Studies of UDP-Galactopyranose Mutase from *Escherichia coli*: An Unusual Role of Reduced FAD in Its Catalysis

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Abstract: The galactofuranose moiety found in many surface constituents of microorganisms is derived from UDP-D-galactopyranose (UDP-Gal*p*) via a unique ring contraction reaction catalyzed by UDP-Gal*p* mutase. This enzyme, which has been isolated from several bacterial sources, is a flavoprotein where the FAD coenzyme is noncovalently bound. Since its catalysis does not appear to involve a redox mechanism, whether the enzyme-bound FAD plays an active role in the reaction mechanism has been obscure. To study this transformation, the corresponding *E. coli* mutase was purified, and the ring contraction product, UDP-Gal*f*, was chemically synthesized. Using UDP-Gal*f* as the substrate, a K_m of 194 μM and a k_{cat} of 1.5 s^{-1} for the catalysis in the reverse direction were obtained. The preference of the reaction toward the pyranose product was confirmed by an equilibrium constant of 0.057 in the forward direction. Interestingly, when the enzyme was reduced by sodium dithionite, its catalytic efficiency was increased by more than 2 orders of magnitude. A comparable rate enhancement was also noted when the flavin coenzyme was selectively reduced by photoreduction in the presence of 5-deazariboflavin under anaerobic conditions. Since mutase with either oxidized or reduced FAD is active, the change of the redox state in FAD appears to affect only the activity, but not the catalytic mechanism. It is conceivable that reduction of FAD may induce a favorable conformational change of the enzyme that may be more conducive to catalysis. It is also possible that the reduced flavin bears a higher electron density at N-1, which may then be used to stabilize the transiently formed oxocarbenium ion intermediates to facilitate catalysis. Whether structural effects, electronic effects, or a combination of both dictates the ability of FAD to enhance the rate of the mutase reaction is an interesting, albeit challenging question. Nevertheless, the present work has provided, for the first time, evidence indicating the active involvement of FAD in regulating the catalytic efficiency of this enzyme.

UDP-D-galactofuranose (**2**, UDP-Gal*f*) is the precursor of the galactofuranose moiety[1] found in a variety of surface constituents of microorganisms.[2] UDP-Gal*f* is biosynthetically derived from UDP-D-galactopyranose (**1**, UDP-Gal*p*) via a unique ring contraction reaction catalyzed by UDP-galactopyranose mutase (Scheme 1).[3] The fact that galactofuranose has been found in many pathogens but not in human tissues makes UDP-Gal*p* mutase an attractive target for therapeutic agents. This enzyme, which has been isolated from several bacterial sources,[3,4] is a flavoprotein, with the flavin adenine dinucleotide (FAD) coenzyme bound noncovalently. Few mechanistic studies of this mutase had been described until a recent report,[5] in which the UDP-Gal*f* and UDP-Gal*p* interconversion was elegantly demonstrated to involve cleavage of the anomeric C−O bond. As illustrated in Scheme 1, the catalysis of the forward reaction is likely to be initiated by distortion of the ring which allows attack of O_4 on C-1 to release UDP, or by elimination of UDP first to form an oxocarbenium ion **3** followed by O_4 attack on C-1. Subsequent ring opening between C-1 and O_5 of the bicycloacetal intermediate **4**, via either **5** or **6**, followed by the rebound of UDP at C-1 leads to the formation of **2**. It must be noted that the proposed mechanism does not involve a redox reaction, nor is there a change in the redox states of the substrate or product. Thus, these two considerations do not clearly ascribe an active role to the enzyme-bound FAD in the reaction mechanism. Hence, the role of FAD in UDP-Gal*p* mutase remains obscure. The present work describes our study on the mutase from *Escherichia coli*, and the initial evidence indicating the active involvement of FAD in regulating the catalytic efficiency of this enzyme.

Results and Discussion

Expression of *glf* Gene and Purification of Glf Protein. The UDP-Gal*p* mutase encoding gene from *E. coli* K-12, *glf*,[3] was amplified by the polymerase chain reaction (PCR) and cloned into the expression vector pET24b(+). The recombinant construct was used to transform *E. coli* BL21 (DE3), and the resulting cells were incubated at 37 °C in LB medium. This *C*-terminal His_6-tagged enzyme was purified to near homogene-

