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A unique catalytic mechanism for UDP-galactopyranose mutase

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The flavoenzyme uridine 5′-diphosphate (UDP)-galactopyranose mutase (UGM) catalyzes the interconversion of UDP-galactopyranose (UDP-Gal*p*) and UDP-galactofuranose (UDP-Gal*f*). The latter is an essential precursor to the cell wall arabinogalactan of *Mycobacterium tuberculosis*. The catalytic mechanism for this enzyme had not been elucidated. Here, we provide evidence for a mechanism in which the flavin cofactor assumes a new role. Specifically, the N5 of the reduced anionic flavin cofactor captures the anomeric position of the galactose residue with release of UDP. Interconversion of the isomers occurs via a flavin-derived iminium ion. To trap this putative intermediate, we treated UGM with radiolabeled UDP-Gal*p* and sodium cyanoborohydride; a radiolabeled flavin-galactose adduct was obtained. Ultraviolet-visible spectroscopy and mass spectrometry indicate that this product is an N5-alkyl flavin. We anticipate that the clarification of the catalytic mechanism for UGM will facilitate the development of anti-mycobacterial agents.

In 1993, the World Health Organization declared tuberculosis a "global health emergency." A third of the world's population is infected with the tubercle bacillus, *M. tuberculosis*[1], and tuberculosis kills ~2 million people annually[2]. Traditional methods used to combat this infection are losing effectiveness owing to the appearance of multi-drug-resistant strains. The unique cell wall of *M. tuberculosis*, which is impenetrable to many antibiotics, is essential to the viability of the organism[3]. A critical component of this cell wall is the arabinogalactan, a polysaccharide chain consisting of arabinofuranose and galactofuranose (Gal*f*) residues[4,5]. The formation of the arabinogalactan is mediated by a series of enzymes including UGM. This flavoenzyme supplies the biosynthetic precursor necessary for incorporation of Gal*f* residues into the cell wall (**Fig. 1a**)[6]. It is essential for mycobacterial growth[7]. Because Gal*f* is not a constituent of mammalian glycoproteins or glycolipids, strategies to inhibit its incorporation may lead to efficacious therapeutics[8].

Although several mechanisms for UGM have been proposed, they have been difficult to reconcile with emerging mechanistic data[9–11]. The role of the noncovalently bound flavin adenine dinucleotide (FAD) cofactor has been especially puzzling. Flavoenzymes typically catalyze transformations that involve transfer of electrons, and their cofactors can exist in several different oxidation and ionic states[12]. They carry out redox chemistry through transformations involving either N5 or C4a of the isoalloxazine ring system[13]. The UGMs from *Escherichia coli* and *Klebsiella pneumoniae* are inactive upon full chemical oxidation; however, the addition of a strong reductant restores enzyme activity[14]. This result is notable in the context of potential flavin-mediated redox chemistry. It is unclear what functional groups in the substrate might undergo reduction or how substrate reduction would facilitate isomerization. In some flavoenzymes, the flavin plays a structural role in facilitating catalysis, though this is rare[15]. These flavoenzymes typically fall into a family of related enzymes[16,17], but UGM is not a known member of this class. In the structure of UGM, which has been determined by X-ray crystallographic analysis[14], the enzyme-bound flavin is localized in the putative active site. Moreover, studies in which UGM was reconstituted with flavin analogs reveal the importance of the N5 locus for UGM catalytic activity[18]. Thus, a catalytic mechanism in which the reduced flavin plays solely a structural role is difficult to envision. Together, these observations suggest that the reduced flavin cofactor may have a new catalytic function. Thus, we sought to explore the role of the flavin cofactor in UGM catalysis.

RESULTS

Mechanistic proposal for UGM

A proposal for the catalytic mechanism of UGM has been advanced in which UDP-galactose isomerization occurs through an imine formed between a lysine side chain of the enzyme and the anomeric position of the galactose residue[19]. This mechanism is consistent with results from a positional isotope exchange experiment showing that the anomeric bond to the UDP moiety is cleaved during catalysis[9]. Despite the notable features of this mechanism, no conserved candidate nucleophile is apparent in the structure of UGM[14]. In contrast, accumulating evidence supports a role for the reduced flavin cofactor in catalysis[14]. We postulate that N5 of the reduced flavin attacks the anomeric position of galactose. Only reduced UGM is catalytically active, and the N5 position is only nucleophilic when the flavin is in the reduced state. If the proposed covalent intermediate is formed, interconversion of the pyranose and furanose forms can proceed via

