Nucleophilic Participation of Reduced Flavin Coenzyme in Mechanism of UDP-galactopyranose Mutase

Received for publication, October 11, 2011, and in revised form, December 1, 2011 Published, JBC Papers in Press, December 20, 2011

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UDP-galactopyranose mutase (UGM) requires reduced FAD (FADred) to catalyze the reversible interconversion of UDP-galactopyranose (UDP-Galp) and UDP-galactofuranose (UDP-Galf). Recent structural and mechanistic studies of UGM have provided evidence for the existence of an FAD-Galp/p adduct as an intermediate in the catalytic cycle. These findings are consistent with Lewis acid/base chemistry involving nucleophilic attack by N5 of FADred at C1 of UDP-Galp/p. In this study, we employed a variety of FAD analogues to characterize the role of FADred in the UGM catalytic cycle using positional isotope exchange (PIX) and linear free energy relationship studies. PIX studies indicated that UGM reconstituted with 5-deaza-FADred is unable to catalyze PIX of the bridging C1–OPα oxygen of UDP-Galp, suggesting a direct role for the FADred N5 atom in this process. In addition, analysis of kinetic linear free energy relationships of kcat versus the nucleophilicity of N5 of FADred gave a slope of ρ = −2.4 ± 0.4. Together, these findings are most consistent with a chemical mechanism for UGM involving an S2-type displacement of UDP from UDP-Galp/p by N5 of FADred.

UDP-galactopyranose mutase (UGM) is a flavoprotein that catalyzes the isomerization of UDP-galactopyranose (UDP-Galp) and UDP-galactofuranose (UDP-Galf). This reaction is essential for many pathogenic species of bacteria, protozoa, and fungi because UDP-Galp serves as the activated Galp donor during cell wall biosynthesis in these organisms (1, 2). Of particular clinical importance is the causative agent of tuberculosis, Mycobacterium tuberculosis, the cell wall of which possesses a galactan chain of ~35 Galp residues that is essential for viability. Given the global prevalence of tuberculosis (World Health Organization Media Center) (4) and the increasing incidence of multidrug-resistant strains (6), UGM has become an attractive drug target because mammalian glycans do not contain Galp residues. UGM has also attracted much attention because it requires a reduced FAD coenzyme (FADred) to catalyze a reversible ring contraction/expansion reaction that is redox-neutral (7). This has raised questions about the catalytic function of the coenzyme during turnover.

Several previous studies have provided important insights into the chemical mechanism of the UGM-catalyzed reaction. Using positional isotope exchange (PIX) studies, Blanchard and co-workers (8) demonstrated that the anomeric C1–OPα bond is broken and reformed during turnover. Using NaCNBH3 as a chemical quenching agent to trap species 9 (Fig. 1), Kiessling and co-workers (9, 10) provided evidence for the intermediacy of the iminium ion (7) in the UGM catalytic cycle. Species 7 is likely derived from the FAD-substrate adducts 6 and 8, where N5 of the flavin is covalently linked to the anomeric carbon of the substrate (9, 10). Structures of reduced UGM determined in the presence of UDP-Galp by saturation transfer difference NMR spectroscopy (11) and x-ray crystallography (10) revealed that N5 of FADred is in close proximity to the anomeric carbon of the substrate, providing compelling evidence for the participation of N5 in nucleophilic attack at C1 of the substrate to form 6 and 8 during turnover.

Three mechanistic hypotheses have been proposed to explain the formation of 6 and 8 (Fig. 1). Generation of these intermediates may occur via nucleophilic attack by N5 of FADred at the anomeric carbon of 1 (or 2) concerted with cleavage of the C1–OPα bond (Fig. 1, path A), which is reminiscent of typical S2-type substitutions. Alternatively, formation of 6 and 8 may take place in a stepwise fashion similar to S1-type substitutions (Fig. 1, path B), where elimination of UDP to produce an oxocarbenium intermediate (such as 4) precedes the nucleophilic attack by N5. It is also possible that the electron-deficient nature of 4 could facilitate single-electron transfer (SET) from FADred to form a radical pair (such as 5 and 10), followed by covalent bond formation to afford 6 and 8 (Fig. 1, path C). Indirect evidence for an oxocarbenium intermediate...
has come in the form of a significant rate reduction observed with UDP-[2-F]Gal (12) and the inability of UGM to displace UDP from the linear substrate analog UDP-galactitol (13).