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Scheme 1

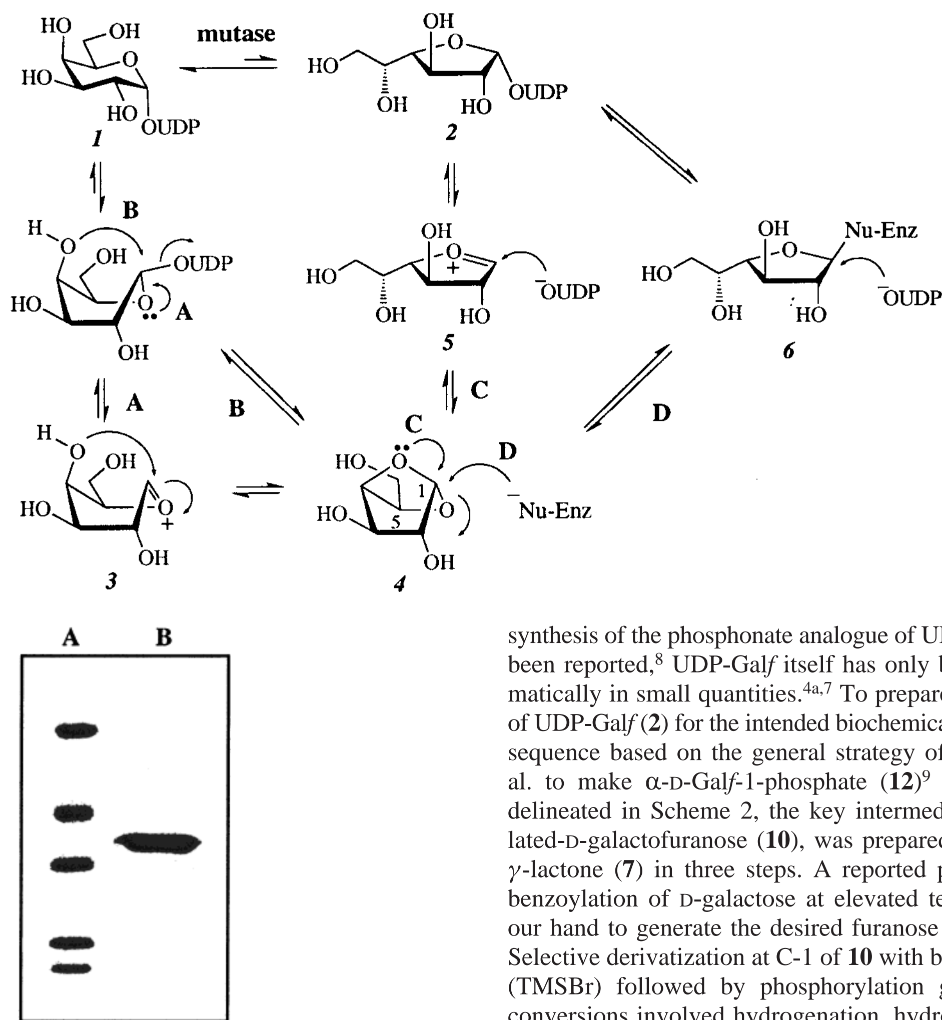

Figure 1. Lane A: Molecular weight marker, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa). Lane B: Purified UDP-galactopyranose mutase.

ity (see Figure 1) using Ni-NTA resin (Qiagen) and found to be stable in phosphate buffers containing 15% glycerol.[6] *N*-Terminal amino acid sequencing confirmed that the first 10 residues (MYDYIIVGSG) of this protein are identical to the translated *glf* sequence. The purified UDP-Gal*p* mutase (Glf) was determined to be a homodimer by judging from a M_r of 72 kDa estimated by gel filtration chromatography (Superdex 200) and a calculated mass of 44 kDa based on the translated peptide sequence. The enzyme exhibits a typical unresolved flavin spectrum with absorbance maxima at 450 and 376 nm. A stoichiometry of 0.52 of bound FAD per protein monomer was estimated by quantitation of FAD released from an enzyme sample of known concentration.

Synthesis of UDP-Gal*f* (2). Since the equilibrium of the interconversion between UDP-Gal*p* (**1**) and UDP-Gal*f* (**2**) catalyzed by UDP-Gal*p* mutase greatly favors the formation of UDP-Gal*p* (**1**),[7] it has been difficult to assay the enzyme activity using commercially available UDP-Gal*p*. This problem, however, can be circumvented by studying the catalysis in the reverse direction using UDP-Gal*f* (**2**) as the substrate. Although synthesis of the phosphonate analogue of UDP-Gal*f* has recently been reported,[8] UDP-Gal*f* itself has only been obtained enzymatically in small quantities.[4a,7] To prepare sufficient amounts of UDP-Gal*f* (**2**) for the intended biochemical studies, a synthetic sequence based on the general strategy of de Lederkremer et al. to make α-D-Gal*f*-1-phosphate (**12**)[9] was developed. As delineated in Scheme 2, the key intermediate, per-*O*-benzoylated-D-galactofuranose (**10**), was prepared from D-galactono-γ-lactone (**7**) in three steps. A reported procedure for direct benzoylation of D-galactose at elevated temperature failed in our hand to generate the desired furanose derivatives **10**.[10−12] Selective derivatization at C-1 of **10** with bromotrimethylsilane (TMSBr) followed by phosphorylation gave **11**. The final conversions involved hydrogenation, hydrolysis, and coupling with uridine phosphomorpholidate in anhydrous pyridine in the presence of 1*H*-tetrazole to afford **2** in 4% overall yield (**7** → **2**).[13] The final product was purified by size exclusion chromatography and reversed phase HPLC. The structure of **2** was confirmed spectroscopically by NMR (^{1}H, ^{13}C, ^{31}P) and high-resolution mass spectra.

Characterization of Glf Protein. The activity of the purified enzyme was assayed by incubating a mixture of UDP-Gal*f* (**2**) and Glf at 37 °C, and analyzing the extent of interconversion by reversed phase HPLC. The equilibrium constant (K_{eq} = [**2**]/[**1**]) was determined to be 0.057, providing the first quantitative measurement of the preference of the reaction toward the pyranose product. A K_m of 194 μM for UDP-Gal*f* and a k_{cat} of 1.5 s^{-1} for the catalysis in the reverse direction (**2** → **1**) were also obtained. None of the common redox cofactors, such as $NAD(P)^+$, NAD(P)H, FAD, or the combination of NAD(P)H and FAD (2 mM each) showed a significant effect on the reaction rate.[14] Likewise, assays performed under anaerobic conditions gave results comparable to those obtained aerobically.