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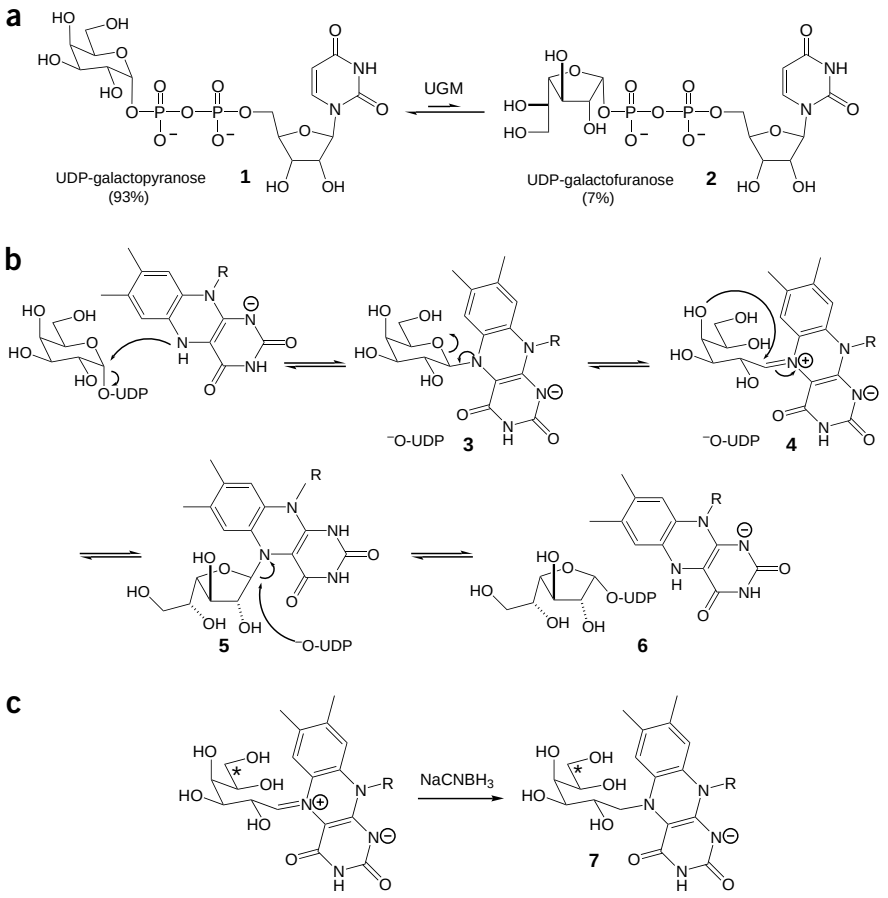

Figure 1 The reaction catalyzed by UGM. (**a**) UGM catalyzes the interconversion of UDP-Gal*p* (**1**) and UDP-Gal*f* (**2**). The pyranose form (**1**) is favored at equilibrium (93:7 ratio). (**b**) Proposal for the catalytic mechanism of UGM. A covalent flavin-galactose intermediate (**3**) is formed through attack of the reduced flavin N5 on the anomeric position of sugar. An S_N1 or S_N2 process could lead to species **3**. Interconversion between pyranose and furanose forms occurs via iminium ion **4**. The precise protonation state of the N1 in each intermediate is unknown. (**c**) Sodium cyanoborohydride is expected to reduce species **4** to yield a N5 flavin adduct **7**. A radiolabeled substrate (asterisk, radiolabel position) can serve as a mechanistic probe.

flavin-derived iminium species **4** (**Fig. 1**). Subsequent displacement of the reduced flavin by UDP to regenerate the glycosyl pyrophosphate linkage would produce the isomeric sugar nucleotide. Our proposed catalytic mechanism outlines a new function for reduced flavin in enzyme catalysis; that is, it mediates iminium ion formation through nucleophilic addition.

