Published in final edited form as:

Biochemistry. 2017 August 01; 56(30): 3983–3992. doi:10.1021/acs.biochem.7b00189.

Conformational Control of UDP-Galactopyranose Mutase Inhibition

Kittikhun Wangkanont^{a,†}, Valerie J. Winton^a, Katrina T. Forest^{a,c,*}, and Laura L. Kiessling^{a,b,*}

^aDepartment of Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA

^bDepartment of Biochemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA

^cDepartment of Bacteriology University of Wisconsin-Madison, Madison, WI, 53706, USA

Abstract

UDP-galactopyranose mutase (Glf or UGM) catalyzes the formation of uridine 5'-diphosphate- α -D-galactofuranose (UDP-Gal β) from UDP-galactopyranose (UDP-Gal α). The enzyme is required for the production of Gal β -containing glycans. UGM is absent in mammals, but members of the *Corynebacterineae* suborder require UGM for cell envelope biosynthesis. The need for UGM in some pathogens has prompted the search for inhibitors that could serve as antibiotic leads. Optimizing inhibitor potency, however, has been challenging. The UGM from *Klebsiella pneumoniae* (KpUGM), which is not required for viability, is more effectively impeded by small-molecule inhibitors than are essential UGMs from species such as *Mycobacterium tuberculosis* or *Corynebacterium diphtheriae*. Why KpUGM is more susceptible to inhibition than other orthologs is not clear. One potential source of difference is UGM ortholog conformation. We previously determined a structure of CdUGM bound to a triazolothiadiazine inhibitor in the open form, but it was unclear whether the small molecule inhibitor bound this form or to the closed form. By varying the terminal tag (CdUGM-His₆ and GSG-CdUGM), we crystallized CdUGM to capture the enzyme in different conformations. These structures reveal a pocket in the active site that can be exploited to augment inhibitor affinity. Moreover, they suggest the inhibitor binds the open form of most prokaryotic UGMs but can bind the closed form of KpUGM. This model and the structures suggest strategies to optimize inhibitor potency by exploiting UGM conformational flexibility.

Graphical abstract

*Corresponding authors: Katrina T. Forest (Tel. 608-265-3566, forest@bact.wisc.edu) and Laura L. Kiessling (Tel. 608-262-0541, kiessling@chem.wisc.edu).

[†]Present address: Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

Supporting Information: Description of the remote sodium ion binding site and the corresponding figure (Figure S1).

Author Contributions: KW, KTF and LLK designed the study and analyzed the results. KW and VJW performed the experiments. KW, KTF, and LLK wrote the manuscript with input from VJW.

Conflict of Interest: The authors declare no competing financial interest.

Introduction

UDP-galactopyranose mutase (UGM) catalyzes the interconversion of UDP-galactopyranose (UDP-Galp) and UDP-galactofuranose (UDP-Galf) (Figure 1).¹⁻³ The latter serves as an activated sugar donor used by glycosyltransferases to synthesize Galf glycoconjugates. Galf residues are critical components of the galactomannan component of the *Aspergillus* cell wall⁴ and the essential galactan layer of the mycobacterial and corynebacterial cell walls.^{5, 6} Additionally, virulence factors of *Leishmania* contain Galf residues.^{7, 8} Although Galf-containing glycans in nematodes have yet to be identified, nematodes encode a UGM, and *Caenorhabditis elegans* expresses a functional UGM.⁹⁻¹¹ In contrast, Galf residues are not found in mammals nor do mammals possess UGM.¹² In many organisms that encode a UGM, deletion or downregulation of UGM production has deleterious consequences,^{9, 13} including lethality in mycobacteria.^{5, 6} Accordingly, UGM inhibitors block the growth of *Mycobacterium* and *Corynebacterium* species.¹⁴⁻¹⁷ These data suggest UGM is an attractive antimicrobial target, especially in *Mycobacterium tuberculosis* and drug-resistant *Corynebacterium diphtheriae*.¹⁸

Effective UGM inhibitors can probe the role of UGM in different organisms¹⁷ and serve as potential antimicrobial leads. Many known inhibitors are UDP-Galf analogs that bind UGM *in vitro* but have not been shown to function in cells.¹⁹⁻²⁶ Our group identified non-substrate analogue 2-aminothiazoles as some of the most potent UGM inhibitors described to date¹⁴ (Figure 1). These compounds, however, exhibited some toxicity to mammalian cells and were difficult to optimize.^{15, 27} Through virtual screening, we found a family of triazolothiadiazine inhibitors (Figure 1) that possess improved physical properties and that are active against *Mycobacterium tuberculosis*.¹⁵ We sought to optimize these inhibitors further by understanding the molecular basis of their affinity for UGM.

