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Review

Structure, mechanism, and dynamics of UDP-galactopyranose mutase

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ABSTRACT

The flavoenzyme UDP-galactopyranose mutase (UGM) is a key enzyme in galactofuranose biosynthesis. The enzyme catalyzes the 6-to-5 ring contraction of UDP-galactopyranose to UDP-galactofuranose. Galactofuranose is absent in humans yet is an essential component of bacterial and fungal cell walls and a cell surface virulence factor in protozoan parasites. Thus, inhibition of galactofuranose biosynthesis is a valid strategy for developing new antimicrobials. UGM is an excellent target in this effort because the product of the UGM reaction represents the first appearance of galactofuranose in the biosynthetic pathway. The UGM reaction is redox neutral, which is atypical for flavoenzymes, motivating intense examination of the chemical mechanism and structural features that tune the flavin for its unique role in catalysis. These studies show that the flavin functions as nucleophile, forming a flavin–sugar adduct that facilitates galactose-ring opening and contraction. The 3-dimensional fold is novel and conserved among all UGMs, however the larger eukaryotic enzymes have additional secondary structure elements that lead to significant differences in quaternary structure, substrate conformation, and conformational flexibility. Here we present a comprehensive review of UGM three-dimensional structure, provide an update on recent developments in understanding the mechanism of the enzyme, and summarize computational studies of active site flexibility.

Introduction

Galactofuranose (Gal*f*) is the five membered ring form of the more common 6-membered ring sugar galactopyranose (Gal*p*). Gal*f* is thermodynamically less stable than Gal*p* because of the strain associated with the 5-membered ring. Nevertheless, it has been known for nearly a century that microorganisms produce Gal*f* in several forms. For example, galactocarolose, an extracellular β-D-(1 → 5)-linked polygalactofuranose produced by *Penicillium charlesii*, was the first polysaccharide shown to contain Gal*f* [1], motivating interest in the underlying biosynthetic pathway. Decades later, it was shown that *P. charlesii* could not use exogenous galactose to produce galactocarolose [2], leading to the discovery of a new nucleotide, UDP-Gal*f*, as the precursor in galactocarolose biosynthesis [3]. Similarly, investigations into the origins of Gal*f* in the T1 antigen of *Salmonella typhimurium* identified UDP-Gal*p* in the biosynthetic pathway and suggested the existence of an enzyme that catalyzes the 6-to-5 ring contraction of UDP-Gal*p* to UDP-Gal*f* [4,5]. Interest in Gal*f* biosynthesis stems in part from the observation that this sugar is not present in mammals; however, it is a major component of cell wall and cell surface glycoconjugates in many bacteria and eukaryotic organisms, including the human pathogens *Mycobacteria tuberculosis*, *Klebsiella pneumoniae*, *Trypanosoma cruzi*, *Leishmania major*, and *Aspergillus fumigatus* [6–10]. Targeting cell wall biosynthesis is an effective and well-established method for combating bacterial infections. Since Gal*f* is absent in humans, the enzymes involved in the biosynthesis of Gal*f* are potential drug targets. At the center of Gal*f* biosynthesis is the enzyme UDP-galactopyranose mutase (UGM).[1] The gene encoding for a UGM enzyme was first cloned from

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[1] *Abbreviations used*: UGM, UDP-galactopyranose mutase; Gal*p*, galactopyranose; Gal*f*, galactofuranose; UDP-Gal*p*, UDP-galactopyranose; UDP-Gal*f*, UDP-galactofuranose; EcUGM, UDP-galactopyranose mutase from *Escherichia coli*; KpUGM, UDP-galactopyranose mutase from *Klebsiella pneumoniae*; DrUGM, UDP-galactopyranose mutase from *Deinococcus radiodurans*; AfUGM, UDP-galactopyranose mutase from *Aspergillus fumigatus*; TcUGM, UDP-galactopyranose mutase from *Trypanosoma cruzi*; bUGM, bacterial UDP-galactopyranose mutase; eUGM, eukaryotic UDP-galactopyranose mutase; RMSD, root mean square deviation; PIX, positional isotope effects; PDB, Protein Data Bank; MD, molecular dynamics.