(6) The wild-type enzyme was reported to be unstable even in the presence of glycerol.[3]

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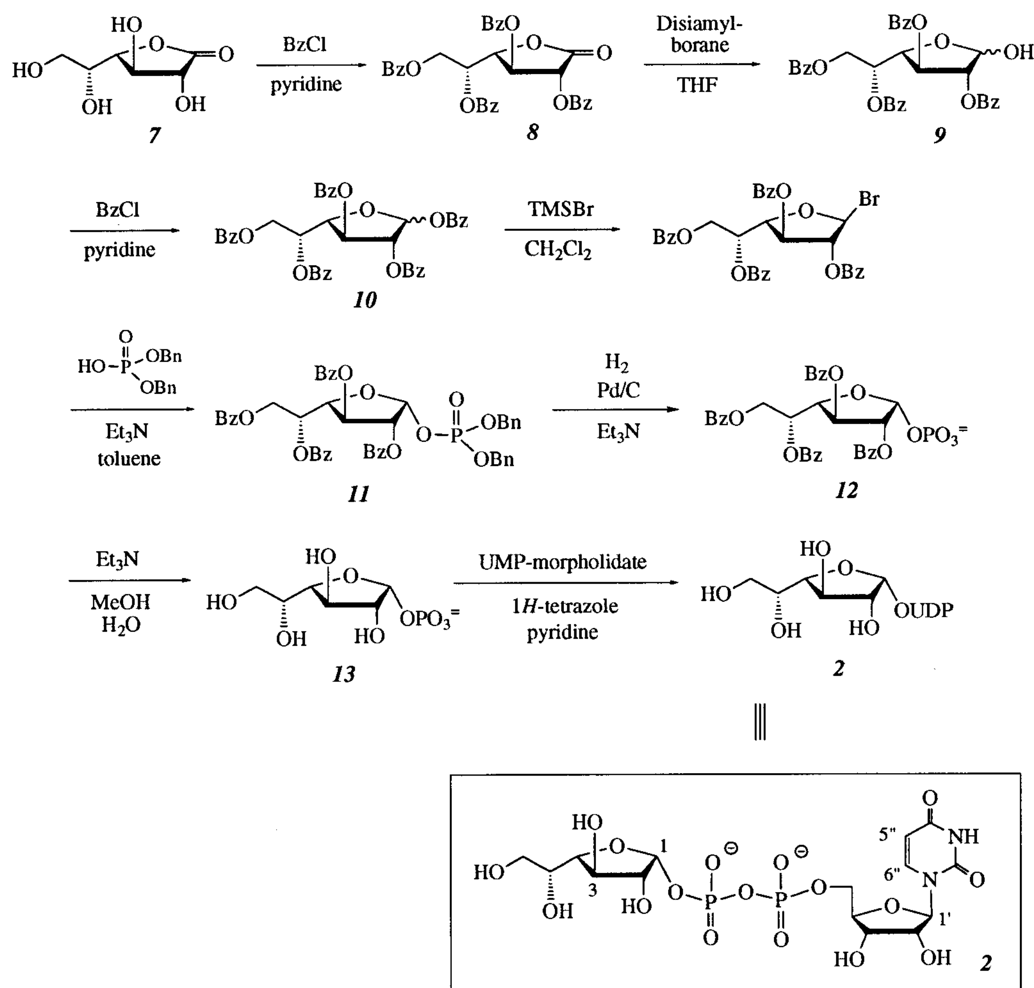
Scheme 2



In addition, no redox-active metal ion was detected by inductively coupled plasma (ICP) analysis of Glf, and dialysis of the enzyme against 10 mM EDTA did not result in any loss of activity. All of these findings suggest that the interconversion between **1** and **2** catalyzed by the mutase is unlikely to be a redox process.

Activity Enhancement Upon Dithionite Reduction. Although none of the above data are indicative of a redox process for the mutase reaction, a mechanism in which substrate oxidation followed by rearrangement and product reduction are coupled to the recycling of FAD between its oxidized and reduced forms remains a viable scenario. If such a redox recycling is indeed the mechanism for Glf, reduction of the enzyme bound FAD should inactivate the mutase since the flavin coenzyme assumes an active role in this postulated oxidation–reduction sequence. To gain further insight into whether the enzyme bound FAD plays a role in the catalysis, the mutase was treated with dithionite to reduce the flavin coenzyme. To our great surprise, when sodium dithionite (20 mM) was included in the assay mixture, the catalytic efficiency (k_{cat}/K_m) of the mutase was increased by more than 2 orders of magnitude (k_{cat} of 27 s^{-1} and K_m of 22 μM). This result clearly indicated that the reduced enzyme is more active than the oxidized enzyme.

It should be noted that in addition to FAD, UDP-Gal*p* mutase also contains two cysteine residues (C16 and C45).[3] Titration with Ellman's reagent (DTNB)[15] revealed that half (~52%) of the thiols in the native enzyme are in the reduced form with the remaining half involved in disulfide linkage. Thus, the increase of catalytic efficiency upon dithionite treatment may be a combined effect of the reduction of FAD and the disulfide linkage. Indeed, after treatment with dithionite, the chromophore of the flavin coenzyme was bleached, and almost all thiols (91%) were in the reduced form. Interestingly, the bleached FAD in the reduced mutase could be readily oxidized by air, and the activity of the resulting enzyme lowered to the same level as the control without dithionite treatment. Because most of the cysteines (85%) remained reduced upon brief exposure to air, the activity enhancement after treatment with dithionite must be largely attributed to the reduction of FAD. This conclusion is consistent with the facts that these cysteines are not conserved among mutases of different origins,[16] and thiol-modifying agents, such as iodoacetamide and iodoacetate, have no inhibitory effect on the enzyme at concentration as high as 20 mM.