We used X-ray crystallography to determine the structure of a triazolothiadiazine inhibitor bound to *Corynebacterium diphtheriae* UGM (CdUGM).¹⁵ The complex adopted an open conformation and not the closed form observed for substrate-bound *Klebsiella pneumoniae* UGM (KpUGM)²⁸ used in the virtual screen. The triazolothiadiazine inhibitor was bound in the active site but not in the orientation of the lowest energy pose in the closed complex. We detected unmodeled electron density peaks under the opened lid, which might represent alternate conformation(s), in which the lid is closed over the active site. Refined occupancies of 0.81 and 0.87 for the inhibitor in each active site of the biological dimer suggest, however, that the closed conformation may be of the unliganded state. These data suggest that the UGM-inhibitor complexes can adopt multiple conformations and raise uncertainty about the preferred inhibitor binding modes.¹⁵ Given that inhibitor affinity differs among UGM

orthologs, we sought to analyze variations in UGM conformation and extrapolate their consequences on inhibitor binding.

All of the small-molecule, heterocyclic UGM inhibitors studied to date are more potent against KpUGM than against other UGM orthologs tested.^{14, 15} For example, the K_i value of the triazolothiadiazine inhibitor against KpUGM is $8 \pm 3 \mu\text{M}$, a value about 4-fold better than that for *M. tuberculosis* (MtUGM) ($31 \pm 18 \mu\text{M}$) and 10-fold better than that for CdUGM ($77 \pm 37 \mu\text{M}$). Other analogs show larger differences, as illustrated by the different K_i values for compound 3 (Figure 1) for KpUGM ($1.1 \pm 0.2 \mu\text{M}$) and CdUGM ($29 \pm 8 \mu\text{M}$) respectively.¹⁵ Indeed, superior inhibitory activity against KpUGM is observed for the triazolothiadiazine inhibitors as well as the 2-aminothiazole inhibitors tested.^{14, 15} The active sites of the orthologous enzymes are similar. Neither sequence variation nor structural studies to date reveal a molecular basis for the affinity differences. Understanding these differences, however, could guide the development of more potent inhibitors that serve as leads for pulmonary diseases caused by non-tuberculous mycobacteria and tuberculosis. Attempts to crystallize KpUGM with inhibitors yielded crystals exhibiting lattice-translocation disorder coupled with translational pseudosymmetry²⁹ (unpublished results). Neither the conformation of the mobile lid in KpUGM nor the inhibitor occupancy could be determined. The challenges that arose in obtaining a structure of KpUGM bound to an inhibitor led us to re-examine the structure of CdUGM. We had obtained a structure of a complex with this ortholog, and we reasoned that using X-ray crystallography to capture its conformational features might illuminate differences between CdUGM and KpUGM.

UGM orthologs are conformationally dynamic. Both prokaryotic and eukaryotic UGMs possess a mobile lid that can close over the active site.^{26, 28, 30} In the open form, the lid lacks secondary structure and is positioned away from the active site. In the closed conformation, the lid adopts a helical conformation that positions a conserved arginine to interact with the substrate UDP-Galp.³¹ Comparison of oxidized and reduced KpUGM structures showed reduction *in crystallo* accompanied a move in the mobile lid toward the substrate.²⁸ Minor variations in lid conformation have also been reported in the structures of other prokaryotic UGMs. Though the position of the conserved arginine in KpUGM²⁸ suggests the residue mainly interacts with the pyrophosphoryl group of the UDP-Galp substrate, in the *Deinococcus radiodurans* (Dr) UGM, the corresponding arginine interacts with the pyrophosphoryl group and the galactopyranose residue.³⁰ Studies of the *M. tuberculosis* UGM with a non-substrate inhibitor suggest there is an allosteric binding pocket near the active site and that occupation of this pocket prevents loop closure.³² Eukaryotic UGMs also adopt multiple conformations, and they have a second mobile flap containing an asparagine residue involved in substrate recognition.^{33, 34} Additionally, molecular dynamics studies indicate a third mobile loop near the active site in the *Trypanosoma cruzi* UGM (TcUGM).³⁴ Dramatic conformational changes are also observed in a histidine-containing loop in eukaryotic UGMs.^{33, 34} These observations highlight the flexibility of UGMs and suggest that progress in understanding UGM conformational variation would advance inhibitor design.

To determine structures of CdUGM in distinct conformations, we sought to crystallize the enzyme in multiple crystal forms. Because residues at protein termini tend to participate in

crystal packing and impose alternative structural restrains, they can dramatically affect crystallization.³⁵ We hypothesized that by varying the tag we could obtain different crystal forms of CdUGM and thereby gain insight into the accessible conformational states of the enzyme. We therefore produced CdUGM variants with a hexahistidine tag at the C-terminus (CdUGM-His₆) or a three-amino acid peptide linker at the N-terminus (GSG-CdUGM). Crystallization of these fusion proteins afforded two new crystal forms of CdUGM. The resulting structures shed light on the conformational dynamics of UGM and provide new information to guide inhibitor development.