Active UGM is not a potent reducing agent

The ability of the reduced flavin N5 to serve as a nucleophile in catalysis is largely unexplored, but the ability of flavins to participate in one-electron transfer is well documented. Therefore, several mechanistic proposals have been advanced for UGM in which the reduced flavin carries out a one-electron reduction of the substrate[18,20]. For example, it has been proposed that the substrate pyrophosphate group undergoes reduction. Moreover, the flavin could act by transferring a single electron to an intermediate sugar oxocarbenium ion[10]. Either of these mechanisms would require that the flavin cofactor of UGM be an outstanding reducing agent[21]. If the protein environment stabilizes the reduced state of the flavin, electron transfer processes of this type should be inaccessible[22]. Therefore, we measured the reduction potential of the enzyme to assess the likelihood of the reduced flavin carrying out a single-electron reduction of the substrate or substrate-derived oxocarbenium ion.

Anaerobic reductive titrations of UGM revealed that the midpoint redox potential is –97 mV at pH 7 versus –219 mV for free FAD (see **Supplementary Fig. 1** online) and this result is consistent with related data determined by Fullerton *et al.*[20]. This deviation corresponds to an increase of ~5.7 kcal mol^{-1} in the thermodynamic stability of the reduced form of UGM-bound flavin. Thus, the reduced flavin in UGM cannot serve as an outstanding reductant; it can only transfer electrons to functional groups that are appropriate acceptors (reduction potential >–97 mV). Consequently, we tested our hypothesis that the reduced flavin functions as a nucleophile.

UGM uses a reduced anionic flavin cofactor

Deprotonation of dihydroflavin at N1 is expected to enhance the nucleophilicity of N5 by increasing electron density of the isoalloxazine ring system[23]. To ascertain the ionic state of the reduced flavin in UGM, we determined its pK_a by fluorescence spectroscopy (see **Supplementary Fig. 1** online). This value is ~6.7, indicating that at physiological pH the majority of the reduced cofactor is in the anionic form. This result, which is consistent with other data[20], suggests that the cofactor exists in a reduced anionic state in catalytically active UGM. Together, the data suggest that N5 has the properties expected of a

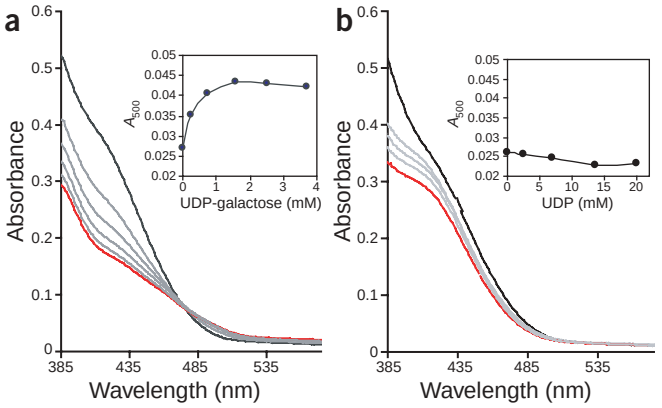

Figure 2 UV-visible difference spectra for titration of UGM with UDP-Gal*p* highlighting the critical region. Solutions with or without UGM were scanned. (**a**,**b**) Concentrated UDP-Gal*p* (**a**) or UDP (**b**) was added and the spectra were determined again (see Methods). Difference spectra were obtained by subtracting the data from the blank sample from the sample with reduced enzyme.

nucleophile that participates in the UGM catalytic mechanism.

Spectroscopic observation of a flavin-iminium ion

Most flavin species give rise to characteristic absorbance spectra; accordingly, we sought to gather spectroscopic evidence for the putative flavin intermediate (**4**, **Fig. 1b**). In a previous study using model compounds, a weak absorbance was identified for a flavin N5 iminium ion near 500 nm[24]. We therefore examined the spectrum of UGM under catalytic conditions. In the presence of dithionite and increasing amounts of UDP-Gal*p*, UGM showed an increase in absorbance at 500 nm, and an isosbestic point at 475 nm was observed (**Fig. 2a**). In contrast, when the enzyme was treated with UDP, no absorbance at 500 nm was detected (**Fig. 2b**). As predicted from our proposed mechanism, the galactose moiety must be present to observe the 500-nm absorbance. The spectroscopic data are consistent with the presence of a flavin-derived iminium ion. On the basis on these results, we sought to trap this transitory species by using hydride reduction to yield isolatable flavin-sugar adduct (**7**; numbering according to **Fig. 1**).

