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Mechanism and Active Site Residues of GDP-Fucose Synthase

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Abstract: L-Fucose, 6-deoxy-L-galactose, is a key component of many important glycoconjugates including the blood group antigens and the Lewis^X ligands. The biosynthesis of GDP-L-fucose begins with the action of a dehydratase that converts GDP-D-mannose into GDP-4-keto-6-deoxy-mannose. The enzyme GDP-fucose synthase, GFS, (also known as GDP-4-keto-6-deoxy-D-mannose epimerase/reductase, GMER) then converts GDP-4-keto-6-deoxy-D-mannose into GDP-L-fucose. The GFS reaction involves epimerizations at both C-3″ and C-5″ followed by an NADPH-dependent reduction of the carbonyl at C-4. This manuscript describes studies that elucidate the order of the epimerization steps and the roles of the active site acid/base residues responsible for the epimerizations. An active site mutant, Cys109Ser, produces GDP-6-deoxy-D-altrose as its major product indicating that C-3″ epimerization occurs first and premature reduction of the GDP-4-keto-6-deoxy-D-altrose intermediate becomes competitive with GDP-L-fucose production. The same mutation results in the appearance of a kinetic isotope effect when [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose is used as a substrate. This indicates that Cys109 is the base responsible for the deprotonation of the substrate at C-3″. The Cys109Ser mutant also catalyzes a rapid wash-in of solvent derived deuterium into the C-5″ position of GDP-fucose in the presence of NADP$^+$. This confirms the order of epimerizations and the role of Cys109. Finally, the inactive His179Gln mutant readily catalyzes the wash-out of deuterium from the C-3″ position of [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose. Together these results strongly implicate an ordered sequence of epimerizations (C-3″ followed by C-5″) and suggest that Cys109 acts as a base and His179 acts as an acid in both epimerization steps.

Introduction

L-Fucose, or 6-deoxy-L-galactose, is found as a key component of many biologically important glycoconjugates in both prokaryotes and eukaryotes.[1,2] In mammals, L-fucose is a terminal sugar in the glycans that comprise the human ABO blood group antigens.[3] It is also an essential component of the cell surface ligands for the selectin proteins.[4] The binding of endothelial selectins to the fucose-containing Lewis^X and sialyl Lewis^X structures on the surface of leukocytes is responsible for initiating the inflammatory response. Humans unable to fucosylate proteins suffer from the immune disorder known as leukocyte adhesion deficiency type II.[5] Conversely, *O*-fucosylation of the Notch receptors is critical for the proper functioning of the Notch signaling pathways and increased levels of fucosylation has been linked to cancer.[6,7] In human parasites such as *Trypanosoma brucei* (the causative agent of African sleeping sickness), fucose biosynthesis is essential for the life cycle of the parasite and the biosynthetic enzymes represent attractive targets for drug development.[8] In many strains of bacteria L-fucose is found as a component of capsular polysaccharides and lipopolysaccharides (LPS).[9] Notably, the human pathogen *Helicobacter pylori* (the causative agent of peptic and duodenal ulcers), expresses the Lewis^X trisaccharide as the major component of its LPS in an effort to evade the host's immune system via a strategy of molecular mimicry.[10]

L-Fucose is biosynthesized as the sugar nucleotide, GDP-L-fucose, in two enzymatic steps from the precursor GDP-D-mannose (Figure 1).[2,11,12] The first step is catalyzed by GDP-mannose 4,6-dehydratase (GMD) and produces GDP-6-deoxy-α-D-*lyxo*-hexos-4-ulose (GDP-6-deoxy-4-keto-mannose).[13,14] This enzyme catalyzes an overall redox neutral dehydration reaction involving an initial NADP$^+$-dependent oxidation at C-4″, followed by an elimination of water between C-5″ and C-6″, and a final reduction at the C-6″ carbon of the resulting enone intermediate.