Materials and Methods

CdUGM-His₆ complexed to sodium citrate

The sequence encoding residues 1-387 of CdUGM was amplified using primers 5'-CGAGCAATTGACCAACAAGGACCATAGATTA-TGTCTGACTTTGATCTGATCGTGGTAGGT-3' and 5'-ATTCGAGCTCTCATTAAATGG-TGATGGTGGTGTATTTTCAGGGCGTCGACAAGCTTGTTAT-3'. The PCR product was then digested and cloned into the MfeI and SalI sites of pMALc5x. The resulting construct coded for CdUGM with a C-terminal His₆ tag with no linker (CdUGM-His₆). CdUGM-His₆ was produced and purified using previously reported protocols.^{15, 36} Protein was dialyzed against 20 mM Tris pH 7.0 and concentrated to 10 mg/mL. The 2-aminothiazole inhibitor¹⁴ was added to a final concentration of 1 mM (from 40 mM stock in isopropanol). This inhibitor has a K_i for CdUGM of 4.9 ± 1.5 μ M, as determined using a previously reported HPLC-based assay³⁷. Crystals were grown by hanging drop vapor diffusion using 2 μ L of protein solution and 2 μ L of the well solution (100 mM sodium citrate pH 5.6, 15% isopropanol, and 16-18% PEG 5000 MME), reaching maximal size within three to four days. Crystals were cryoprotected by briefly soaking in the well solution containing 1 mM 2-aminothiazole and 20% ethylene glycol before vitrification and storage in liquid nitrogen. Single crystal X-ray diffraction data were collected at beamline 21-ID-F (LS-CAT) at Argonne National Laboratory using a MAR225 CCD detector and processed using HKL2000.³⁸ Molecular replacement was performed with Phaser³⁹ using a previously reported KpUGM monomer structure in an open conformation (PDB 2BI7).⁴⁰ Restraints for small molecule ligands were generated using eLBOW.⁴¹ Model adjustment and refinement were performed with Coot and phenix.refine, respectively.^{42, 43} The model was validated using MolProbity.⁴⁴ Structure figures were generated with PyMOL (v. 1.7, Schrödinger LLC). Data collection and refinement statistics are shown in Table 1. During refinement, the Na⁺-O bond length was restrained to 2.43 Å with tolerance of 0.25 Å.

To determine the sodium ion identity, crystals were grown as described above then briefly washed and soaked for 24 hours in 100 mM potassium citrate pH 5.6, 15% isopropanol, and 18% PEG 5000 MME without the 2-aminothiazole inhibitor. Cryoprotection was carried out as described above, with potassium citrate replacing sodium citrate. Single crystal X-ray diffraction data were collected at beamline 14-1 at Stanford Synchrotron Radiation Lightsource using a MAR325 CCD detector (Table 1). These data were analyzed using difference Fourier techniques; no refinement was carried out.

The coordinates and structure factors for CdUGM-His₆ in complex with sodium citrate have been deposited to the Protein Data Bank under the accession code 5BR7. Structure factors for the potassium citrate-soaked crystal of CdUGM-His₆ crystal were appended as the second data set under the same accession code.

GSG-CdUGM complexed to UDP

Site-directed mutagenesis was performed on a previously reported CdUGM construct (G-CdUGM)¹⁵ to add a short linker (SerGly) after the glycine of the tobacco etch virus (TEV) cleavage site for more efficient cleavage (GSG-CdUGM). The primers used were 5'-AGCGAAAACCTGTATTTTCAGGGTAGCGGCATG-TCTGACTTTGATCTGATCGTGGTAG-3' and 5'-CTACCACGATCAGATCAAAGTCAG-ACATGCCGCTACCCTGAAAATACAGGTTTTTCGCT-3'. GSG-CdUGM was purified using previously reported protocols.^{15, 36} Protein was dialyzed against 20 mM Tris pH 7.0 and concentrated to 10 mg/mL. UDP-galactopyranose (UDP-Galp) was added to a final concentration of 10 mM (from 100 mM stock in water). Crystals were grown by hanging drop vapor diffusion using 2 µL of protein solution and 2 µL of the well solution (100 mM Bis-Tris pH 5.5, 200 mM lithium sulfate, and 21% PEG 3350) and reached maximal size in 1-2 weeks. Cryoprotection was achieved by brief soaking in the well solution with PEG 3350 supplemented to 35%. Crystals were then vitrified and stored in liquid nitrogen. Single crystal X-ray diffraction data were collected at beamline 21-ID-G (LS-CAT) at Argonne National Laboratory using a MAR300 CCD detector. Data processing, phasing, and refinement were carried out using the procedures and programs described for CdUGM-His₆. Molecular replacement was performed using a structure of *Mycobacterium smegmatis* UGM (MsUGM) in closed conformation (PDB 5EQD) because the opened KpUGM structure (PDB 2BI7)⁴⁰ used as a search model did not yield a molecular replacement solution. During refinement, torsional non-crystallographic symmetry restraints were applied to improve the geometry of chain B. Several combinations of translation-libration-screw (TLS) groups suggested by the TLSMD server were initially examined^{45, 46}. The optimal refinement strategy with lowest R-factor and R-free involves splitting chain B into four TLS groups (residues 2-62, 63-235, 236-316, and 317-386) with chain A as a single TLS group. Data collection and refinement statistics are shown in Table 1. The coordinates and structure factors of GSG-CdUGM have been deposited under the accession code 5EQF.

