to enhance the inherent substrate promiscuity by structure-based enzyme engineering (Barton *et al.*, 2002; Moretti & Thorson, 2007).

GlmU, an SNT that utilizes UTP and GlcNAc-1-P as its natural substrates, synthesizes UDP-GlcNAc, which forms an important element of the cell wall of several bacteria. Given this important role, GlmU is an established target for antimicrobial drugs, as supported by several investigations (Burton *et al.*, 2006; Mochalkin *et al.*, 2008; Pereira *et al.*, 2009). Previously, we have characterized GlmU from *Mycobacterium tuberculosis* (GlmU^{Mtb}; Parikh *et al.*, 2009). GlmU^{Mtb} is a bifunctional enzyme with independent acetyltransferase and uridylyltransferase active sites in the C-terminal and N-terminal domains, respectively (Verma *et al.*, 2009). In previous studies, we attempted to understand the structure–function relationships that explain the catalytic mechanisms and their regulation in this molecule (Jagtap *et al.*, 2012, 2013). As several SNTs possess wide substrate utilization, in this study we set out to characterize the substrate specificity of the uridylyltransferase reaction using biochemical and crystallographic studies. These showed that the enzyme is highly specific for its natural substrates UTP and GlcNAc-1-P. Its inability to effectively utilize other nucleotides and sugar phosphates does not make it a suitable candidate for the commercial biosynthesis of diverse sugar nucleotides; it can use CTP, but only marginally. However, based on crystallographic studies, we report an unusual conformation of bound ATP that may provide a platform for the *de novo* design of an inhibitor against GlmU. In the crystal structure reported here, we find ATP bound along with GlcNAc-1-P and Mg²⁺ ions at the active site. Unusually, the presence of three instead of two Mg²⁺ ions and a distorted conformation of the ATP molecule arrests the enzyme in an inactive pre-catalytic state. GlcNAc-1-P, three Mg²⁺ ions and ATP occupy the active site and render it inactive for catalysis. This molecular complex can thus be used as a ‘lead’ for the development of an inhibitor against the enzyme that interferes with the uridylyltransferase activity. Besides this, an analysis of the bound Mg²⁺ ions suggests an inhibitory mechanism noted for RNA polymerases (Wang *et al.*, 2006).

2. Materials and methods

2.1. Cloning, mutagenesis and purification of GlmU^{Mtb}

Cloning, protein expression and purification of GlmU^{Mtb} were performed as described previously (Verma *et al.*, 2009).

2.2. Uridylyltransferase assays

Malachite green assays were employed for uridylyltransferase assays (Jagtap *et al.*, 2012).

Assays were performed in a 10 µl volume containing 25 mM HEPES buffer pH 7.6, 5 mM MgCl₂, 1 mM DTT with 1 mM sugar-1-phosphate (GlcNAc-1-P, Glc-1-P and Man-1-P), 2 mM NTP (UTP, ATP, GTP, CTP and TTP), 0.04 U thermostable inorganic pyrophosphatase (TIPP) and subsaturating concentrations of GlmU^{Mtb} for 30 min at 30°C. Reactions were terminated by incubation at 65°C for 30 min and the product formed was estimated using a malachite green phosphate assay kit according to the manufacturer’s recommendation.

2.3. Crystal structure of GlmU in complex with ligands of the uridylyltransferase reaction

The wild-type GlmU^{Mtb} protein was purified and crystallization was carried as described previously (Parikh *et al.*, 2009). Crystals of GlmU in complex with ligands (ATP and GlcNAc-1-P) and three Mg²⁺ ions were obtained by soaking apo GlmU^{Mtb} crystals in soaking solution consisting of 10% PEG 8000, 100 mM HEPES pH 7.5,

Table 1

Data-collection and refinement statistics for GlmU^{Mtb}[GlcNAc-1-P:ATP].