In this study, we employed PIX and linear free energy relationships (LFERs) to further investigate the role of N5 of FAD\textsubscript{red} during UGM catalysis. To determine whether N5 is indeed necessary for cleavage of the anomeric bond of UDP-Gal, PIX was monitored for the double-labeled UDP-Gal\textsubscript{p} substrate in the presence of UGM reconstituted with reduced 5-deaza-FAD. The results were then corroborated by considering the perturbations to the rate of isomerization imposed upon changing the nucleophilicity of N5. Together, the results of these experiments are most consistent with an $S_N^{2}$-type displacement of UDP from the substrate by N5 of FAD\textsubscript{red} (Fig. 1, path A).

**EXPERIMENTAL PROCEDURES**

**UGM Expression and Purification**—The glf gene encoding UGM was introduced into a pET-24b\textsuperscript{+} vector to generate the recombinant pQZ-1 plasmid (7). The plasmid was then transformed into *Escherichia coli* BL21 Star\textsuperscript{TM} (DE3) for overexpression of UGM as a C-terminal His\textsubscript{6} fusion protein. Cells were cultured in LB medium with 50 mg/liter kanamycin at 37 °C until the absorbance at 600 nm reached 0.6. Protein overexpression was induced by the addition of isopropyl β-d-thiogalactopyranoside to 0.1 mM, and the culture was allowed to incubate for an additional 18 h at 18 °C. The cells were then harvested by centrifugation, disrupted by sonication, and purified using nickel-nitrilotriacetic acid resin (Qiagen). UGM was purified as the holoenzyme with a bright yellow color and a distinct UV-visible absorption profile characteristic of tightly bound FAD. The purified enzyme was then flash-frozen in storage buffer (100 mM potassium Pi (pH 7.5) containing 15% glycerol) and stored at −80 °C.

**Preparation of Double-labeled UDP-Galp**—Double-labeled UDP-Galp was prepared according to published methods (8), where the double labels were introduced by incubating 100 mg of d-[1-13C]galactose (99%; Cambridge Isotope Laboratories) in 200 \(\mu\)l of \(\text{H}_2^{18}\text{O}\) (95%, normalized; Fluka) at 55 °C for 2 days. This led to ~80% incorporation of \(^{18}\text{O}\) at C1 as determined by mass spectrometry. A solution of 80 mM double-labeled galac-
Mechanism of UDP-galactopyranose Mutase
tose was then treated with pyruvate kinase and galactose kinase
in the presence of 125 mM phosphoenolpyruvate and 6 mM ATP
in 50 mM Tris buffer (pH 7.5) to generate [1-13C,1-18O]galac-
tose 1-phosphate. The enzymes were removed by filtration
(YM-10 membrane) following complete consumption of the
labeled galactose as monitored by TLC. The filtrate was adjusted
to pH 8.5 and treated with galactose-1-phosphate uridy-
lytransferase, UDP-glucose pyrophosphorylase, and inorganic
pyrophosphatase in the presence of 0.6 mM UDP-glucose and
100 mM UTP to form UDP-[1-13C,1-18O]Galp. The reaction
was monitored by HPLC (see below for conditions). Enzymes
were then removed by filtration (YM-10 membrane), and the
product was purified using a DEAE-cellulose column eluted
with a 0–0.1 M gradient of NH4HCO3 in water. The isolated
UDP-Galp was determined to be 95% pure based on HPLC
analysis. The extent of isotopic double-label incorporation into
the UDP-Galp product was determined by 1H NMR and 13C
NMR spectrometries and confirmed by high resolution electro-
spray ionization mass spectrometry (negative-ion mode), dem-
31P NMR spectroscopies as

demonstrating peaks at m/z 566.0518 and 568.0530, correspon-
ding to the 16O (calculated m/z 566.0511) and 18O (calculated m/z
568.0553) isotopologues, respectively.