Results

Structure of CdUGM-His₆

To obtain structures of CdUGM in various conformations to contrast with the triazolothiadiazine-bound structure, two new CdUGM fusion proteins were crystallized. The first, CdUGM-His₆, afforded tetragonal crystals with one molecule in the asymmetric unit, corresponding to one half of the UGM dimer. Each crystallographically equivalent monomer is in an open conformation (Figure 2A). In this structure, the space where the substrate normally binds is occupied by an unexpected ligand—citrate. As expected for an open conformation, the mobile lid is not helical. The conserved arginine (R176) that normally contacts the pyrophosphoryl group of the substrate does not interact with the citrate ion

(Figure 2A). A comparison with the structure of the CdUGM–triazolothiadiazine complex (PDB 4XGK) gave an overall C α RMSD of 0.88 over 386 residues. Thus, the conformation of the citrate-bound CdUGM and the small molecule-bound CdUGM are similar (Figure 2B).

The citrate ion was identified on the basis of its shape and bonding partners and is located in the active site cavity adjacent to the FAD cofactor (Figure 3A). Citrate, which was present in the crystallization buffer, is anionic like the UDP-Galp substrate. We analyzed the structure to evaluate features of the active site that may be exploited for future inhibitor design. The citrate ion forms salt bridges with UGM residues R288 and H290 and numerous ordered water molecules, thereby participating in a hydrogen-bonding network with the active site residues. A strong density feature was observed bridging the citrate ion and the oxidized FAD (Figure 3B). The absence of peaks in the mF_o-DF_c map suggested the possibility that this density was the oxygen atom of a water molecule; however, irregular octahedral coordination geometry, bond lengths of approximately 2.4 Å⁴⁷ (Figure 3A), and proximity to the negatively charged citrate ion suggest that the peak corresponds to a sodium ion. An additional distal sodium ion binding site was also identified (Figure 2A and S1). A potassium citrate buffer yielded no crystals, suggesting that the sodium ions were important for crystallization. When crystals grown with sodium citrate were soaked in potassium citrate buffer, a loss of electron density was observed for the active site sodium and citrate ions, as judged by the difference Fourier map (Figure 3C). These data suggest that it is a sodium ion coordinated by FAD, citrate ion, and the backbone carbonyl of A60. The likely presence of a cation near the flavin cofactor is intriguing, as the transition state for catalysis may involve flavin capture of an oxocarbenium ion.^{1, 48}

GSG-CdUGM bound to UDP

A second CdUGM variant, GSG-CdUGM, was generated from cleavage of an N-terminal fusion protein. The wild-type enzyme bearing an N-terminal glycine-serine-glycine sequence crystallized in its dimeric state in the presence of UDP-Galp (Figure 4). The electron density of chain A is well-defined. Chain B has elevated B factors and several weak side chain densities, although the backbone density could be unambiguously traced. Both monomers are in a closed conformation with the lid residues adopting helical structures. During the course of crystallization, UDP-Galp was apparently converted to UDP. We considered the possibility that Galp was disordered, but the B factor for the β phosphate is not elevated relative to the α phosphate and the electron density difference maps around UDP revealed no evidence for the presence of galactose. The observed hydrolysis is consistent with the mechanistic proposal that UDP functions as a leaving group in the interconversion of UDP-Galp and UDP-Galf.¹