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	<i>H</i> 3
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 79.27, <i>c</i> = 276.73, <i>α</i> = <i>β</i> = 90, <i>γ</i> = 120
Resolution (Å)	47.82–1.98 (2.10–1.98)
<i>R</i> _{merge} (%)	8.9 (46.2)
<i>I</i> / <i>σ(I)</i>	29.64 (4.04)
Completeness (%)	99.6 (98.7)
Refinement	
Resolution range (Å)	47.82–1.98
No. of reflections	40730
<i>R</i> _{work}	0.1589 (0.199)
<i>R</i> _{free}	0.2005 (0.265)
No. of atoms	
Protein	3445
Ligand/ion	80
Water	474
Average <i>B</i> factors (Å ²)	
Protein	32.9
Ligand/ion	40.6
Water	31.5
R.m.s.d.	
Bond lengths (Å)	0.0266
Bond angles (°)	2.1569

20 mM MgCl₂, 4 mM CoCl₂ and substrates: 10 mM ATP and 10 mM GlcNAc-1-P at 4°C. The ligand-bound crystals were cryoprotected in a cryosolution consisting of 20% ethylene glycol, 15% PEG 8000, 100 mM HEPES pH 7.5, 20 mM MgCl₂, 4 mM CoCl₂ prior to X-ray diffraction data collection.

2.4. Data collection, structure determination and refinement

X-ray diffraction data for ligand-bound GlmU crystals were collected using an in-house Rigaku MicroMax-007 HF X-ray source at a wavelength of 1.54 Å (Cu Kα). The data were indexed, integrated and scaled using the *XDS* program package. The crystals contained one molecule per asymmetric unit and belonged to space group *H*3 (No. 146). The structure was determined by the molecular-replacement method using *Auto-Rickshaw* (Panjikar *et al.*, 2005). The previously determined structure of apo GlmU^{Mtb} (in a hexagonal space group; PDB entry 3dj4; Parikh *et al.*, 2009) was used as a search model. The coordinates for ligands were obtained from the Protein Data Bank (PDB). The library parameters for these ligands were generated using the *PRODRG* server (Schüttelkopf & van Aalten, 2004). The initial model was subjected to several rounds of manual building in *Coot* (Emsley & Cowtan, 2004), and the initial refinement used *REFMAC5* (in *CCP4*) employing rigid-body refinement followed by several rounds of restrained refinement (Murshudov *et al.*, 2011). Following the initial structure refinement, ligands were fitted into the electron density and the structure of the complex was further refined using *REFMAC5*. The quality of the final model was assessed using *PROCHECK* (Laskowski *et al.*, 1993). The final refinement statistics are listed in Table 1. The electron density for Mg²⁺ ions was differentiated from that of water molecules based on analysis of the coordination geometries (Harding, 2001). CoCl₂ was included in the crystallization experiments as it was necessary for crystal growth. To determine whether any of the metal-binding sites were occupied by Co²⁺ ions, we examined anomalous dispersion by cobalt, which absorbs X-rays at a wavelength of 1.54 Å. The data processed without merging Bijvoet pairs, *i.e.* with the anomalous contribution, were combined with the phases of the search model to locate the position of the cobalt ion(s) in the asymmetric unit. The absence of any peaks in the anomalous difference Fourier map suggested that none of the metal-binding sites were occupied by Co²⁺.

ions (data not shown). The atomic coordinates and structure factors for the structure presented here have been deposited in the PDB (<http://www.rcsb.org>) with accession code 4k6r.

3. Results and discussion

3.1. Biochemical studies to evaluate substrate specificity in GlmU

In order to probe the innate promiscuity of GlmU^{Mtb} for the usage of various nucleotides and sugar phosphates, we commenced enzyme kinetics studies in the presence of five commonly used nucleotides and three different sugar phosphates. The nucleotidyltransfer activity of the enzyme towards various nucleotides showed that the enzyme is highly specific for UTP. Of the other nucleotides tested, only marginal activity with CTP was detected, while no activity was found with TTP, ATP and GTP (Fig. 1*a*). Of the three sugar phosphates tested, the enzyme displayed activity only for the substrate GlcNAc-1-P, and was inactive in the assays using glucose 1-phosphate and mannose 1-phosphate (Fig. 1*b*).

Few SNTs have been found to have the inherently broad substrate specificity that makes them suitable for the commercial synthesis of a

Figure 1

A nucleotidyltransfer assay for GlmU^{Mtb} was carried out for different nucleotides and sugar phosphates. (a) The ability of GlmU^{Mtb} to utilize different nucleotides (2 mM) is shown. The maximum enzymatic activity was observed for UTP, which is the natural substrate; considering this as 100%, the activities of the other nucleotides are shown as relative percentage activity. Modified malachite green assays were carried out in triplicate and error bars are shown. (b) Similarly, to test for its ability to use different sugars, assays were carried out with UTP as the substrate. Uridyltransfer assays were carried out with three sugar phosphates (indicated) at 2 mM concentration. The maximum activity was observed with the natural substrate GlcNAc-1-P; considering this as 100%, the products formed when glucose-1-P and mannose-1-P were used are shown.