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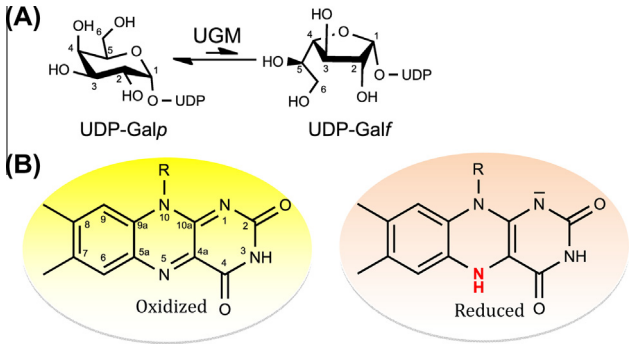

Scheme 1. Reaction catalyzed by UGM (A) and structures of the oxidized and reduced flavin cofactor (B).

Escherichia coli in 1996 and given the name *glf* [11], paving the way for detailed structure–function studies that have continued to this day and are the subject of this review.

UGM is flavoenzyme that catalyzes the interconversion of UDP-Gal*p* and UDP-Gal*f* (Scheme 1A). The equilibrium of the UGM-catalyzed reaction favors UDP-Gal*p* by the ratio of 11:1 because of the aforementioned ring strain associated with galactofuranose [11]. Following the cloning of the UGM gene from *E. coli* [11], UGMs from other bacteria, fungi, and parasites have been identified [6,12–14]. Deletion of the gene encoding for UGM in *M. tuberculosis* demonstrated that this enzyme is essential for growth, whereas in *A. fumigatus* and *L. major*, UGM is an important virulence factor [15–17]. Validation of UGM as a drug target has prompted extensive structural and mechanistic studies leading to the elucidation of the chemical mechanism. UGMs have a novel 3-dimensional structure that tunes the flavin cofactor to function as a nucleophile and a scaffold in catalysis. Here, we review the chemical mechanism of UGMs and the structural changes that are required for activity. These unique mechanistic features can be exploited for the development of specific drugs against several bacterial and eukaryotic human pathogens.

Chemical mechanisms of UGM

Catalytic mechanism of UGM, a noncanonical flavoenzyme

Nassau et al. discovered that *E. coli* UGM is a flavoenzyme [11], and indeed all UGMs characterized to date contain flavin adenine dinucleotide (FAD). Flavoenzymes typically catalyze oxidation–reduction reactions with the flavin serving as the redox center, and thus, the role of FAD in the redox neutral UGM reaction was enigmatic (Scheme 1A). Several mechanistic routes for the interconversion of UDP-Gal*p* to UDP-Gal*f* were initially tested in prokaryotic UGMs using a battery of chemical probes. It was shown that the enzyme was active with UDP-2-F-Gal*p* and UDP-3-F-Gal*f*, eliminating the possibility of oxidation of Gal*p* at the 2-OH or 3-OH moiety [18,19]. Further characterization of the recombinant *E. coli* protein showed that the enzyme was active when the flavin was in the oxidized form, but significantly more active when the enzyme was chemically reduced with dithionite (Scheme 1B)[20]. Subsequent studies showed that only the reduced enzyme exhibits catalytic activity, and the spurious activity attributed to the oxidized enzyme in fact originated from a subpopulation of reduced protein that had persisted in the enzyme preparation [21]. The presence of reduced UGM during purification was clearly established in the UGM from *A. fumigatus*, as the recombinant protein purified under aerobic conditions remarkably stabilizes 50% of the FAD in the reduced form [22].