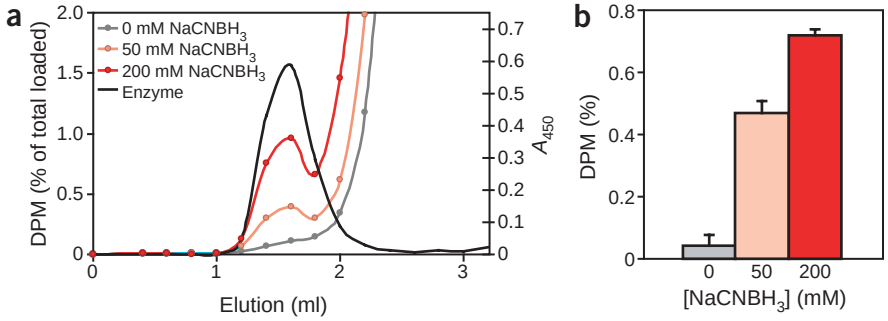

Figure 3 Treatment of UGM with UDP-Gal*p*-6-[^{3}H] in the presence of $NaCNBH_3$. (**a**) Samples containing sodium dithionite, purified UGM, unlabeled UDP-Gal*p* and UDP-Gal*p*-6-[^{3}H] were treated with $NaCNBH_3$ (see Methods) and the products were separated by gel filtration. Fractions were analyzed for tritium signal (disintegrations per minute, DPM). In a separate experiment, the elution profile of UGM was determined by monitoring its A_{450}. (**b**) Enzyme samples containing sodium dithionite, tritiated and unlabeled UDP-Gal*p* were treated with varying amounts of $NaCNBH_3$. After acid-promoted precipitation of UGM, the soluble sample was applied to a reverse phase column and the fractions were analyzed. Data are plotted as % DPM, which represents the percent of radioactivity eluted with methanol compared to the total (eluted plus flowthrough).

Trapping the flavin-iminium ion with $NaCNBH_3$

Iminium ions are common intermediates in many enzymatic reactions. Such transient species are prone to reduction in the presence of a hydride source, a process that would 'trap' the enzyme, thereby preventing substrate turnover (**Fig. 1c**). Indeed, when UGM was treated with sodium cyanoborohydride or sodium borohydride, the apparent rate of enzyme interconversion diminished to nearly zero (see **Supplementary Fig. 2** online). To determine whether the observed decrease in rate was due to reduction of the iminium ion (**4**), we used a radiolabeled substrate (UDP-Gal*p* with ^{3}H at the C6 position, UDP-Gal*p*-6-[^{3}H]) (**Fig. 1c**). If the purported covalent intermediate is trapped, the radiolabel should become covalently affixed to the flavin cofactor. Under these circumstances, the radiolabel should coelute with the enzyme when subjected to chromatography. Accordingly, UGM was exposed to the tritium-labeled substrate in the presence of sodium dithionite, which generates the active form of the enzyme, and sodium cyanoborohydride, which should trap the iminium ion. The reaction products were separated by gel filtration. In the absence of a hydride source, all of the radiolabel elutes with the substrate, UDP-Gal*p*. In contrast, radiolabel was detected in the fraction containing the enzyme when sodium cyanoborohydride was present (**Fig. 3a**). At the highest concentration of sodium cyanoborohydride (200 mM), ~1% of the total radiolabel seems to associate with the enzyme. Based on the ratio of labeled to unlabeled UDP-Gal*p*, this value indicates that ~20% of the enzyme is trapped. These data implicate a covalent intermediate that is captured only in the presence of a hydride source.

To determine whether the radiolabel is linked covalently to the enzyme or to the noncovalently bound flavin cofactor, we further analyzed the radioactive eluant containing enzyme. The eluant was exposed to acid to denature and precipitate the protein, thereby liberating the flavin cofactor. After this treatment, the radiolabel was found in the flavin-containing supernatant (see **Supplementary Fig. 3** online). We carried out reverse-phase chromatography on this supernatant. We first tested whether unreacted flavin and UDP-Gal*p* could be separated on a C18 reverse-phase column (Sep-Pak cartridge; see **Supplementary Fig. 4** online). When the sample was loaded and the column washed with buffer, UDP-Gal*p* was not retained but the flavin was. The flavin eluted from the column upon treatment with methanol (see **Supplementary Fig. 4** online). Given these results, we reasoned that a flavin adduct should also be retained on the column. We therefore carried out C18 reverse-phase chromatography on samples derived from the reaction of UGM, UDP-Gal*p*-6-[^{3}H], dithionite, and sodium cyanoborohydride (**Fig. 3b**). Under these conditions, the methanol eluant was radioactive, suggesting that the tritiated galactose was associated with the flavin. When sodium cyanoborohydride was omitted from the reaction, little or no radioactivity was detected in the methanol eluant. Together these data indicate that the substrate-derived galactose residue is indeed associated with the flavin and not with the enzyme. Thus, a covalent galactose-flavin adduct forms under catalytic conditions.