wide range of sugar nucleotides (Mizanur *et al.*, 2004; Gronwald *et al.*, 2008; Kotake *et al.*, 2004; Pereira *et al.*, 2009; Mizanur & Pohl, 2009). Such enzymes are usually engineered to further broaden their substrate utilization, so that a single enzyme can be employed for the synthesis of a diverse set of molecules for industrial production. One such SNT that has been evaluated for its commercial viability and studied extensively is RmlA (glucose-1-phosphate thymidyltransferase). It was found to utilize all five of the major nucleotides, *i.e.* ATP, GTP, TTP, UTP and CTP, and 30 different sugar-phosphate

Figure 2

(a) The structure of GlmU^{Mtb}[GlcNAc-1-P:ATP] is shown. The acetyltransferase and uridyltransferase active sites are marked A and U. The inset shows an enlarged view of the uridyltransfer active site, with unbiased electron density for the ligands in an $F_o - F_c$ map contoured at 2σ . The map was generated using $F_o - F_c$ coefficients and phases calculated before including the ligands and Mg²⁺ ions (but after initial refinement of the protein atoms alone). Modelled into the density are ATP, GlcNAc-1-P and the three Mg²⁺ ions, which are labelled Mg_A²⁺, Mg_B²⁺ and Mg_C²⁺. (b) A structural comparison of GlmU^{Mtb}[GlcNAc-1-P:ATP] with GlmU^{Mtb}[GlcNAc-1-P:UTP] is shown as ribbons coloured purple and pink, respectively. The nucleotides ATP and UTP bound at the active site are shown as sticks coloured blue and magenta, respectively. The loop regions that show a conformational change are indicated.

derivatives (Pereira *et al.*, 2009). The high promiscuity in the substrate utilization of wild-type RmlA renders it ideal for commercial chemoenzymatic synthesis. However, the higher enzymatic activities found with pyrimidine nucleotides compared with purine nucleotides necessitated enzyme engineering in RmlA. Mutations created to alter the pyrimidine/purine bias were also successful (Pereira *et al.*, 2009). Based on these studies, it appears that only a few of the pyrophosphorylase class of enzymes can utilize a broad range of substrates with varying affinity and reaction rates.

3.2. Crystal structure of GlmU in complex with ATP and GlcNAc-1-phosphate

We have previously determined structures of GlmU^{Mtb} bound to its natural substrates and in complex with one of the substrates,

GlcNAc-1-P (Jagtap *et al.*, 2013; PDB entries 4hcq and 4g87). These structures show stabilizing interactions for the nucleotide base similar to those found in the structure of the related SNT RmlA (Moretti *et al.*, 2011; Blankenf eldt *et al.*, 2000). RmlA employs dTTP and Glc-1-P as natural substrates. Based on structural and biochemical studies, it was shown that a conserved glutamine (corresponding to Gln83 in GlmU^{Mtb}) is responsible for its specificity towards the pyrimidine base, *i.e.* dTTP (Jagtap *et al.*, 2013; Blankenf eldt *et al.*, 2000). However, RmlA was found to utilize all five nucleotides. Intriguingly, despite conserving the identical glutamine, Gln83, at the active site, GlmU^{Mtb} shows a completely different nucleotide preference; it can only use UTP (and marginally CTP) in the reaction (Fig. 1*a*). In order to understand the molecular basis of the bias for UTP, we resorted to structure determination using X-ray crystallography in the presence of other nucleotides. For this, we soaked apo crystals of GlmU (as