Preparation of 7/8-Substituted FAD Analogues—The 7/8-
substituted FAD analogues used in this study were prepared
according to published methods (14). The identity of each com-
31P NMR spectroscopies as

pound was verified by 1H NMR and 31P NMR spectrometries as
well as by high resolution electrospray ionization mass
spectrometry.

Preparation of Apo-UGM and Reconstitution with Other
FAD Analogues—FAD was removed from the purified enzyme
by the addition of a 2.6-fold volume excess of 3 M KBr in 20%
glycerol to a 10 mg/ml enzyme stock in storage buffer (15). The
resulting mixture was incubated on ice for 2 min before precip-
31P NMR spectroscopies as

itating the enzyme by the addition of a 1.8-fold volume excess
of saturated ammonium sulfate (pH 2.5). The enzyme precipitate
was pelleted by centrifugation at 18,000 g for 10 min. The
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clear yellow supernatant containing released FAD was
decanted, and the protein pellet was redissolved in 4 ml of storage
buffer. The high salt treatment was repeated a second time,
and the resulting apo-UGM was dialyzed against storage buffer.
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Reconstitution of UGM with FAD analogues was carried out by
incubating a 2.5 mg/ml solution of apo-UGM with an excess of
flavin cofactor for 15 min at room temperature. The reconsti-
31P NMR spectroscopies as

tuted enzyme/cofactor solution was then diluted with storage
buffer to a concentration compatible with the enzyme assays. In
the LFER studies, the molar ratio of cofactor to apo-UGM was
~100:1. Enzyme concentrations were determined by the Brad-
ford assay using bovine serum albumin as the protein standard.

PIX EQUILIBRATION BY APOENZYME IS NOT ACCELERATED IN THE
PRESENCE OF 5-DEAZAFLAVIN—As described previously (8), the C1
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13C NMR signal of UDP-Galp is split into a doublet by the adjacent β-phosphate and demonstrates an upfield shift of
~0.03 ppm when the anomeric oxygen is replaced with 18O. The
31P NMR spectroscopies as

resulting two doublets were deconvoluted and integrated using Varian VnmrJ software and a multicomponent fit with a
Lorentzian line shapes. A 13C NMR example spectrum is pro-
duced in supplemental Fig. S2 along with the deconvolution. 13C
NMR and mass spectrometry results indicated that the double-
31P NMR spectroscopies as

labeled UDP-Galp (11) was prepared with >99% incorporation of
13C at C1 and 77 ± 4% incorporation of 18O at the bridging
C1–OPβ position. All remaining atoms in the substrate were at
31P NMR spectroscopies as

natural isotopic abundance.

PIX experiments were conducted under reducing conditions
(7 mM sodium dithionite) in NMR tubes. The change in the
fraction (f) of 18O versus 16O bound to 13C was monitored
versus time (t) by 13C NMR spectroscopy. (13C NMR stack plots
are shown in supplemental Fig. S2.) Complete scrambling of the
Pβ oxygens (Fig. 2) predicts the fraction of 18O at the bridging
C1–OPβ position to ultimately reach an equilibrium value of
f eq = 0.26 (the product of the initial 18O enrichment in our
double-labeled UDP-Galp substrate and the statistical factor of
one-third). In the presence of 10 μM UGM reconstituted with
FADred, PIX scrambling (11 ≈ 12) was >90% complete within
20 min of incubation. In contrast, PIX was considerably slower
in the presence of either 15 μM apo-UGM or UGM reconsti-
tuted with 5-deaza-FAD\text{red} (Fig. 3), occurring over a time scale
of \( \sim 100 \) times faster when UGM was reconstituted with FAD\text{red}. Time courses were determined in separate
experiments leading to the difference in the ordinate intercepts (see
supplemental data for details). Reactions were run at 27 °C in potassium P₅ (pH 7.5), 10% D₂O, and 7 mM Na₂S₂O₄ with 15 \( \mu \)M enzyme. Progress curves were obtained by fitting Equation 1 to the observed time courses using nonlinear regression.