Characterization of the reduced flavin adduct

The expected product of the reduction of the flavin-derived iminium ion is the N5-alkylated flavin (**7**), a species with an expected half-life of <1 min (ref. 25) in an oxidative environment. Thus, reaction conditions were stringently controlled during the acquisition of UV-visible spectroscopy and mass spectrometry data. After acid-promoted precipitation of the enzyme, high-performance liquid chromatography (HPLC) analysis of the soluble products revealed a shoulder on the front end of the flavin peak. This shoulder was observed only for samples exposed to sodium cyanoborohydride (**Fig. 4a**). The UV-visible spectrum of the FAD-containing peak showed signature absorbances at 260, 370 and 450 nm. In contrast, in the spectrum of the material eluting immediately before FAD, a broad band at ~340 nm is present (**Fig. 4b**). This shift in absorbance is characteristic of N5-monoalkylated flavins[26]. Furthermore, analysis of this material by mass spectrometry indicated the presence of a species of mass 951 Da (**Fig. 4c**). This mass corresponds to that expected for the flavin adduct (**7**). Together, our results implicate a flavin-derived iminium ion (**4**) as a key catalytic intermediate.

DISCUSSION

Our data provide support for a catalytic mechanism for UGM in which isomerization of the nucleotide-sugar substrate proceeds via flavin-derived iminium ion (**4**; **Fig 1b**). Little information regarding

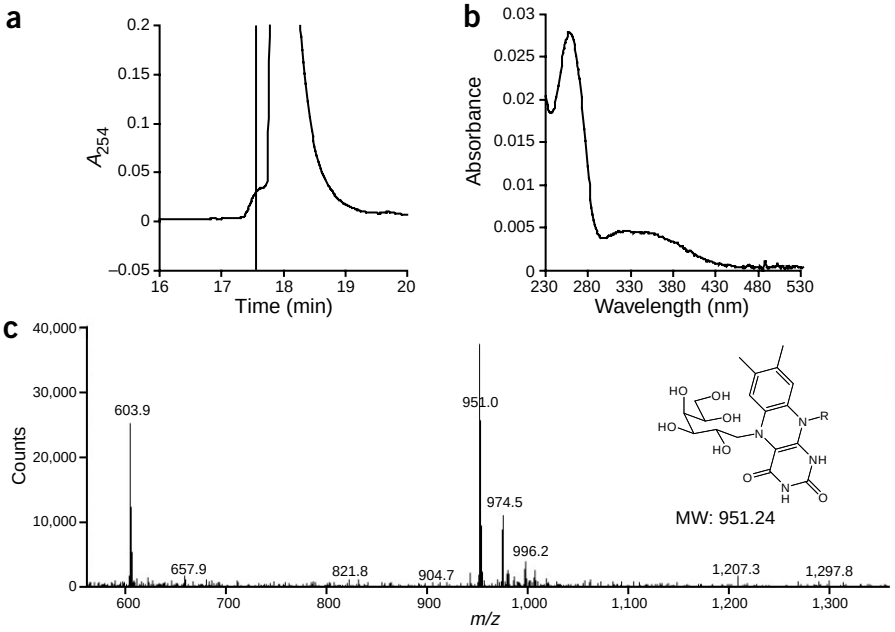

Figure 4 Spectroscopic analysis of the products of reactions containing UGM, UDP-Gal*p* and $NaCNBH_3$. (**a**) Liquid chromatographic analysis of reaction products containing UGM, sodium dithionite, UDP-Gal*p* and sodium cyanoborohydride (see Methods). Black vertical line, R_f of the product analyzed by UV-visible spectroscopy and mass spectrometry. (**b**) UV-visible spectrum of the peak shoulder reveals the product eluting at this R_f exhibits a characteristic shift at 340 nm indicative of an N5-monoalkylated flavin. (**c**) Mass spectral analysis (positive mode) of material eluting at this R_f indicates a species with the predicted *m/z* of species **7** (**Fig. 1**), which corresponds to a molecular mass of 951.24. Also detected are peaks at 974.5 and 996.2, which correspond to the expected masses for $[M + Na]^+$ and $[M + 2Na]^+$. The mass at 603.9 probably corresponds to the decomposition product from cleavage of the pyrophosphate bond in species **7**.

the formation or intermediacy of a flavin-derived iminium ion in catalysis exists, yet related species have been observed. Cyanide has been used to trap a flavin iminium adduct formed in the reaction of D-amino acid oxidase with a non-natural nitroalkane anion substrate[27]. These data suggest that a flavin-derived iminium ion, which would be susceptible to capture by cyanide as well as by a hydride source, is formed when this substrate is processed by the oxidase. Finally, the formation of flavin-iminium ion species has been observed in studies of flavin reactivity[25,28].