Photoacceleration. To further study the role of reduced FAD in enhancing the activity of mutase, the flavin coenzyme was selectively reduced by photoreduction of mutase in the presence of 5-deazariboflavin under anaerobic conditions at room temperature.[17] It was found that the specific activity of the bleached

(14) NAD(P)H was reported to be required for the activity of mutase isolated from *Klebsiella pneumoniae*.[4a]

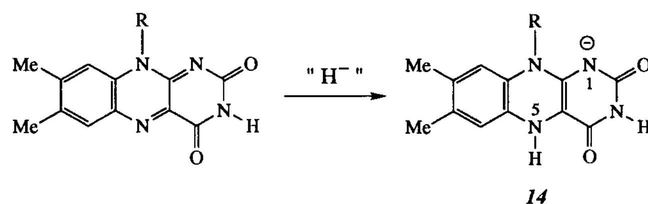
(15) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49–60.

(16) The mutase from *Mycobacterium tuberculosis* contains no cysteine residues at all,[4b] and that from *Klebsiella pneumoniae* serotype O1 contains six cysteines.[4a] Since these cysteine residues are not conserved, they are unlikely to play active roles in the proposed mechanism.

enzyme was 29-fold higher than that of the control without illumination. The magnitude of enhancement is comparable to that observed in the case of dithionite reduction (36-fold enhancement at room temperature). Most importantly, the free thiol content of the photoreduced Glf was determined to be 51%, which is essentially the same as that of the native enzyme. These data firmly established that reduction of the enzyme-bound FAD is the most significant factor in the large enhancement of UDP-Gal*p* mutase activity. Since mutase with either oxidized or reduced FAD is active, this fact strongly suggests that the change of the redox state of FAD affects only the activity, but not the catalytic mechanism.

Several enzymes that contain oxidized flavin and yet catalyze reactions involving no net redox chemistry are known. These include chorismate synthase,[18] acetolactate synthase,[19] mandelonitrile lyase,[20] tartronate-semialdehyde synthase,[21] and YerE.[13] While the catalytic roles for the flavin in these enzymes remain uncertain, it has been shown that chorismate synthase, which catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate, requires a reduced FMN for activity. Recently, the reduced flavin coenzyme in chorismate synthase has been demonstrated to be involved in the catalytic cycle mediating a radical mechanism.[22] A similar pathway for UDP-Gal*p* mutase invoking radicals appears unlikely since both the oxidized and reduced enzymes are active.

Proposed Mechanism of Acceleration. Overall, the observed significant effect of the reduced enzyme-bound FAD on the rate of UDP-Gal*p* mutase illustrates an interesting phenomenon. One can propose that reduction of FAD, which involves transformation of the coenzyme from a highly conjugated planar frame to a bent butterfly structure, may induce a conformational change within the enzyme that may be more conducive to catalysis. Likewise, it is also possible that the reduced flavin imparts a more negative character at N-1 (**14**), which may then be used



to stabilize the transiently formed oxocarbenium ion intermediates (**3** and/or **5**) to facilitate catalysis. A comparison of the circular dichroism (CD) spectra of the native and the reduced enzyme failed to discern a major change of the enzyme conformation associated with the bleaching of FAD (Figure 2). Thus, this makes the charge stabilization theory more appealing. Still, one cannot fully discount the possibility that the invoked favorable conformational change may be highly localized in the active site and hence may not be detected by CD. Therefore, understanding whether it is structural or electronic effects (or a combination of both) that dictate the novel capacity of reduced FAD for mutase rate enhancement is clearly a challenging task for future exploration.

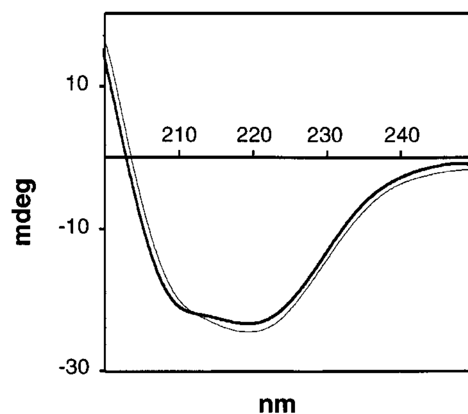


Figure 2. Circular dichroism spectra of Glf (11 μM) in 50 mM potassium phosphate buffer, pH 7.6 (bold line), and Glf (11 μM) in the same buffer containing 20 mM sodium dithionite (plain line).

In summary, the coenzyme FAD in UDP-galactopyranose mutase plays an unusual role in regulating the catalytic efficiency of this enzyme. Further studies should reveal whether this is a unique characteristic of UDP-Gal*p* mutase or if this is just the first example of an enzyme utilizing flavin in a nonclassical way to achieve optimal activity.

Experimental Section

General. Unless otherwise specified, all chemicals were purchased from Aldrich or Sigma, and used without further purification. NMR spectra were recorded on Varian 200, 300, or 500 MHz spectrometers. ^{13}C NMR spectra were recorded with proton broad-band decoupling and the reported spin couplings are from phosphorus. ^{31}P NMR spectra were recorded with proton decoupling and externally referenced with 85% phosphoric acid. The *J*-values are given in Hz. For NMR spectra recorded in D_2O, *t*-BuOH was added as an internal reference; the chemical shifts are 1.27 and 31.2 ppm for ^{1}H NMR and ^{13}C NMR, respectively.[23] All UV–visible spectra were taken on a Beckman DU650 spectrophotometer. Fast-atom bombardment (FAB) and electrospray ionization (ESI) mass spectra were recorded by the MS facility at the Department of Chemistry of the University of Minnesota. High-performance liquid chromatography (HPLC) analysis and purification was conducted with a Hewlett-Packard 1090A system equipped with a photodiode array detector. To detect nucleotide sugar, the detector wavelength was set at 262 nm. Circular dichroism spectra were recorded on a Jasco 710 instrument. Analytical thin-layer chromatography was carried out on Merck silica gel 60 G-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% in EtOH). ICP analysis was performed by the Research Analytical Laboratory at the Department of Soil, Water, and Climate, of the University of Minnesota. *N*-Terminal amino acid sequencing was performed by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota.