Figure 3

The unusual conformation of ATP seen in GlmU^{Mtb}[GlcNAc-1-P:ATP]. (a) The regular octahedral hexacoordination geometry of the three metal ions, Mg_B²⁺, Mg_{B'}²⁺ and Mg_C²⁺, is shown. Interactions made by Mg_{B'}²⁺ and Mg_C²⁺ appear important in positioning P α away from the nucleophile. (b) Substrates bound at the active site of GlmU^{Mtb}[GlcNAc-1-P:UTP] (reported in Jagtap *et al.*, 2013), which is the catalytically active form. UTP and GlcNAc-1-P are shown in sticks with C atoms coloured yellow. An asterisk indicates the attacking nucleophile of GlcNAc-1-P. Magnesium ions bound to both structures are shown as green spheres. Coordination interactions of Mg_{B'}²⁺ with the triphosphate of UTP are shown. (c) GlmU^{Mtb}[GlcNAc-1-P:ATP] is in a catalytically inactive state. A superposition with the active GlmU^{Mtb}[GlcNAc-1-P:UTP] is shown. Nucleotides are marked and shown as sticks with C atoms coloured cyan for ATP and yellow for UTP. In the unusual ATP conformation, P α of UTP (marked P α -U) is in a catalytically competent position optimal for nucleophilic attack. Magnesium ions bound to both structures are shown as spheres; those in dark green correspond to those of GlmU^{Mtb}[GlcNAc-1-P:ATP] and those in light green correspond to those of GlmU^{Mtb}[GlcNAc-1-P:UTP]. (d) ATP bound in an atypical conformation at the active site is shown as sticks with C atoms coloured cyan. Residues making interactions with ATP are shown in stick representation; those forming the hydrophobic pocket are shown with C atoms coloured green. Residues Thr18 and Arg19 that interact with the γ -phosphate are shown in sticks with their C atoms coloured yellow. The hydrogen-bond interaction made by Gln83 with the ribose sugar is shown as a blue broken line. Ala14, Pro16, His58 and Pro86 forming a hydrophobic pocket around the adenine base are shown in sticks with their C atoms coloured green. (e) With the natural substrate, *i.e.* UTP, Gln83 interacts with the uracil base. UDP-GlcNAc and pyrophosphate at the active site of GlmU^{Mtb} (PDB entry 4g87) are shown as sticks with their C atoms coloured cyan. Hydrogen-bond interactions of Gln83 with the uracil base are shown as blue broken lines. Residues Thr18 and Arg19 that interact with the γ -phosphate are shown in sticks with their C atoms coloured yellow. (f) As (c) but in a different orientation. Here, the magnesium ions are labelled. Mg_{B'}²⁺ in GlmU^{Mtb}[GlcNAc-1-P:ATP] is displaced by 2.1 Å from the corresponding Mg_{B'}²⁺ in GlmU^{Mtb}[GlcNAc-1-P:UTP].

described in §2) in a solution containing purine nucleotides (either ATP or GTP), GlcNAc-1-P and MgCl_2 .

The crystal structure of GlmU^{Mtb} determined from crystals soaked in a solution containing GTP and GlcNAc-1-P showed clear electron density for GlcNAc-1-P but not for GTP (data not shown). This observation is in line with our biochemical studies and is consistent with the inability of the enzyme to perform the nucleotidyltransfer reaction with GTP. Strikingly different results were found after similar soaking experiments carried out with ATP. Clear unbiased electron density could be observed for both GlcNAc-1-P and ATP (Fig. 2*a*) and, except for a slight change in the orientation of two loop regions (Fig. 2*b*), the structure of the active site and the uridyltransferase domain remained unaltered with respect to the structures of the complexes of the enzyme with its natural substrates and with one of the substrates, GlcNAc-1-P (PDB entries 4hcq and 4g87). More remarkably, in the new structure, termed $\text{GlmU}^{\text{Mtb}}[\text{GlcNAc-1-P:ATP}]$, an unusual conformation was observed for ATP bound at the active site. This new binding pose was completely different from those of nucleotides observed thus far in available structures of SNTs. This atypical conformation of ATP appears to be stabilized by two Mg^{2+} ions, Mg_B^{2+} and Mg_C^{2+} , as shown in Fig. 3(*a*). Fig. 3(*b*) shows the way that UTP, the natural substrate, is stabilized by Mg_A^{2+} and Mg_B^{2+} . The conformation of ATP observed in the present structure positions its P_α 6.15 Å away from the attacking nucleophile of GlcNAc-1-P (Fig. 3*c*). At this distance, the nucleophile would be unable to carry out a nucleophilic attack and hence the nucleotidyltransfer reaction would not occur. Therefore, although ATP binds at the active site, its atypical conformation precludes its participation in the reaction and results in an inactive enzyme–nucleotide complex. With ATP bound at the active site in a different conformation, Gln83 (which interacts with the uracil base in the UTP-bound structure) does not interact with the nucleotide base; instead, it makes a hydrogen-bond interaction with the ribose sugar of the nucleotide (Figs. 3*d* and 3*e*). Apart from this, the nucleotide base occupies a pocket lined by hydrophobic groups. In both the ATP-bound and UTP-bound forms the triphosphate moiety makes similar active-site interactions with the Thr18 and Arg19 residues (Figs. 3*d* and 3*e*). The structural findings and biochemical data taken together indicate that ATP is capable of binding to the active site; however, it is not utilized for product

formation because of the catalytically incompetent conformation that it adopts. Thus, the possibility exists that ATP competing with UTP, the canonical substrate, is a mechanism for regulating the activity of the enzyme; further comprehensive studies, including evaluation of the enzymatic activity or its inhibition in the presence of ATP, would be needed to investigate such possibilities.