The iminium adduct (**4**) could form by several different mechanisms, the simplest of which is nucleophilic attack (either S_N1 or S_N2). An alternative possibility is that compound **4** is generated by the transfer of a single electron from the reduced flavin to an intermediate galactose oxocarbenium ion followed by diradical coupling[10]. Although the reduction potential of the cyclic galactose oxocarbenium ion is not known, the value for the related compound, protonated formaldehyde, has been determined to be –1,180 mV (ref. 21). We determined that the midpoint reduction potential of UGM is –97 mV, indicating that the flavin in UGM is not a strong enough reductant to reduce the oxocarbenium ion. These data support the proposed role for the reduced flavin cofactor as a nucleophile in UGM catalysis.

The N5 of reduced flavin is formally a part of a vinylogous hydrazine. Hydrazines are outstanding nucleophiles, a characteristic that is often attributed to the α effect (nucleophilicity is enhanced at sites in which the adjacent atom has an unshared lone pair)[29,30]. Indeed, the N5 position of dihydroflavins is nucleophilic. It can attack arene epoxides to form a series of unstable N5 adducts[31]. In the flavin ring system, the nucleophilicity of N5 should be tunable by controlling the relative orientation of the lone pairs of N5 and N10 and the protonation state of N1. Our data indicate that the N1 position is deprotonated, which should enhance the nucleophilicity of the isoalloxazine system. Information on the conformation of the reduced flavin may also clarify how UGM tunes the nucleophilicity of its flavin cofactor.

The catalytic role of the flavin observed here has parallels to the chemistry mediated by the cofactor tetrahydrofolate (see **Supplementary Fig. 5** online). Tetrahydrofolate and its derivatives capture formaldehyde and formate in a benign state and facilitate enzyme-catalyzed transfer of one-carbon units. Tetrahydrofolate contains several amino groups, but N5 is the most nucleophilic, and it attacks formaldehyde to generate the iminium species[32]. Despite the structural similarity of tetrahydrofolate and dihydro or reduced flavin, the analogous process using flavin N5 had not previously been observed in enzyme-catalyzed reactions. Notably, some redox chemistry is more accessible for flavin than tetrahydrofolate cofactors[33,34]; thus, reducing or oxidizing conditions as well as the conformation of the cofactor[35] within the enzyme can be used to modulate the tendency of flavin to act as a nucleophile. We anticipate the identification of other flavoenzymes that catalyze reactions through similar intermediates.

The role of galactofuranose residues in pathogenic microorganisms provides impetus for the development of inhibitors of galactofuranose metabolism[8]. Our data support a unique mechanism for UGM that may be used to guide the design of therapeutics. Specifically, compounds that can bind UGM and exploit the nucleophilicity of the flavin N5 should function as antimycobacterial agents. The identification of inhibitors of UGM would also provide the means to address the importance of galactofuranose residues in a wide range of organisms.

METHODS

Reagents. All chemicals used were reagent-grade and purchased from either Sigma or Fisher Scientific unless otherwise noted. Deoxyoligonucleotide primers for PCR were obtained from the University of Wisconsin Biotechnology facility. Restriction endonucleases, polymerases, and chitin resin were purchased from either New England Biolabs or Promega. Nickel-nitrilotriacetic acid (Ni-NTA) resin was obtained from Qiagen and IPTG from Research Organics. UDP-Gal*f* was synthesized as described[36].