We previously found differences in the conformations of oxidized and reduced KpUGM – substrate complexes, raising the possibility that reduction of FAD triggers lid movement onto the active site occupied by UDP-Galp.²⁸ However, in the oxidized GSG-CdUGM structure, both monomers adopt a closed conformation. Each active site contains UDP, a putative intermediate in the catalytic mechanism. When the GSG-CdUGM structure is superimposed with the catalytically active, reduced, closed KpUGM,²⁸ the positions of

active site residues are conserved (Figure 5). Underneath the closed helical lids, UDP fits snugly in the active site of GSG-CdUGM (Figure 5A) with uracil ring stacking onto the π face of Y157. Residues I174 and L177, along with F98 and I154, generate a hydrophobic box around the uracil moiety. The N3H of uracil forms a hydrogen bond with the backbone carbonyl of F153, and the O8 is recognized by the amide side chains of N278 and N280. The hydroxyl group of T158 forms hydrogen bonds with both O7 of uracil and 2-hydroxyl group of ribose. The indole NH of W162 engages in hydrogen bonding with the 3'-hydroxyl group of ribose. The pyrophosphoryl group is recognized by the side chains of R176 (from the lid), Y187, R288, Y326 and Y364. Upon superposition of UDP-Galp-bound closed KpUGM onto the structure of GSG-CdUGM, the UDP-Galp substrate from the KpUGM structure fits well into the pocket of closed GSG-CdUGM (Figure 5B). Moreover, the superimposed anomeric carbon of Galp is oriented as expected for catalysis: it is 3.4 Å away from the nucleophilic nitrogen of FAD, which is comparable to the 3.6 Å difference observed in KpUGM. Thus, we conclude that the GSG-CdUGM adopts a conformation similar to that of other catalytically active UGMs.

Discussion

Despite the availability of dozens of UGM structures, the only structure determined with a non-substrate analog bound is that of CdUGM.¹⁵ Here, our crystallization of CdUGM with unique termini resulted in two new crystal forms that provide information about the relationship between UGM conformation and ligand binding. The structure of CdUGM-His₆ identifies an unexpected citrate-Na⁺-FAD complex, which binds to the open form of CdUGM. In the structure of CD-UGM bound to triazolothiadiazine inhibitor 2,¹⁵ the enzyme also adopts an open conformation, but the presence of residual density hinted that the inhibitor might also bind the lid-closed conformation of CdUGM.¹⁵ The conformation of the UGM complex is similar to that previously found for the enzyme bound to the triazolothiadiazine inhibitor.¹⁵ Thus, the available data support the relevance of the open form of the complex. At the pH of the crystallization buffer, citrate exists predominantly as a dianion; therefore, it is of note that the mobile lid does not descend to allow the conserved arginine to neutralize the charge. Similarly, with carboxylate 2, the mobile lid remains open. Interaction of the mobile loop arginine with the pyrophosphate moiety may be more favorable, thereby favoring lid closure upon substrate binding.

The structure of the CdUGM-His₆ – sodium citrate complex features an active site pocket that could be targeted by citrate-like small molecules. We therefore estimated the citrate dissociation constant. At a citrate concentration of 100 mM, the citrate was not at fully occupancy in the crystallized complex. The dissociation constant (K_d) can be estimated using a simple equilibrium model and expressing [protein] and [protein:ligand] in occupancy terms ($K_d = [\text{CdUGM-His}_6] [\text{citrate}] / [\text{CdUGM-His}_6\text{-citrate}] = 0.08 \times 100 \text{ mM} / 0.92 = 8.7 \text{ mM}$), which provides a value in the range of 10 mM. This estimated K_d is in the range relevant for molecular fragments that can result in useful ligands. This value is also the order of the intracellular citrate concentration in *Escherichia coli* (ranging from 2-20 mM depending on growth media).⁴⁹ Cellular citrate therefore has the potential to influence UGM conformation and regulate the enzyme's activity.

The citrate-bound structure could facilitate the design of novel inhibitors because citrate can serve as a steric and electronic probes of protein-small molecule interactions.⁵⁰ In the context of the CdUGM complex, either the triazolothiadiazine inhibitor or the citrate ion can form a salt bridge to the guanidinium group of R288 (Figure 6), but each occupies a slightly different region of the active site. Specifically, the small citrate ion binds deeper into the active site than does the larger inhibitor, suggesting triazolothiadiazine analogs that extend into the citrate-binding site could bind more tightly. Indeed, this possibility is supported by the increased efficacy of small-molecule inhibitors in which the carboxylate is replaced by a larger *N*-acylsulfonamide group.¹⁶

The binding site identified in the CdUGM citrate complex differs from that occupied by an allosteric inhibitor identified recently. Specifically, a small-molecule heterocycle (MS-208) that differs in structure from the inhibitors described herein was reported to inhibit MtUGM ($K_i \sim 400 \mu\text{M}$).³² The kinetics of inhibition together with computational and mutagenesis studies suggested that MS-208 does not compete directly with the substrate UDP-Gal p but rather binds in an allosteric site. The presence of multiple subsites highlights the flexibility of UGM.