Blanchard's group demonstrated that the anomeric bond was broken during catalysis using positional isotope effects (PIX) [23], which was later supported by work from Liu's group [18]. Cleavage of the anomeric bond suggested a number of possible mechanisms for UGMs (Scheme 2). One mechanism predicts the formation of 1,4-anhydrogalactopyranose (Scheme 2A) [18,23]. However, activity was not detected when reduced UGM was incubated with 1,4-anhydrogalactopyranose in the presence of UDP, eliminating this species from consideration as an intermediate in the UGM reaction [24]. A mechanism involving a single-electron transfer step during catalysis was supported by potentiometric studies that showed that the flavin semiquinone was stabilized in the presence of substrate [25]. In addition, replacement of the FAD with 5-deaza-FAD, a flavin analog restricted to a net 2-electron process, resulted in inactive UGM. The lack of activity of UGM reconstituted with 5-deaza-FAD was initially interpreted as supporting an electron transfer step in the catalytic cycle (Scheme 2B)[26].

A major breakthrough in our understanding of the mechanism of action of UGM came when Kiessling's group isolated an FAD-galactose covalent intermediate [27]. The covalent intermediate formed between the $N5_{FAD}$ and $C1_{Gal\,p}$ was proposed and later validated by isolation and characterization by mass spectrometry and NMR in bacterial UGM (bUGM) and by UV/vis spectrophotometry and mass spectrometry in eukaryotic UGM (eUGM) [28–30]. The flavin–sugar adduct is important in ring opening and activation of the $C1_{Gal\,p}$ [27]. In this process, the flavin also functions as a molecular scaffold, providing the structural constraints required for ring contraction (Scheme 2B) [31]. Formation of this intermediate made mechanistic sense and was consistent with the breaking of the anomeric bond determined from PIX studies and the lack of activity observed with the enzyme reconstituted with 5-deaza-FAD.

Although identification of the flavin covalent intermediate was a landmark in UGM research, the mechanistic steps leading to formation of this intermediate were not completely understood. Formation of the flavin–sugar adduct was proposed to occur by direct attack of the $N5_{FAD}$ to the $C1_{Gal\,p}$ in an S_N2-type mechanism (Scheme 2B) [27,28]. Alternatively, the intermediate could form by attack of the $N5_{FAD}$ to an oxocarbenium galactose intermediate, in an S_N1-type mechanism [32]. In addition, it was proposed that a single electron transfer step from the flavin to the oxocarbenium intermediate would lead to the formation of a flavin semiquinone and a sugar radical. In this mechanism, the flavin–sugar adduct forms by recombination of the radical pair (Scheme 2B) [26]. Recently, Liu and coworkers probed the mechanism using flavin analogs with different nucleophilicities at the $N5_{FAD}$. The kinetic linear free energy relationship resulted in a slope of $\rho = -2.45$, consistent with a direct attack of the $N5_{FAD}$ in an S_N2-type mechanism [33]. In addition, a flavin-iminium intermediate was observed during time-resolved spectroscopy of the reaction of UDP-Gal*f* with reduced *T. cruzi* UGM (TcUGM) without the formation of a flavin semiquinone intermediate, inconsistent with a single electron transfer mechanism [30]. Furthermore, the structures of the complex of UGM with UDP-Gal*p* clearly show that the $N5_{FAD}$ is the proper distance for direct attack of the $C1_{Gal\,p}$ (described in detailed in the next section).

Recent experimental studies have provided insight into the rate-limiting step. Analysis of the kinetics of eUGMs showed that formation of the flavin iminium ion is very fast ($\sim 300\ s^{-1}$) compared to the k_{cat} ($\sim 12\ s^{-1}$ for TcUGM). In addition, viscosity effects studies demonstrated that product release is not rate limiting. This led to the proposal that ring contraction is the rate-determining step (Scheme 3, g and h) [30]. In order to produce the flavin-iminium ion, the $N5_{FAD}$ must be deprotonated. Although this aspect of the reaction has not been elucidated by biochemical approaches, recent quantum mechanical calculations provided insights into this and other steps in the UGM reaction [34]. Hybrid quantum/classical calculations performed at the density functional theory