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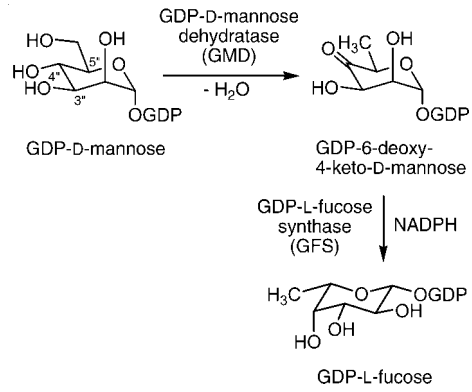

Figure 1. Biosynthesis of GDP-L-fucose from GDP-D-mannose.

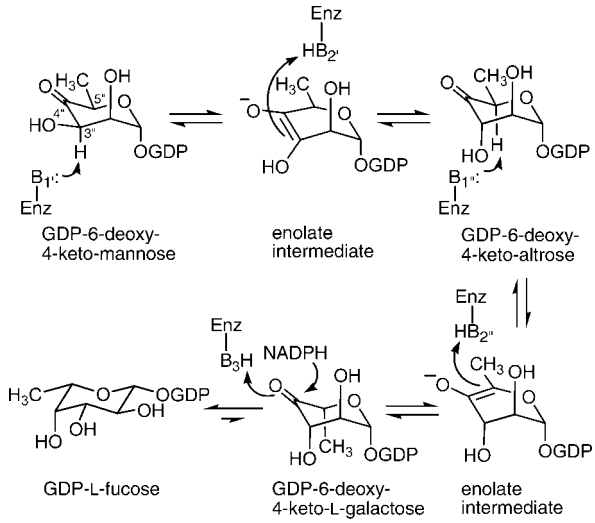

Figure 2. Putative mechanism for the reaction catalyzed by GDP-L-fucose synthase. Accurate conformations are not portrayed in this figure. B_1, B_2, and B_3 represent active site acid/base residues.

This step introduces the characteristic 6-methyl group of fucose as well as the 4-keto functionality that is required for the subsequent epimerizations. The second step in the biosynthesis is catalyzed GDP-fucose synthase or GFS (also known as GDP-4-keto-6-deoxy-D-mannose epimerase/reductase or GMER).[15−20] GFS is remarkable in that it is able to catalyze three distinct reactions within a single active site. These include inversions of stereochemistry at both C-3″ and C-5″, as well as an NADPH-dependent reduction of the ketone functionality at C-4.

A putative mechanism for the reaction catalyzed by GFS is shown in Figure 2. An initial deprotonation at C-3″ generates an enolate intermediate that is reprotonated on the opposite face to give the C-3″ epimer, GDP-6-deoxy-α-D-*arabino*-hexos-4-ulose (GDP-6-deoxy-4-keto-altrose). A similar deprotonation/reprotonation sequence then inverts the stereochemistry at C-5″ to give GDP-6-deoxy-α-L-*xylo*-hexos-4-ulose (GDP-6-deoxy-4-keto-L-galactose). Finally, an NADPH-dependent reduction converts the C-4″ ketone into a hydroxyl group and generates GDP-L-fucose. Studies that support this mechanism include the observation that GDP-6-deoxy-4-keto-mannose bearing a tritium label at C-3″ loses its label to solvent during catalysis.[20] This indicates that the inversions of stereochemistry occur via deprotonation/reprotonation events accompanied by solvent isotope incorporation. This is also consistent with the observation that GFS can catalyze the epimerization steps in the absence of NADPH.[17] Further studies have shown that GFS catalyzes the stereospecific transfer of the *pro-S* hydride from NADPH to the C-4″ position of the sugar nucleotide.[17] When deuterated NADPH was employed, the reaction was slowed by a kinetic isotope effect (KIE) on V_{max} of 1.4, indicating that reduction was only partially rate-limiting during catalysis.