The molecular basis of our observation that triazolothiadiazine inhibitors 2 and 3, as well as our other non-substrate inhibitors, are more effective against KpUGM than other orthologs is unclear. We considered the possibility that different inhibitors might bind open and/or closed forms and that protein conformational equilibrium could influence inhibitor potency. With the GSG-CdUGM structure obtained herein, we were poised to compare the closed form of CdUGM with that of KpUGM. To this end, we superimposed the closed structure of KpUGM containing the predicted lowest energy orientation of an inhibitor¹⁵ onto the closed GSG-CdUGM structure. As predicted from conservation of the active site, no obvious clashes were detected with CdUGM (Figure 7A).¹⁵

The orientation of the triazolothiadiazine inhibitor **2** inhibitor in the CdUGM complex¹⁵ was not predicted by computational docking to be the most favorable (of lowest energy) (Figure 7A). To examine how this inhibitor orientation might impact binding, we superimposed the crystallographically determined inhibitor orientation on the closed CSG-CdUGM structure (Figure 7B). The inhibitor occupies a space that would clash with the position of lid residue R176 (Figure 7B). This conserved positively charged side chain^{31,3}, is present in all UGM orthologs and interacts with the substrate through hydrogen bonding to galactose hydroxyl groups and/or the pyrophosphoryl group.^{26, 28, 30, 33, 51} This “dynamic arginine (R176)”³¹ may adopt an alternate side chain rotamer to alleviate the steric clash with the triazolothiadiazine inhibitor (Figure 7B). Although the flexibility of R176 could differ between UGMs, our data suggest that it is flexible in both KpUGM and CdUGM. Specifically, in the closed KpUGM structure (PDB 3INT), the temperature factors of the closed lid and R176 were higher than those in the rest of the protein. Similarly in the CSG-CdUGM, two side chain rotamers for R176 were present in the open structure (Figure 2A). In contrast, distinct differences in the position of a different residue, Y364, were detected in closed conformations of CdUGM and KpUGM. In GSG-CdUGM, this residue was in the active site, such that the side chain phenol ring was twisted almost perpendicular to its orientation in the inhibitor-bound open structure (Figure 7B). In this way, Y364 may prevent

the inhibitor from binding CdUGM in the closed conformation. A similar inward-oriented position of the Y364 equivalent was observed in the closed structure of MtUGM²⁶. Notably, no such conformational change of the corresponding residue (Y349) was detected in KpUGM (Figure 7C)²⁸. We also noted that N173 in CdUGM (N177 in MtUGM) was replaced in KpUGM with an isoleucine that may favorably interact with the hydrophobic chlorophenyl ring of the inhibitor to increase affinity (Figure 7C). These data suggest that the orientation of the CdUGM complex determined by X-ray crystallography (PDB 4XGK), with the triazolothiadiazine inhibitor binding to the open form of CdUGM, reflects the mode of inhibitor binding to *C. diphtheriae* and *M. tuberculosis*. These results highlight the relevance of the CdUGM-inhibitor complex for advancing inhibitor design.

Often the most efficacious inhibitors are those that bind the closed form over the open form of an enzyme. For example, the inhibitor imatinib targets Abl kinase over other closely related kinases because imatinib interacts with the conformation of Abl in which the activation loop is closed.⁵² In analogy, inhibitor **2** may bind the closed form of KpUGM but the open form of CdUGM, MtUGM, or other UGM orthologs. Though we cannot rule out binding to the open form of the KpUGM, we expect that binding to the closed complex would be of higher affinity than binding to the open state. All of the known X-ray structures of prokaryotic UGM orthologs (CdUGM, MtUGM, and DrUGM,³⁰ with the exception of KpUGM, indicate a Y364 undergoes conformational change. This change is coupled to lid closing and would preclude inhibitor binding to the closed state.

The amino acid residue differences between prokaryotic and eukaryotic UGM active sites are large, and further study is needed to understand molecular basis for differences in inhibitor binding between prokaryotic and eukaryotic enzymes.³¹ For example, there are at least two mobile lids covering the active site in eukaryotic UGM, compared to one in prokaryotic UGMs³¹, which complicates the analysis of the basis for differences in inhibitor affinity. Still, the potencies of 2-aminothiazole small-molecule inhibitors against the *C. elegans* UGM are superior to those observed for CdUGM and MtUGM and similar to those observed against KpUGM.¹¹ Consistent with the prediction from the analysis here, the residue corresponding Y364 in eukaryotic UGMs (AfUGM³³ and TcUGM³⁴) is similar to KpUGM in that no dramatic change in position is observed in the closed conformation of the eukaryotic UGMs.