\[ f = f_{eq} + \Delta f \exp(-k_{eq}t) \]  

where \( f_{eq} \) is the final value after complete equilibration of the exchangeable oxygens, and \( \Delta f \) is the difference between the initial value of \( f \) and \( f_{eq} \). The first-order rate constant
\( (k_{\text{ PIX}}) \) describes the approach to the PIX equilibrium under the experimental conditions and is equivalent to the positional exchange rate (17) normalized for the total initial substrate concentration, which was held constant and saturating in the experiments. The parameters \( k_{\text{ PIX}} \) and \( \Delta f \) were both allowed to float during nonlinear fitting, whereas \( f_{eq} \) was fixed at 0.26.

The observed values of \( k_{\text{ PIX}} \) for apo-UGM and UGM reconstituted with 5-deaza-FAD\text{red} were 0.0015 \( \pm \) 0.0001 and 0.0017 \( \pm \) 0.0001 min\(^{-1}\), respectively, \( \sim 100 \)-fold smaller than the \( k_{\text{ PIX}} \) for UGM reconstituted with FAD\text{red}. These values of \( k_{\text{ PIX}} \) are significantly different from zero (\( p < 0.0001 \)); however, they are not significantly different from one another (\( p > 0.3 \)). No PIX scrambling was observed over a 24-h period when UGM was omitted from the reaction solution. Additional details regarding data fitting and statistical analysis are provided in the supplemental data.

**FAD Analogues Yield Negative Value in LFER Studies**—Following reconstitution of apo-UGM with each FAD analogue (Fig. 4A), the turnover number \( (k_{\text{cat}}) \) for conversion of UDP-Gal\text{f} to UDP-Gal\text{p} (Fig. 1, 2 \( \rightarrow \) 1) was determined from plots of the initial rate \( (v_i) \) (12, 15) versus substrate concentration \( (s_i) \) according to the structural relation (Equation 2),

\[ v_i = k_{\text{cat}} e_0/(K_m + s_i) \]  

where \( e_0 \) is the total enzyme concentration, and \( K_m \) is the Michaelis constant. Controls were also performed with ratios of 1000:1 for the 8-methoxy-FAD and 7-chloro-FAD analogues versus apo-UGM. No significant differences were observed in the fitted kinetic parameters, implying that the...
Mechanism of UDP-galactopyranose Mutase

variations in $k_{cat}$ were not a result of subsaturating flavin concentrations. The resulting plot of $\log_{10}(k_{cat})$ versus the sum of the substituent constants at positions 7 and 8 of the FAD analogues, denoted $\sigma_m$ and $\sigma_p$, respectively, is shown in Fig. 4B. The values of $\sigma_m$ and $\sigma_p$ are based on the ionization of phenylacetic acid in water (18); however, substituent constants based on the ionization of benzoic acid in water gave similar results. On the basis of an analysis of variance, we found no evidence for either unequal expression of meta versus para effects in the LFER, i.e. $\rho_m \neq \rho_p$ (19), or non-additivity, i.e. higher order terms of $\sigma$. The value of the susceptibility factor ($\rho$) estimated from the linear correlation was $-2.4 \pm 0.4$ and is significantly different from zero ($p < 0.01$). Additional details regarding data fitting and statistical analysis are provided in the supplemental data.