Recently, employing crystal structures, we have explained the catalytic mechanism of uridyl transfer by GlmU (Jagtap *et al.*, 2013). This mechanism, elucidated based on the $\text{GlmU}^{\text{Mtb}}[\text{GlcNAc-1-P:UTP}]$ structure determined with natural substrates, requires two Mg^{2+} ions, Mg_A^{2+} and Mg_B^{2+} , to catalyze the reaction (Fig. 3*b*). Mg_A^{2+} is known to activate the nucleophile and Mg_B^{2+} , the second metal ion, makes interactions with the triphosphate moiety of UTP and is likely to stabilize the transition state during the uridyltransfer reaction and also to participate in product release following the reaction (Jagtap *et al.*, 2013). In this way, the two metal ions efficiently accomplish the nucleotidyltransfer reaction. By contrast, in the current structure, $\text{GlmU}^{\text{Mtb}}[\text{GlcNAc-1-P:ATP}]$, three Mg^{2+} ions were surprisingly found at the active site, which is not in line with the known ‘two-metal-ion’ catalytic mechanism required to catalyze the reaction (Jagtap *et al.*, 2013). Interestingly, in the new $\text{GlmU}^{\text{Mtb}}[\text{GlcNAc-1-P:ATP}]$ structure the position of Mg_A^{2+} is conserved, indicating that if P_α were not displaced then the reaction could occur. A comparison shows that the second metal does not bind at site B but occupies a new site 2.15 Å away; this metal is hence termed $\text{Mg}_\text{B'}^{2+}$ (Figs. 3*a* and 3*f*). In this position $\text{Mg}_\text{B'}^{2+}$ is not capable of making an interaction with the attacking phosphate group of GlcNAc-1-P. $\text{Mg}_\text{B'}^{2+}$ makes one coordination interaction with the P_α of ATP and five more with water molecules to satisfy an octahedral geometry. Such a displacement in the position of Mg_B^{2+} was also observed in the crystal structures of RNA polymerase determined (in complex with various nucleotides) at higher and lower Mg^{2+} concentrations (Fig. 4). Together with a displaced Mg^{2+} , the nucleotide was also bound in an alternative conformation in this structure. These studies associated the displacement of Mg^{2+} and the alternative conformation of UTP with an inactive state of the RNA polymerase. The displacement of Mg^{2+} from a catalytically competent position to an inactive position was suggested to inhibit the activity of RNA polymerase (Wang *et al.*, 2006). In $\text{GlmU}^{\text{Mtb}}[\text{GlcNAc-1-P:ATP}]$, the third metal ion, Mg_C^{2+} , is also stabilized by one coordination interaction with P_α and five coordination interactions with water molecules. Its coordination by P_α results in the stabilization of an inactive conformation of ATP.

Overall, the entire metal–substrate complex renders the enzyme catalytically inactive. This structure could therefore be an initial ‘lead’ which can be further developed into an inhibitor acting at the uridyltransfer active site of the enzyme. Given that GlmU is an actively pursued target for antimicrobial drug discovery, these studies provide an alternate direction to the efforts towards structure-based drug design.

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Figure 4
A displaced Mg_B^{2+} is concurrent with the structure of an inactive RNA polymerase. Superposition of the structures of RNA polymerase determined at low (PDB entry 2nvz) and high Mg^{2+} concentrations (PDB entry 2yu9) in complex with UTP (Wang *et al.*, 2006). C atoms of UTP are coloured purple in the latter and yellow in the former (also indicated as high and low). Similarly, the corresponding Mg^{2+} ions are shown as light (low concentration) and dark (high concentration) green spheres. The two metal ions, Mg_A^{2+} and Mg_B^{2+} , are indicated and the displacement of Mg_B^{2+} is shown by a dashed line.

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