The role of UGM cofactor reduction is another key issue for the enzyme's conformation and therefore inhibition. Reduction of the FAD cofactor is required for UGM activity³. The isoalloxazine ring of oxidized FAD is planar but upon reduction adopts a puckered, butterfly conformation. In crystals of KpUGM in complex with the substrate UDP-Galp, the flavin could be reduced within the same crystal form, a process that accompanied further closure of the lid²⁸. Our structure of oxidized GSG-CdUGM in complex with UDP indicates the inactive enzyme can adopt a closed conformation analogous to that observed for the catalytically competent KpUGM. Other closed UGM orthologs have been crystallized with oxidized FAD either with or without bound substrates, including DrUGM³⁰, EcUGM,³ AfUGM⁵¹, and TcUGM³⁴. These findings suggest that the energy barrier for conformational change is small, regardless of the FAD oxidation state. Thus, low barrier conformational change could be universal attribute of UGMs, raising the possibility that inhibitors could

target specific UGM conformational states. Inhibitor trapping of UGM in an inactive conformation could be an effective inhibition strategy.³² Indeed, many kinase inhibitors bind to and stabilize an inactive conformation of their target enzymes⁵³⁻⁵⁶. Our data thus far suggest inhibitors that can occupy the closed, oxidized state of the enzyme might be especially effective. They would not only prevent substrate binding but also limit the access of reducing agents to the isoalloxazine ring thereby preventing generation of the catalytically competent state.

Efforts to understand UGM structural dynamics are opening new possibilities for improving inhibitor potency. The dramatic variation in UGM lid conformations³¹ suggests that inhibitors could be generated that specifically block particular UGM orthologs. Such inhibitors will be useful for targeting pathogens uniquely. Moreover, optimizing and identifying small molecules that can be accommodated in the closed, oxidized form of UGM should lead to next-generation inhibitors with enhanced efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817). Use of the Stanford Synchrotron Radiation Lightsource (SLAC National Accelerator Laboratory) is supported by the DOE Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research and by the NIH NIGMS (grant P41GM103393). We thank Dr. Annie Héroux (Brookhaven National Laboratory) for measuring preliminary X-ray diffraction data at beamline X25 of the National Synchrotron Light Source, which is supported principally by the DOE Offices of Biological and Environmental Research and Basic Energy Sciences, the National Center for Research Resources (P41RR012408) and the NIGMS (P41GM103473). We thank Dr. Robert A. Brown and Virginia A. Kincaid for helpful comments during manuscript preparation.

Funding: This research was supported by National Institutes of Allergy and Infectious Disease R01 AI063596 and R01 AI126592 (to LLK) and by National Science Foundation Grant IOS1353674 (to KTF and H. Goodrich-Blair). KW was supported by a fellowship from the Development and Promotion of Science and Technology Talents Project of Thailand. VJW was supported by an NSF Graduate Research Fellowship (DGE-1256259).

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Abbreviations

UGM	UDP-galactopyranose mutase
UDP	uridine 5'-diphosphate
Gal^f	galactofuranose
Gal^p	galactopyranose
EG 5000 MME	polyethylene glycol 5,000 monomethyl ether
FAD	Flavin adenine dinucleotide

Figure 1.

UGM catalyzes the interconversion of UDP-Galp and UDP-Galf, and the enzyme can be inhibited by 2-aminothiazoles, such as **1**, or triazolothiadiazines, such as **2** and **3**.

Figure 2.

(A) Structure of CdUGM-His₆ in complex with sodium (purple) citrate (green). The enzyme is in an open conformation, as noted by the relationship of the mobile lid (arrows) to the oxidized FAD-containing active site (square). The circle indicates the position of a remote sodium ion-binding site. (B) Structure of the CdUGM–triazolothiadiazine complex (PDB 4XGK). The open conformation of the mobile lid is indicated with arrows.

Figure 3.

Depictions of the active site of the CdUGM-His₆ complex with citrate (A) The sodium ion (purple) is complexed to the flavin (dark grey) and citrate (green). (B) The green mesh corresponds to the $mF_o - DF_c$ map contoured at 3σ after the citrate ion was removed and structure refined with simulated annealing. (C) Structure factor amplitudes collected from a crystal soaked in potassium citrate-containing buffer lead to electron density decreases. The red mesh corresponds to an $mF_o - DF_c$ difference Fourier map contoured at -3σ .

Figure 4. Structure of dimeric GSG-CdUGM. The closed lids form short helices covering the active sites (chain A in grey, lid boxed, and chain B in wheat, lid obscured).

Figure 5.