Construction of the *glf* Expression Plasmid. The sequence of the *glf* gene in *Escherichia coli* has been reported,[3] and this information allowed the design of oligonucleotide primers complementary to the sequence at each end of the gene. The start primer, 5′-TGTTTTGCT-GAGGAT**CATATG**TACGATTATATCATT-3′, contained a *Nde*I restriction site (in bold) and the codons for the first six amino acid residues of Glf. The halt primer, 5′-TTACAAGATAGA**CTCGAG**ATCCG-TACTCATTATATT-3′, introduced a *Xho*I restriction site (in bold) which replaced the stop codon of the *glf* gene. These primers were used to amplify the *glf* gene from the genomic DNA of *E. coli* C600 (ATCC 23724) by PCR. The PCR-amplified fragment was purified, digested with *Nde*I and *Xho*I and ligated into *Nde*I/*Xho*I sites of the transcription vector, pET-24b(+) (Novagen) to give pQZ-1. This recombinant plasmid was isolated and was used to transform *E. coli* DH5α. Positive clones were identified by digestion of the plasmid DNA

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with *Nde*I and *Xho*I and visualization of the excised insert by staining an agarose gel of the DNA with ethidium bromide after electrophoresis. The plasmid DNA from positive clones was used to transform *E. coli* BL21 (DE3). The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al.[24]

Expression and Purification of UDP-galactopyranose Mutase. *E. coli* BL21 (DE3)/pQZ-1 was grown in Luria-Bertani (LB) broth supplemented with kanamycin (50 μg/mL). Six 1 L batches were inoculated with an overnight culture (1 mL each), and the cells were grown at 37 °C for 18 h with vigorous agitation. The cells were harvested by centrifugation (5000*g*, 10 min), washed with 50 mM potassium phosphate buffer (pH 7.0), collected again by centrifugation (5000*g*, 10 min), and stored at −80 °C. A typical yield was approximately 60 g (wet weight) of cells/6 L of culture. Isopropyl β-D-thiogalactoside (IPTG) was found to have little effect on the level of overexpression, and thus was not added. The *C*-terminal His_6-tagged protein was purified to near homogeneity using Ni-NTA resin (Qiagen), according to the protocols recommended by the manufacturer. The purified mutase (Glf) was found to be stable in phosphate buffers containing 15% glycerol. Neither reducing agent (such as dithiothreitol) nor protease inhibitor was included in any purification steps. The yield was about 100 mg of the pure protein from 6 L of culture.

Protein Assay. Protein concentrations were routinely determined by the Bradford method[25] using bovine serum albumin as the standard. This assay was calibrated by comparing the results from quantitative amino acid analysis performed on aliquots of the same sample by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. The purity of the enzyme was assessed by SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out in the discontinuous buffer system of Laemmli,[26] and the separating gel and stacking gel were 12 and 4% polyacrylamide, respectively. Prior to electrophoresis, protein samples were heated in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.0025% bromophenol blue. Electrophoresis of the heated samples was run in 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS (pH 8.3) at 25 mA. Gels were stained with Coomassie blue and destained with acetic acid-ethanol−water (15:20:165 by volume).

Molecular Weight Determination. The molecular weight of the native enzyme was determined by gel filtration chromatography performed on a Pharmacia FPLC Superdex S-200 HR 10/30 column eluted with 50 mM potassium phosphate buffer (pH 7.6) containing 0.15 M NaCl. The column was calibrated by separate chromatographic runs with the following protein standards: cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (160 kDa), β-amylase (200 kDa), and blue dextran (2000 kDa). Each standard was dissolved in 50 mM potassium phosphate buffer (pH 7.6) containing 0.15 M NaCl and injected separately to determine the retention time. The data were analyzed by the method of Andrews.[27] The subunit molecular mass was estimated by SDS-PAGE as described by Laemmli.[26] Protein standards included trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), and bovine albumin (66 kDa).

Quantitation of Bound FAD. The stoichiometry of bound FAD per subunit of the mutase was estimated by measuring the quantity of released FAD from a denatured mutase sample of known concentration. In this experiment, a sample of mutase (94 μM) in 100 mM potassium phosphate buffer, pH 7.6, was denatured by boiling for 10 min in a foil-covered centrifuge tube. The precipitate was removed by centrifugation, and portions of the supernatant were used for the measurement of absorption at 450 nm against a blank that consisted of the final dialysis buffer. An extinction coefficient of 11 300 M^{-1} cm^{-1} was used for the calculation.