The structure of GFS from *Escherichia coli* in complex with $NADP^+$ and NADPH has been solved by X-ray crystallography; however, no structures with a bound GDP-sugar are available.[16,18,19] The structures clearly show that GFS is a member of the short chain dehydrogenase reductase (SDR) family of enzymes,[21,22] and that its fold closely resembles that of UDP-galactose 4-epimerase,[23] an enzyme that also catalyzes redox chemistry at the C-4″ position of a sugar nucleotide. GFS bears the characteristic Ser-Tyr-Lys catalytic triad that is a hallmark of the SDR family members and is responsible for promoting the hydride transfer step.[24] From both the structural analysis of GFS and literature precedence with other sugar nucleotide-modifying SDR enzymes,[25−27] Tyr136 of this triad is strongly implicated as the catalytic acid that protonates the C4 carbonyl during the final reduction step (B_3 in Figure 2). Two additional active site residues, Cys109 and His179, which are not conserved throughout the SDR family have been implicated as the acid/bases involved in promoting the inversions of stereochemistry (B_1 and B_2 in Figure 2). While the absence of bound substrate complicates this assignment, modeling studies suggest these are the only two residues that would be in an appropriate position to play these roles.[16,18,19] In addition, site directed mutations of the Tyr136, Cys109, and His179 residues lead to enzymes with dramatically lowered activities indicating that they are all crucially important for efficient catalysis.[16]

Further insight into the GDP-fucose synthase reaction has been gained from studies on the related enzyme GDP-mannose 3,5-epimerase involved in vitamin C biosynthesis in plants (Figure 3).[28−31] This enzyme can convert GDP-D-mannose into

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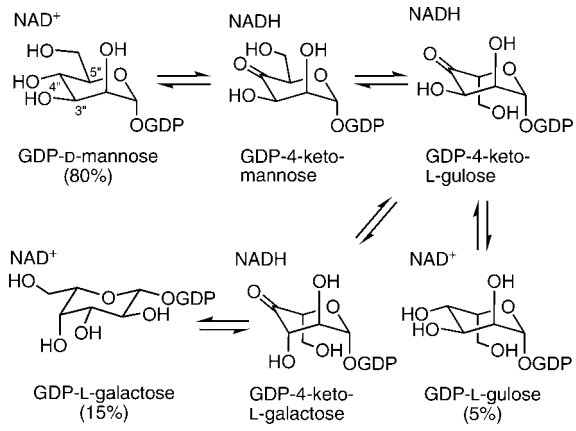

Figure 3. Reactions catalyzed by the enzyme GDP-mannose 3,5-epimerase.

an equilibrium mixture of GDP-D-mannose (80%), GDP-L-gulose (5%), and GDP-L-galactose (15%) via reversible epimerizations at both the C-3″ and C-5″ positions. This enzyme is also a member of the SDR family of enzymes and shares 24% sequence identity with GFS. The proposed mechanism for this reaction involves an initial oxidation at C-4″ by a tightly bound NAD^+ cofactor. Subsequent epimerizations take place first at C-5″ and then at C-3. Reduction of the C-4″ carbonyl can occur with any of the three ketone intermediates leading to the equilibrium mixture of the three epimeric GDP-hexoses. The ordering of the epimerization steps in this enzyme is supported by the observation that the enzyme generates GDP-L-gulose as a product (C-5″ epimerization only), but does not produce GDP-D-talose (C-3″ epimerization only).[28,29]

Despite the current level of understanding of the mechanism of the GDP-fucose synthase reaction, several key aspects remain unknown. The order of epimerization steps (C-3″ first or C-5″ first) is not known and thus the nature of the true intermediate formed during catalysis is unclear (C-3″ first is shown in Figure 2). The identity and precise roles of the acid/base residues that promote the inversions of stereochemistry is not fully understood. While it is clear that the two residues, Cys109 and His179, fulfill at least some of these duties, it is quite conceivable that as many as four active site residues could be required to catalyze two distinct epimerizations (B_1′/B_1″ and B_2′/B_2″ in Figure 2 could all be unique residues). This manuscript presents evidence in favor of a mechanism involving C-3″ inversion followed by C-5″ inversion with GDP-6-deoxy-4-keto-altrose as an intermediate (Figure 2). Cys109 is implicated as the basic residue responsible for deprotonation at both C-3″ and C-5″ (B_1′/B_1″ in Figure 2) and His179 is implicated as the acidic residue responsible for protonation at both C-3″ and C-5″ (B_2′/B_2″ in Figure 2).