Comparison of the conformations of oxidized GSG-CdUGM bound to UDP and reduced KpUGM bound to UDP-Galp. (A) Stereo view of active site of GSG-CdUGM (chain A, grey) with bound UDP (cyan). All bond distances shown are ~ 3.1 Å. (B) Oxidized UGM-UDP complex adopts a closed conformation similar to catalytic conformation. Stereo view of position of UDP-Galp (cyan) from a structure of catalytically active KpUGM (PDB 3INT) superimposed onto GSG-CdUGM (grey) with bound UDP (not shown).

Figure 6. Structure of the triazolothiadiazine inhibitor (orange) from a previously reported co-crystal structure with CdUGM (PDB 4XGK) superimposed onto the structure CdUGM-His₆ (grey) in complex with sodium citrate (purple and green, respectively). The clash between the carboxylates from the inhibitor and citrate is indicated with a zigzag line.

Figure 7.

A comparison of known and docked small-molecule inhibitor complexes highlighting Y364 (black) movement. All superpositions were performed using protein α -carbons with refinement to optimize the fit. Changes in the lid conformation between open and closed forms are denoted with asterisks (closed form, panel A) or a row of arrows (open form, panel B). (A) Computational model of triazolothiadiazine inhibitor (orange) complex derived from the closed form of KpUGM (PDB 3INT) superimposed on the structure of the closed form of GSG-CdUGM (grey). (B) Orientation of the inhibitor (orange) in the crystalline CdUGM complex (light blue, PDB 4XGK), which is in the open form. Superimposed on this complex is the closed form of GSG-CdUGM (grey). A likely alternative rotamer for R176 is shown. The Y364 (black) that clashes with the inhibitor is highlighted. (C) Superposition of the closed GSG-CdUGM (grey) structure with the closed form of KpUGM (green) (PDB 3INT) with the crystallized pose of the triazolothiadiazine inhibitor (orange). CdUGM residue numbering is shown.

Table 1
Data collection and refinement statistics

Data collection statistics	CdUGM-His ₆ -sodium citrate complex	CdUGM-His ₆ soaked in potassium citrate buffer	GSG-CdUGM – UDP complex
Wavelength (Å)	0.97872	1.18080	0.97856
Resolution range (Å) [*]	46.72 - 1.95 (2.02 - 1.95)	49.01 – 2.35 (2.43 - 2.35)	29.1 - 2.15 (2.23 – 2.15)
Space group	P 4 ₁ 2 ₁ 2	P 4 ₁ 2 ₁ 2	C2
Unit cell	98.3 98.3 126.2	98.0 98.0 126.3	115.6 82.7 108.6 90 112.6 90
Total reflections	1,075,063 (89,525)	378,033 (33,333)	376,968 (26,894)
Unique reflections	45,492 (4,454)	26,343 (2,584)	50,316 (4,637)
Multiplicity	23.6 (20.1)	14.4 (12.9)	7.5 (5.8)
Completeness (%)	99.9 (99.6)	100.0 (100.0)	97.5 (90.5)
Mean I/σ(I)	36.3 (3.6)	24.0 (3.0)	27.4 (2.8)
Wilson B-factor (Å ²)	29.3	37.0	41.9
R-merge	0.103 (0.756)	0.127 (0.971)	0.064 (0.419)
R-meas	0.105 (0.775)	0.123 (>1)	0.069 (0.458)
R-pim	0.020 (0.164)	0.035 (0.280)	0.025 (0.181)
Highest shell CC1/2	(0.923)	(0.801)	(0.956)
Refinement statistics			
Resolution range (Å) [*]	- 1.95 (2.02 -1.95)		28.25 – 2.15 (2.22 – 2.15)
R-factor	0.1528 (0.1982)		0.1765 (0.2485)
R-free (10%)	0.1895 (0.2267)		0.2281 (0.3009)
Number of atoms			
protein	3302		6,344
FAD	53		106
UDP	-		50
Citrate	13		-
Na ⁺	2		-
water	458		228
Protein residues	386		771
RMSD (bonds, Å)	0.008		0.008
RMSD (angles, °)	1.05		1.192
Est. coordinate error (ML, Å)	0.17		0.25
Ramachandran favored (%)	98.8		98.4
Ramachandran outliers (%)	0		0
Average Isotropic B-factor(Å ²)			
Chain A			
protein	31.6		48.1
FAD	26.5		52.1
UDP	-		37.4
Citrate	32.3		-

Data collection statistics	CdUGM-His ₆ -sodium citrate complex	CdUGM-His ₆ soaked in potassium citrate buffer	GSG-CdUGM – UDP complex
Na ⁺	30.2		-
water	41.2		49.6
Chain B			
protein	-		69.6
FAD	-		85.3
UDP	-		56.8
water	-		51.7

* Statistics for the highest-resolution shell are shown in parentheses.