Synthesis of UDP-Gal*f* (2). 2,3,5,6-Tetra-*O*-benzoyl-D-galactono-1,4-lactone (8). To a solution of D-galactono-1,4-lactone (**7**, 10 g, 56 mmol) in pyridine (150 mL) was slowly added excess benzoyl chloride (40 mL) at 0 °C. The resulting mixture was stirred for 3 h at room temperature and was then poured into ice water to quench the reaction. The aqueous layer was decanted, and the remaining syrup was dissolved in ethyl acetate (300 mL). The organic solution was washed with dilute sodium bicarbonate and brine and dried over magnesium sulfate. The solvent was removed under reduced pressure, and the residue was chromatographed (ethyl acetate/hexanes = 1:5) on silica gel to give **8** in 72% yield (24 g). ^{1}H NMR of **8** is identical to the reported data.[10]

2,3,5,6-Tetra-*O*-benzoyl-D-galactofuranose (9). Disiamylborane was prepared by adding 2-methyl-2-butene (2.0 M in THF, 40 mL) to a solution of borane-dimethyl sulfide complex (10 M borane, 4 mL) at 0 °C under nitrogen atmosphere. After stirring for 3 h, 2,3,5,6-tetra-*O*-benzoyl-D-galactono-1,4-lactone (**8**, 5.94 g, 10 mmol) in THF (15 mL) was added to this disiamylborane solution, and the resulting mixture was allowed to react for an additional 18 h at room temperature. Water (15 mL) was slowly added, and the mixture was stirred for 1 h to quench the reaction. After removal of solvent under reduced pressure, the organic residue was purified by silica gel column chromatography with ethyl acetate/hexanes (1:3) as the eluent. The product **9** was isolated as a mixture of α and β isomers (4.7 g, 79%). ^{1}H NMR of **9** is identical to the reported data.[10]

Penta-*O*-benzoyl-D-galactofuranose (10). 2,3,5,6-Tetra-*O*-benzoyl-D-galactofuranose (**9**, 10.1 g, 17 mmol) was dissolved in pyridine (80 mL) and cooled with an ice−water bath. Excess benzoyl chloride (30 mL) was slowly added to this solution and the resulting mixture was stirred for an additional 3 h at 0 °C. The mixture was then poured into ice water, and the aqueous layer was decanted. The remaining syrup was dissolved in ethyl acetate (300 mL) and washed with dilute sodium bicarbonate and brine and dried over sodium sulfate. After the solvent was removed under reduced pressure, the crude product was chromatographed on silica gel to afford the desired product **10** in 84% yield (9.98 g). ^{1}H and ^{13}C NMR of **10** are identical to the reported data.[12]

Dibenzyl 2,3,5,6-Tetra-*O*-benzoyl-α-D-galactofuranosyl-1-phosphate (11). To a solution of **10** (3 g, 4.3 mmol) in anhydrous CH_2Cl_2 (25 mL) was slowly added excess bromotrimethylsilane (6 mL) at 0 °C. The cooling bath was removed 1 h later, and the reaction was stirred for 24 h at room temperature. The mixture was evaporated to dryness under reduced pressure, and the residue was mixed with dibenzyl phosphate (1.8 g, 6.5 mmol) and Et_3N (895 μL) in anhydrous toluene (10 mL). After stirring overnight at room temperature, the mixture was evaporated to dryness under reduced pressure. The crude product was chromatographed on silica gel (ethyl acetate/toluene = 1:9) to give the product **11** in 50% yield (1.79 g). ^{1}H NMR ($CDCl_3$) δ 4.63 (1H, dd, J = 12.0, 6.3), 4.74 (1H, dd, J = 6.9, 4.5), 4.77 (1H, dd, J = 12.0, 4.5), 4.85 (1H, dd, J = 11.7, 7.8), 4.96 (1H, dd, J = 11.7, 6.9), 4.99 (1H, dd, J = 12.0, 8.7), 5.06 (1H, dd, J = 11.7, 7.5), 5.76 (1H, ddd, J = 7.5, 4.5, 1.8), 5.86 (1H, dt, J = 6.3, 4.5), 6.19 (1H, t, J = 6.9), 6.38 (1H, dd, J = 6.0, 4.5), 7.0−8.2 (m, aromatic-Hs). ^{13}C NMR ($CDCl_3$) δ 62.8, 69.4 (d, J = 5), 69.5 (d, J = 5), 70.8, 73.4, 76.6 (d, J = 6), 80.0, 97.7 (d, J = 4), and the aromatic signals 127−134, 165.5, 165.6, 165.7, 165.9. ^{31}P NMR ($CDCl_3$) δ −1.8.

Triethylammonium 2,3,5,6-Tetra-*O*-benzoyl-α-D-galactofuranosyl-1-phosphate (12). Compound **11** (1.65 g, 2 mmol) in a mixture of ethyl acetate (20 mL) and Et_3N (1.6 mL) was hydrogenated overnight in the presence of catalytic amount of palladium (10%) on charcoal. After being filtered through a Celite pad, the solution was concentrated to dryness to yield **12** as a monotriethylammonium salt (1.41 g, 98% yield). ^{1}H NMR ($CDCl_3$) δ 1.15 (9H, t, J = 7.2), 2.89 (6H, q, J = 7.2), 4.59 (1H, dd, J = 12.0, 4.8), 4.75 (1H, dd, J = 12.0, 4.2), 4.88 (1H, dd, J = 12.0, 4.2), 5.67 (1H, ddd, J = 7.2, 4.5, 0.9), 5.85 (1H, dt, J = 6.6, 4.2), 6.17 (2H, m), 7.26−7.35 (8H, m), 7.37−7.49 (4H, m), 7.85−8.18 (8H, m). ^{13}C NMR ($CDCl_3$) δ 8.5, 45.3, 63.1, 71.5, 74.3, 76.6 (d, J = 6), 78.7, 96.0 (d, J = 4), and the aromatic signals 127−134, 165.5, 165.8, 165.9, 166.0.