Results

Enzyme Preparation and Kinetic Analysis. Genes encoding for the recombinant versions of the *E. coli* GDP-mannose 2,4-dehydratase (GMD) and GDP-fucose synthase (GFS) bearing N-terminal hexahistidine tags were expressed in *E. coli* and the resulting enzymes were purified by metal affinity chromatography. In past studies, the importance of Cys109 in the GFS reaction was probed by generating the Cys109Ala mutant and demonstrating that it suffered from a 7000-fold decrease in enzymatic activity.[16] In this study, a more conservative change was desired and the Cys109Ser mutant was prepared. It was anticipated that if Cys109 were acting as a catalytic acid/base residue, the Cys109Ser mutant would retain sufficient residual activity to allow for thorough kinetic analysis and product studies. Literature precedence indicates that such a replacement of a cysteine acid/base catalyst typically leads to a mutant that retains 0.1−1.0% of the wild type activity.[28,32,33] To study the importance of His179, it was not possible to make such a conservative mutation and the His179Gln mutant was generated.

The substrate for GFS, GDP-6-deoxy-α-D-*lyxo*-hexos-4-ulose (GDP-6-deoxy-4-keto-mannose), was generated by treatment of GDP-mannose with GMD at room temperature.[15] Due to the sensitive nature of this compound toward decomposition, only minimal purification was employed, and the material was freshly prepared before each use. The reaction was monitored by mass spectral analysis until complete and the enzyme was removed by ultrafiltration. The product was concentrated by lyophilization and then used directly in experiments with GFS.

Kinetic analysis of the GFS reaction was performed by directly monitoring the disappearance of NADPH chromophore at 340 nm. The wild type enzyme followed Michaelis−Menten kinetics with values of $k_{cat} = 0.65 \pm 0.02\ s^{-1}$ and $K_{M(GDP\text{-}Man)} = 2.2 \pm 0.2\ \mu M$ (saturating NADPH, see Supporting Information). The Cys109Ser mutant also followed Michaelis−Menten kinetics with values of $k_{cat} = 0.0012 \pm 0.0001\ s^{-1}$ and $K_{M(GDP\text{-}Man)} = 0.9 \pm 0.2\ \mu M$ (saturating NADPH). The 540-fold decrease in the value of k_{cat} for this mutant is consistent with the notion that the residue is involved in acid/base catalysis. When the His179Gln mutant was subjected to kinetic analysis, extremely low levels of activity could be detected, however, it was not possible to accurately measure kinetic constants using the direct assay. It was estimated that the value of k_{cat} had been reduced by at least 10 000-fold by this mutation. This indicates that His179 is a key catalytic residue and is consistent with past studies showing that the His179Asn mutant suffered from a 1 000-fold decrease in the value of k_{cat}.[16]

Product Analysis in the Wild Type and Cys109Ser Reactions. In order to determine the specificity of the reactions catalyzed by the wild type and Cys109Ser synthases, product analyses were performed. Freshly prepared GDP-6-deoxy-4-keto-mannose was incubated with each of the proteins in the presence of excess NADPH. The reactions were run to completion as monitored by mass spectrometry, and the GDP-hexose produced was isolated using ion-exchange chromatography. In the case of the reaction catalyzed by wild type GFS, GDP-fucose was identified as the sole product using ^1H spectroscopy.[34] This assignment is clear from the presence of a signal due to the anomeric proton of a sugar nucleotide (C-1″) at a chemical shift corresponding to that of GDP-fucose (doublet of doublets at 4.90 ppm in Figure 4b). The upfield shift of this signal and large $J_{H1,H2}$ value is expected for GDP-β-L-fucose that adopts a $^1C_4$ conformation in solution. This finding highlights the high specificity in the reduction step of the GFS reaction. The enzyme will not reduce the carbonyl of the starting material or the singly inverted intermediate at any significant rate and only delivers a hydride once both inversions of stereochemistry have taken place.