Enzyme preparation. The gene encoding UGM, *glf*, was amplified by carrying out PCR on the genomic DNA isolated from *K. pneumoniae* serotype 01 (American Type Culture Collection). A sequence encoding a His_6 tag was added to the 3′ end of the amplified gene by PCR and the resulting DNA ligated into a pGEM-Teasy (Promega) vector with ampicillin resistance. The final construct was transformed into ER2566 *E. coli* cells (New England Biolabs). Transformed cells were grown to an A_{600} of 1.0–1.2 in Terrific Broth and 200 mg l^{-1} ampicillin at 37 °C. Cultures were then induced with 0.5 mM IPTG and grown overnight at 15 °C. Cells were harvested by centrifugation at 3,100*g*, and resuspended in sodium phosphate buffer (50 mM sodium phosphate, pH 8, 20 mM imidazole, 300 mM NaCl). Cells were disrupted via treatment with lysozyme, ribonuclease A, Triton X-100 and sonication (Branson 450 sonifier). The soluble fraction was applied to a Ni-NTA column and washed with above resuspension buffer. Purified UGM was eluted

with a solution of 250 mM imidazole, pH 8. A G-50 Sephadex column was used to desalt the final preparation. Typical yields were 10–15 mg l^{-1} purified enzyme.

UV-visible spectroscopy. All spectroscopy was done using a Varian Cary 50 UV-visible spectrophotometer. In identical cuvettes, solutions containing either 20 mM sodium dithionite in 50 mM sodium phosphate buffer, pH 7, with 150 μM UGM or without enzyme were measured using the scan mode. Concentrated UDP-Gal*p* or UDP was titrated into both cuvettes and measured again. Difference spectra were obtained by subtracting the data from the blank sample from the sample with reduced enzyme.

Radiolabeling studies. Samples (500 μl) contained 20 mM sodium dithionite, 96 μM purified UGM, 0.5 mM UDP-galactopyranose (cold), and 20 μCi of UDP-Gal*p*-6-[^{3}H] in 500 mM sodium phosphate buffer, pH 7. Solid sodium cyanoborohydride was added to appropriate samples, and they were allowed to incubate for ~20 min. Solutions were then separated using 3.5-inch Sephadex G-50 columns equilibrated with 20 mM sodium dithionite in 500 mM sodium phosphate buffer, pH 7. Fractions were collected and analyzed for tritium signal (**Fig. 3**). In a separate experiment, purified UGM and UDP-Gal*p* were separated on the same column to determine R_f values of these standards. Data were plotted as an elution profile. In another experiment, C18 Sep-Pak cartridges (Waters) were equilibrated with 10 ml of methanol followed by 50 ml of sodium phosphate buffer (100 mM sodium phosphate, pH 6). Samples (described above) were treated with HCl to denature protein. Soluble fractions were applied to Sep-Pak cartridge and washed with 50 ml of sodium phosphate buffer (100 mM sodium phosphate, pH 6) before elution with 10 ml of methanol (**Fig. 3b**; see **Supplementary Fig. 4** online). Elution and flowthrough samples were analyzed for tritium signal. Retention of radioactivity was determined by the amount of radioactivity eluted with methanol divided by the total radioactivity (amount eluted plus amount washed through the column). To determine the amount of enzyme undergoing hydride reduction, the percentage of labeled UDP-galactose was compared with the percentage of enzyme radiolabeled. All chromatography solutions contained 20 mM sodium dithionite to minimize the oxidation of intermediates.

HPLC and LC-MS analysis. Samples (125 μl) containing 20 mM sodium dithio-nite, 300 μM UGM, 0.5 mM UDP-galactopyranose, and 50 mM sodium cyanoborohydride in sodium phosphate buffer (500 mM sodium phosphate, pH 7) were acid-denatured with HCl and centrifuged at 9,300*g* for 8 min. For HPLC and UV-visible analysis, soluble fractions were filtered and injected (50 μl) onto a C18 column (Varian Microsorb, 4.6 × 250 mm) equilibrated with acetate buffer (5 mM ammonium acetate, pH 6). A linear gradient of methanol (15–85% (v/v)) over 40 min eluted the flavin species at ~17 min (**Fig. 4a**,**b**). UV-visible spectra from the photodiode array detector were collected for all time points. For liquid chromatography and mass spectrometry (LC-MS) analysis, soluble fractions were filtered and injected (40 μl) on a Shimadzu LC-MS containing a C18 column (Supelco Discovery, 2.1 × 150 mm) equilibrated with 0.4% (v/v) formic acid. A linear gradient of acetonitrile and 0.2% (v/v) formic acid (15–85% (v/v)) over 40 min eluted the flavin species at ~10 min. Positive-ion mass spectral data was collected for all time points after 3 min (**Fig. 4c**).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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