DISCUSSION

Evidence for the cleavage of the anomeric UDP-Galp bond during turnover by UGM was first provided by Blanchard and co-workers (8) using PIX. In this study, incubation of UGM with UDP-[1-13C]Galp enriched with 18O at the bridging oxygen between C1 and the $\beta$-phosphate (11) was monitored by 13C NMR spectroscopy. When 18O is replaced with 16O at the bridging position in the double-labeled substrate during turnover (see 12), a well resolved downfield shift of the 13C1 resonance occurs, with the change in the ratio of 13C–16O and 13C–18O signals indicative of bond cleavage and reformation during catalysis (Fig. 2). It has also been shown that UGM reconstituted with 5-deaza-FAD does not catalyze interconversion of UDP-Galf and UDP-Galp (15). This observation was initially interpreted as support for a mechanism involving SET (Fig. 1, path C) because 5-deaza-FAD is restricted to 2-electron processes (20). Nevertheless, this indirect evidence does not exclude the possibility of a nucleophilic role for N5 of FADred and, in particular, an $SN_2$-type process if it is required for expulsion of the UDP moiety.

As an initial test of the hypothesis that N5 is directly involved in the cleavage of the anomeric bond of UDP-Galp to form the flavin-substrate adduct (6 and 8), we considered the ability of UGM to utilize 5-deaza-FAD to catalyze PIX of the $P_{\mu}$ oxygens in UDP-Galp. Reconstitution of apo-UGM with 5-deaza-FAD showed no effect on an observable low background rate of PIX despite the comparable binding affinities of 5-deaza-FAD and FADred (15). Therefore, the observation that 5-deaza-FAD is unable to substitute for FADred in catalyzing PIX of the $P_{\mu}$ oxygens implies that N5 of FADred is necessary for cleavage of the anomeric C1–OP$_{\mu}$ bond. The background PIX is most likely due to residual (<1%) holo-UGM in the apo-UGM preparation, which is consistent with the tight binding of FADred to UGM ($K_{d} < 10$ nm) (15) and the observed formation of UDP-Galf in all reactions (see supplemental data). It should also be noted that PIX would be independent of SET if the reaction were to proceed via the oxocarbenium ion intermediate 4 (Fig. 1, paths B and C). Thus, the inability of reduced 5-deaza-FAD to promote anomeric bond cleavage/reformation of 1 and 2 cannot be ascribed to an inability to facilitate SET and is more likely due to the absence of N5 serving as the nucleophile in C1–OP$_{\mu}$ bond cleavage during the concerted attack at C1 of the substrate (Fig. 1, path A).

To characterize the role of N5 of FADred during the UGM-catalyzed isomerization more closely, we next examined the kinetic LFERs associated with changes in the nucleophilicity of N5. If adduct 6 (or 8) is formed via an $SN_2$-type substitution (Fig. 1, path A), and if this step is at least partially rate-limiting to steady-state turnover, then changes in the nucleophilicity of N5 should be reflected in the rate of steady-state turnover. In contrast, the rate of steady-state turnover is expected to be much less sensitive to the nucleophilicity of N5 if adduct formation proceeds by an $S_N_1$ or a SET pathway (Fig. 1, paths B and C), where formation of the oxocarbenium species (4) would be substantially rate-limiting. Thus, as the electron density at N5 of FADred is decreased by substitution of the isoalloxazine moiety, a decrease in the steady-state reaction rate is expected for mechanism A, whereas little or no effect is expected for mechanisms B and C.