In the case of the Cys109Ser mutant, a different outcome was observed in that a mixture of two products were formed (Figure 4a). Examination of the ^1H spectrum (Figure 4c)

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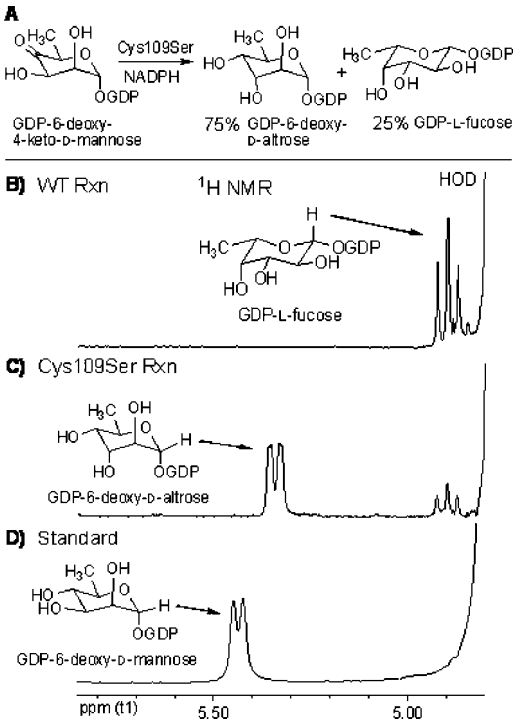

Figure 4. Product analysis of the reaction catalyzed by the Cys109Ser mutant. (A) Structures of the reaction products. (B) ^{1}H spectrum of GDP-L-fucose obtained from a reaction with the wild type enzyme. (C) ^{1}H spectrum of products obtained from a reaction with the Cys109Ser mutant. (D) ^{1}H spectrum of a GDP-6-deoxy-D-mannose standard.

indicated that only 25% of the product was GDP-fucose (H-1″, 4.90 ppm) and that the remaining 75% was an isomer (H-1″, 5.34 ppm). Reasonable possibilities for the structure of this isomer include GDP-6-deoxy-D-mannose (reduction of starting material), GDP-6-deoxy-D-altrose (epimerization at C-3″ followed by reduction) or GDP-6-deoxy-L-gulose (epimerization at C-5″ followed by reduction). We have found that it is possible to prepare an authentic sample of GDP-6-deoxy-D-mannose by treating GDP-6-deoxy-4-keto-mannose with GDP-mannose 4,6-dehydratase and excess NADPH. Apparently the ″tightly bound″ $NADP^+$ cofactor in this enzyme will slowly exchange with the reduced cofactor in solution and then reduce the C-4″ carbonyl of its normal reaction product. ^{1}H analysis of GDP-6-deoxy-D-mannose prepared in this fashion showed an anomeric proton shifted downfield from that of the unidentified isomer (H-1″, 5.43 ppm, Figure 4d). The observed chemical shift is similar to that of GDP-mannose and is expected for a sugar nucleotide in a $^4C_1$ conformation. This indicates that the unidentified isomer did not result from the direct reduction of the starting material and that a single epimerization had occurred prior to reduction. The structural elucidation of the isomer by an analysis of coupling constants or NOE measurements is complicated by the fact that the species will have at least two substituents in an axial orientation and will therefore adopt both the $^1C_4$ and $^4C_1$ conformations in solution. These conformations will rapidly interconvert on the ^{1}H time scale and a time-averaged spectrum will be observed (this is consistent with the midrange chemical shift at 5.34 ppm). Instead, the site of epimerization was deduced using an isotopic wash-out experiment using either [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose or [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose. [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose can easily be prepared by carrying out the GMD reaction in buffer prepared with D_2O since it has been well-established that the dehydratase