Bis(triethylammonium) α-D-Galactofuranosyl-1-phosphate (13). The protected galactofuranosyl-1-phosphate salt (**12**, 0.71 g, 0.84 mmol) was kept in a solution of $MeOH/H_2O/Et_3N$ (5:2:1, 32 mL) at 32 °C for 3 days. The resulting mixture was concentrated to dryness under reduced pressure, and the residue was chromatographed on Sephadex LH-20 (1.5 × 45 cm) using MeOH as the eluent. Fractions containing the product were pooled and evaporated to dryness to yield **13** as a bis-triethylammonium salt (341 mg, 88% yield). ^{1}H NMR (D_2O) δ 1.30 (18H, t, J = 7.2), 3.23 (12H, q, J = 7.2), 3.68 (1H, dd, J = 11.7, 7.2), 3.76 (1H, dd, J = 11.7, 4.2), 3.78−3.91 (2H, m), 4.18 (1H, ddd, J =

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8.4, 4.2, 2.4), 4.29 (1H, dd, $J = 8.4, 7.2$), 5.57 (1H, t, $J = 4.5$). ^{13}C NMR (D_2O) δ 9.8, 48.2, 63.9, 73.4, 75.1, 78.1 (d, $J = 8$), 83.1, 98.4 (d, $J = 6$). ^{31}P (D_2O) NMR δ 0.8.

UDP-Gal*f* (2). The triethylammonium salt of α-D-galactofuranosyl phosphate (**13**, 200 mg, 0.43 mmol) was dried by coevaporating with anhydrous pyridine (3 mL) a few times. To this residue was added UMP-morpholidate (760 mg, 1.1 mmol) in anhydrous pyridine (3 mL), and the solution was evaporated again to dryness under vacuum. This was followed by the addition of 1*H*-tetrazole (100 mg, 1.4 mmol) in anhydrous pyridine (5 mL), and the resulting solution was stirred at room temperature for 40 h. Removal of the solvent under vacuum gave a solid residue which was dissolved in methanol and chromatographed on a Sephadex LH-20 column (2.5 × 120 cm) using methanol as the eluent. Fractions containing the desired product, judging by TLC analysis ($EtOH:NH_4OH:H_2O = 5:3:1$), were pooled and evaporated to dryness under reduced pressure. Compound **2** was further purified by HPLC using a C_{18} column (Alltech, 10 × 250 mm). The eluent was 1.5% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8, and the flow rate was 5.0 mL/min. The retention time of **2** under these conditions was 4.7 min. ^{1}H NMR (D_2O) δ 1.30 (18H, t, $J = 7.2$, Et_3N-Me), 3.23 (12H, q, $J = 7.5$, Et_3N-CH_2), 3.65 (1H, dd, $J = 11.4, 6.9$, 6-H), 3.74 (1H, dd, $J = 11.7, 4.2$, 6-H), 3.76−3.87 (2H, m, 4-H and 5-H), 4.17 (1H, ddd, $J = 8.4, 4.2, 2.7$, 2-H), 4.21−4.33 (4H, m, 3-H, 4′-H, and 5′-H_2), 4.37−4.43 (2H, m, 2′-H and 3′-H), 5.66 (1H, dd, $J = 5.9, 4.2$, 1-H), 6.00 (1H, d, $J = 8.4$, 5″-H), 6.01 (1H, d, $J = 3.3$, 1′-H), 8.00 (1H, $J = 8.4$, 6″-H). ^{13}C NMR (D_2O) δ 9.8 (Et_3N-Me), 48.2 (Et_3N-CH_2), 63.7 (C-6), 66.5 (d, $J = 6$, C-5′), 71.3 (C-2′), 73.7 (C-5), 75.1 (C-3), 75.3 (C-3′), 78.2 (d, $J = 7$, C-2), 83.3 (C-4), 84.9 (d, $J = 9$, C-4′), 89.9 (C-1′), 99.2 (d, $J = 6$, C-1), 104.2 (C-5″), 143.2 (C-6″), 153.4 (C-2″), 167.8 (C-4″). ^{31}P NMR (D_2O) δ −12.2 (d, $J = 20.8$), −11.0 (d, $J = 20.8$). Negative ion HRFABMS calcd for $C_{15}H_{23}N_2O_{17}P_2$ $[M - H]^-$ 565.0472, found *m/z* 565.0499.

Enzyme Assay. The activity of the purified enzyme was assayed by incubating a mixture of UDP-Gal*f* (**2**, 1 mM) and an appropriate amount of the mutase in 30 μL of 100 mM potassium phosphate buffer (pH 7.5) for 2 min at 37 °C. Dithiothreitol was initially included in the assay mixture but was later found unnecessary and was omitted. The reaction was analyzed by HPLC using a C_{18} column (Microsorb-MV, Varian, 4.6 × 250 mm) which was eluted with 1.5% acetonitrile in 50 mM triethylammonium acetate buffer, pH 6.8. The detector was set at 262 nm, and the flow rate was 1.0 mL/min. Baseline resolution was achieved, and the retention times for UDP-Gal*f* (**2**) and UDP-Gal*p* (**1**) were 7.8 and 6.3 min, respectively. The extent of conversion was determined by comparing the integration of the substrate and product peaks. The conversion was routinely controlled to be within 30% by properly adjusting the enzyme concentration.

For assays conducted under reducing conditions, the incubation was performed in sealed vials in the presence of freshly prepared sodium dithionite (20 mM). For assessing the effects of the exogenous cofactor on catalysis, 2 mM of each cofactor being tested was included in the incubation mixture.