To test this hypothesis, several FAD analogues (Fig. 4A) containing electron-withdrawing or electron-donating groups at positions 7 and/or 8 of the isoalloxazine moiety (meta and para, respectively, to the N5 position) were chemoenzymatically synthesized according to published procedures (14). The nucleophilicity of N5 is represented as the sum of the $\sigma_m$ and $\sigma_p$ substituent constants for the substituent at the meta and para positions, respectively, of the FAD analogues. These substituent constants are based on the ionization of phenylacetic acid in water (18). Except for the para-methoxy substituent, these values are nearly identical to the Hammett substituent constants obtained for ionization of benzoic acid in water (21). In the case of $p$-methoxy, $\sigma_m$ is significantly more negative with benzoic acid likely due to hydrogen bond stabilization of a trans-quinoidal resonance structure in H$_2$O (22). Although a better correlation was obtained with the values of $\sigma_m$ and $\sigma_p$ based on ionization of phenylacetic acid, the same conclusions were drawn with those based on benzoic acid (see supplemental data). Previous studies of many flavoenzymes (14, 19, 23, 24) and model systems (25) reconstituted with 7- and 8-substituted flavin analogues have established a precedent for significant LFERs correlating Hammett substituent constants with a variety of parameters related to flavin structure and reactivity.

A linear correlation of $\log_{10}(k_{cat})$ for the UGM-catalyzed reaction versus the sum of $\sigma_m$ and $\sigma_p$ for the FAD analogues was observed with a slope of $-2.4$ as shown in the Hammett plot of Fig. 4B. This implies that the rate of steady-state turnover by UGM is indeed sensitive to the electron density at N5 of FADred. The large negative $\rho$ value suggests a substantial decrease in electron density on the flavin in the transition state of the step(s) that limits steady-state turnover. For the conversion of 2 to 1, formation of adduct 8 via an $SN_2$-type reaction (2 $\rightarrow$ 8) and/or formation of the iminium species (8 $\rightarrow$ 7) would involve substantial loss of electron density from the flavin N5 center. If formation of 8 (Fig. 1) were to occur via an $S_N_1$ process, the expectation would be a $\rho$ value approximating zero. Similarly, rate-determining product dissociation in either mechanism would also be expected to yield a $\rho$ value near zero.
Alone, the results of our LFER studies cannot distinguish between rate-limiting SN₂-type adduct formation (2 → 8) and iminium formation (8 → 7). However, our PIX studies strongly suggest that if an SN₁-type mechanism was operative, then cleavage of the anomic bond to form an oxocarbonium intermediate (4) would be energetically demanding and contribute significantly to limiting $k_{cat}$. Because this step is significantly impaired when N5 of FAD$^{red}$ is absent in the active site of UGM, the most consistent interpretation of our PIX and LFER studies is that adduct formation (2 → 8) occurs by an SN₂-type reaction that is mediated by N5 of FAD$^{red}$.

In summary, 5-deaza-FAD is not able to support PIX of the β-phosphate oxygens of UDP-Galp, and $k_{cat}$ for the isomerization of UDP-Galf$^{f}$ to UDP-Galp exhibits a negative LFER with respect to decreasing electron density at N5 of FAD$^{red}$. These observations represent a direct experimental evaluation of nucleophilic participation by FAD$^{red}$ during UGM catalysis. Together, they support a mechanism in which the covalent FAD$^{red}$-Galp$^{f}$ intermediates (6/8) are formed through a concerted SN₂-displacement involving N5 of FAD$^{red}$ as the nucleophile. Thus, as has been characterized recently in several other flavoenzymes (14, 26–28), UGM appears to utilize reduced flavin in a covalent manner to mediate chemical transformations that do not involve redox chemistry, illustrating the catalytic versatility of the ubiquitous flavin coenzyme (29). Finally, these findings help explain the pharmacologically unsatisfactory results obtained with UGM inhibitors that mimic a transition state for generation of an oxocarbonium intermediate (5, 30–32). Therefore, analogues that specifically target the nucleophilic addition may offer more promising leads for developing inhibitors of UGM activity.

Acknowledgments—We thank Dr. Kenji Itoh for preparing UDP-Galf and Drs. Ben Shoulders, Yasushi Ogawara, and Eita Sasaki for helpful suggestions and discussions. We also gratefully acknowledge Steve Sorey for assistance in collecting PIX data.

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Mechanism of UDP-galactopyranose Mutase

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