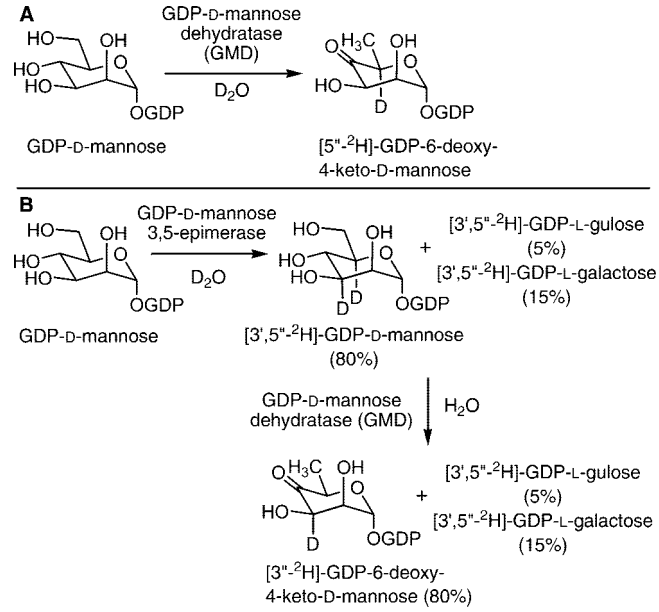

Figure 5. (A) Preparation of [5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose. (B) The preparation of [3″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose.

reaction is accompanied by solvent isotope incorporation at C-5″ (Figure 5a).[35,36] Lyophilization and resuspension in buffer prepared with H_2O prepares the substrate for the wash-out experiment. In order to prepare [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose, a sample of GDP-mannose was extensively incubated with GDP-mannose 3,5-epimerase (Figure 3) in D_2O to generate a mixture of [3″,5″-^{2}H]-GDP-D-mannose (80%), [3″,5″-^{2}H]-GDP-L-gulose (5%), and [3″,5″-^{2}H]-GDP-L-galactose (15%) (Figure 5b). This mixture was isolated by size-exclusion chromatography and then treated with GMD in a buffer prepared with H_2O. GMD was found only to accept [3″,5″-^{2}H]-GDP-D-mannose as a substrate and therefore generated a sample of [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose (80%) contaminated with [3″,5″-^{2}H]-GDP-L-gulose (5%), and [3″,5″-^{2}H]-GDP-L-galactose (15%) that was used directly in the wash-out experiment. Both labeled substrates were treated with the Cys109Ser mutant and excess NADPH and the resulting products were analyzed by mass spectrometry. In the case of [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose (m/z = 587), no deuterium was detected in the spectrum of the reduced products (m/z = 588), whereas with [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose (m/z = 587), 75% of the resulting product retained one deuterium label (m/z = 589). Since solvent isotope incorporation accompanies an inversion of stereochemistry,[20] it is clear that the isomer formed by the Cys109Ser mutant is GDP-6-deoxy-D-altrose (epimerization at C-3″ followed by reduction). This indicates GFS first inverts the stereochemistry at C-3″ and then inverts the stereochemistry at C-5″ during normal catalysis (Figure 2). With the Cys109Ser mutant, premature reduction of the intermediate can occur before the second epimerization takes place. The observation that premature reduction is competitive with GDP-fucose formation indicates that Cys109 plays a key role in the second epimerization step.

Kinetic Isotope Effects in the Wild Type and Cys109Ser Reactions. In order to probe the role of Cys109 as an acid/base residue in the GFS reaction, kinetic isotope effects (KIEs) were

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measured with deuterated versions of GDP-fucose. It was anticipated that any steps requiring this residue would be slowed in the Cys109Ser mutant and this may result in them becoming rate-determining. [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose and [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose (containing 5% [3″,5″-^{2}H]-GDP-L-gulose and 15% [3″,5″-^{2}H]-GDP-L-galactose) were used to probe whether deprotonation at either C-5″ or C-3″ are rate-determining steps. The labeled compounds were prepared as described previously (Figure 5), and the unlabeled counterparts were simultaneously prepared using identical conditions and reagents, but with H_2O instead of D_2O. Control reactions (monitored by mass spectrometry) showed that the deuterium labels in the starting materials did not exchange with solvent at any significant rate either in the absence or the presence of GFS/NADPH. With the wild type GFS, the KIEs on the values of k_{cat} for [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose and [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose were found to be $k_H/k_D = 1.0 \pm 0.1$ and $k_H/k_D = 1.0 \pm 0.1$, respectively. The absence of a primary KIE with either substrate indicates that the deprotonations at C-3″ and C-5″ are not rate-determining steps in the normal GFS reaction.