Determination of Kinetic Parameters. To determine the kinetic parameters of the native enzyme, a series of samples containing the purified mutase (480 nM) and UDP-Gal*f* (0.10, 0.125, 0.167, 0.25, 0.50, and 1.0 mM) in a total volume of 30 μL of 100 mM potassium phosphate (pH 7.6) were prepared. Each sample was incubated at 37 °C for 1.5 min and frozen with liquid nitrogen to terminate the reaction. The contents of these samples were analyzed by HPLC as described above, and the ratio of product and substrate peak was used to determine the activity. The kinetic parameters were deduced by fitting these data to the Michaelis−Menten equation. The same procedure was also used to study the reduced enzyme. The incubation mixture in this case contained 4.8 nM enzyme, 20 mM fresh prepared sodium dithionite, and appropriate concentration of UDP-Gal*f* (10, 12.5, 16.7, 25, 50, 100, 200, and 500 μM) in 100 μL of 100 mM potassium phosphate buffer (pH 7.6).

Equilibrium Constant. The equilibrium constant, K_{eq}, was calculated from the ratio of product to substrate at equilibrium. The product and substrate ratios were determined for both the forward and reverse directions by integration of the corresponding peaks from the HPLC chromatogram. For the reverse reaction, 1 mM UDP-Gal*f* was mixed with 8 μM purified mutase in 200 μL of 100 mM potassium phosphate buffer (pH 7.6). The incubation was allowed to reach equilibrium as determined by the constant product/substrate ratio by HPLC. The same procedure was repeated with a 200 μL incubation of 1 mM UDP-Gal*p* and 8 μM enzyme in 100 mM potassium phosphate buffer (pH 7.6).

Preparation of 5-Deazariboflavin. 5-Deazariboflavin required for the photoreduction experiment was synthesized according to a reported procedure,[28] and characterized by NMR. ^{1}H NMR (DMSO-d_6) δ 2.32 (3H, s), 2.44 (3H, s), 3.44 (1H, br), 3.60 (3H, br), 4.21 (1H, br), 4.46 (1H, s, exchangeable), 4.63 (1H, d, $J = 13.8$), 4.78 (1H, d, $J = 5.7$, exch.), 4.87 (1H, s, exch.), 4.99 (1H, m), 5.11 (1H, d, $J = 3.9$, exch.), 7.86 (1H, s), 7.94 (1H, s), 8.86 (1H, s), 11.0 (1H, s, exch.). ^{13}C NMR (DMSO-d_6) δ 19.1, 21.4, 47.7, 63.9, 70.0, 73.4, 74.1, 114.2, 118.4, 120.1, 131.0, 134.1, 140.4, 141.5, 146.4, 156.8, 158.0, 162.7.

Photoreduction. To selectively reduce the enzyme bound flavin coenzyme, a mixture of 180 nM mutase, 23 μM 5-deazariboflavin, and 12 mM EDTA in a total volume of 120 μL of 100 mM potassium phosphate buffer (pH 7.6) was prepared. This reaction mixture was made anaerobic through a Schlenk line with repeated cycles between vacuum and argon on ice. To this mixture was added 30 μL of anaerobic 5 mM UDP-Gal*f* solution (in 100 mM potassium phosphate buffer, pH 7.6), and photoreduction was carried out promptly by irradiating this sample with a slide projector light bulb (400 W, 82 V) controlled by a variable autotransformer. The light source was approximately 10 cm away from the sample which was kept in a water bath at room temperature. The total incubation time was 2 min, and the total illumination time was 1 min, with 10 s cooling intervals after every 10 s illumination. Excess 5-deazariboflavin was used to ensure complete reduction of the bound FAD. A control experiment was performed in parallel without illumination.

Thiol Titration. All experiments were run in buffers saturated with nitrogen gas. A typical experiment involved the addition of the denaturing solution consisting of 1 mM EDTA and 6.4 M guanidine hydrochloride in 80 μL of 100 mM potassium phosphate buffer (pH 7.3) to a solution of the mutase in 10 μL of 50 mM phosphate buffer (pH 7.6). The exposed thiols in the denatured enzyme were titrated by adding 10 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to the solution and measuring the change in absorbance at 412 nm ($\epsilon_{412} = 13\,700$ M^{-1} cm^{-1}) against a control without DTNB. The absorption of DTNB alone at 412 nm measured from a separate experiment was subtracted. To study the effect of photoreduction of Glf, a mixture of 110 μM enzyme, 20 μM 5-deazariboflavin, and 5 mM EDTA in 200 μL of 100 mM potassium phosphate buffer (pH 7.6) was illuminated for 15 s under anaerobic conditions, which resulted in complete bleaching of the flavin chromophore. To this solution was added 1.6 mL of the above denaturing solution and the exposed thiols were similarly titrated. To determine the effect of dithionite reduction on Glf, the purified enzyme (210 μM) was treated with 5 mM sodium dithionite in 100 μL of potassium phosphate buffer (50 mM, pH 7.6). To this solution was added 800 μL of the above denaturing solution and the resulting mixture was loaded onto a 10DG desalting column (Bio-Rad) preequilibrated with EDTA (1 mM) and guanidine hydrochloride (6.0 M) in potassium phosphate buffer (50 mM, pH 7.3). The protein was eluted using the same solution. The protein fractions were pooled, and the exposed thiols in the denatured enzyme were titrated. For the air reoxidized enzyme, the denaturing solution was added immediately after the bleached mutase recovered its absorption at 450 nm. The resulting mixture was desalted and thiols were similarly titrated.

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Note Added in Proof. A synthesis of **2** was recently reported (Tsvetkov, Y. E.; Nikolaev, A. V. *J. Chem. Soc. Perkin. Trans. I* **2000**, 889−891).

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