In the case of the Cys109Ser mutant, the KIE on the value of k_{cat} for [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose was found to be 1.0 ± 0.1. The absence of any significant KIE with this substrate was expected since the major product with this mutant is GDP-6-deoxy-D-altrose that is formed in a process that does not involve deprotonation at C-5. However, when [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose was tested with Cys109Ser, the KIE on the value of k_{cat} was found to be 1.5 ± 0.1 (see Supporting Information). This suggests that deprotonation at C-3″ has become a partially rate-determining step with this mutant and a masked primary KIE is observed. This observation strongly supports the notion that Cys109 serves as the basic residue involved in the deprotonation at C-3″ in the first step of the reaction ($B_{1'}$ in Figure 2).

Solvent Deuterium Exchange into GDP-Fucose. Information on the order of epimerizations and on the role of the active site residues can also be obtained by monitoring solvent-derived deuterium incorporation into the product, GDP-fucose. When GDP-fucose and $NADP^+$ are incubated with GFS, no apparent reaction is observed since the equilibrium of the GFS reaction strongly favors these products. Nevertheless, it is expected that the reverse reactions are still kinetically accessible and that the oxidation and epimerization steps may still take place. To observe these processes, a sample containing GDP-fucose and $NADP^+$ was incubated with GFS in a buffer prepared from D_2O and the reaction was monitored by ^{1}H spectroscopy. An examination of the spectrum taken before the addition of enzyme shows all four signals due to the H-2″−H-5″ protons in the region between 3.5 and 3.8 ppm (Figure 6a). After treatment with wild type GFS the signals due to H-3″ and H-5″ readily disappear due to exchange of these protons with solvent-derived deuterium (Figure 6b). The incorporation of two deuterium atoms is also observed in purified samples analyzed by mass spectrometry. Monitoring the time course of this exchange shows that H-5″ exchanges roughly twice as fast as H-3″ (not shown in Figure 6). The deuterium incorporation results show that the reverse reaction is kinetically accessible despite the thermodynamics favoring GDP-fucose production.

Quite a different result was obtained when the Cys109Ser mutant was subjected to the same experiment. In this case, H-5″ readily exchanged with solvent deuterium whereas H-3″ did not, even after extended incubations (Figure 6c). When the

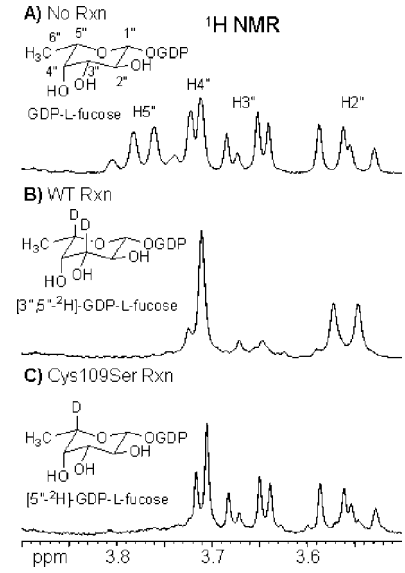

Figure 6. ^{1}H spectra showing the wash-in of solvent derived deuterium into GDP-L-fucose. (A) GDP-L-fucose before treatment with enzyme. (B) [3″,5″-^{2}H]-GDP-L-fucose formed after treatment of GDP-L-fucose with wild type GFS and $NADP^+$ in D_2O. (C) [5″-^{2}H]-GDP-L-fucose formed after treatment of GDP-L-fucose with the Cys109Ser mutant and $NADP^+$ in D_2O.

His179Gln mutant was examined, no appreciable deuterium incorporation into product could be detected under similar conditions. These results are consistent with the notion that C-5″ is epimerized after C-3″ in the normal reaction direction, and therefore the C-5″ proton is the first to exchange with solvent deuterium in the reverse direction. The base that deprotonates the C-5″ position of the GDP-6-deoxy-4-keto-L-galactose intermediate is assigned to His179 that normally functions as an acid in the forward reaction direction ($B_{2''}$ in Figure 2). Since this residue is unperturbed in the Cys109Ser mutant, C-5″ deprotonation and exchange may readily occur. This exchange likely occurs during the lifetime of the enol(ate) intermediate and does not involve epimerization at C-5″ since Cys109 ($B_{1''}$ in Figure 2) has been mutated. In the case of the His179Gln mutant, it is likely that the reversible oxidation of GDP-fucose can still occur, but no deprotonation events take place because the appropriate basic residue is missing.

Solvent Isotope Exchange into GDP-6-deoxy-4-keto-mannose with His179Gln. In all of the experiments discussed thus far, the His179Gln mutant has been essentially inactive toward catalysis. This could either be due to the mutation of a key catalytic residue or a more dramatic structural perturbation that effectively destroys the active site of the enzyme. The observation of partial reactions with this mutant would support the former notion and therefore isotopic washout experiments were performed. A sample of bis-labeled [3″,5″-^{2}H]-GDP-6-deoxy-4-keto-mannose was generated in a similar fashion as the 3″-labeled substrate (Figure 5b) with the exception that the GMD reaction was also carried out in D_2O. This material was incubated with His179Gln and NADPH in a buffer prepared with H_2O and the reaction was monitored by mass spectroscopy. Under these conditions the GFS activity of this mutant was so low that no GDP-fucose formed during the incubations. However, it was clear that the mutant was capable of catalyzing the wash-out of deuterium from the bis-labeled material and control reactions demonstrated that this process was not due to a nonenzymatic background reaction. The loss of deuterium was

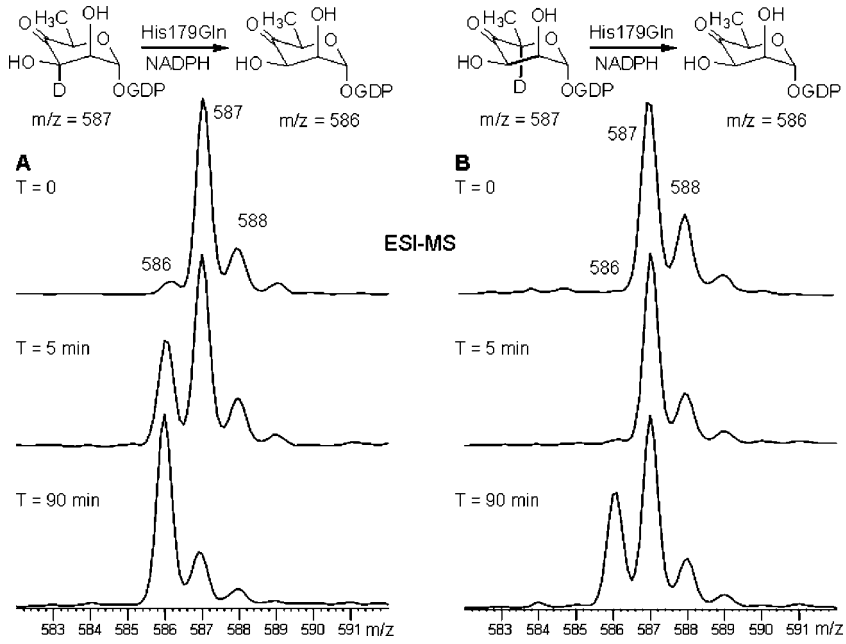

Figure 7. ESI mass spectra showing the His179Gln catalyzed wash-out of deuterium from labeled GDP-6-deoxy-4-keto-D-mannose. (A) The reaction of [3″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose in H_2O monitored as a function of time. (B) The reaction of [5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose in H_2O monitored as a function of time.

biphasic and one deuterium was lost about 20 times faster than the other. In order to determine which deuterium was washed-out more readily, samples of [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose and [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose were individually treated with the His179Gln mutant and NADPH. In the case of [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose, mass spectral analysis indicated that ~35% of the label had been lost after 5 min of incubation (Figure 7a). With [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose, a similar extent of isotopic wash-out required 90 min of incubation under identical conditions (Figure 7b). The observation that the His179Gln mutant catalyzes isotope exchange into starting material indicates that its active site has not been dramatically perturbed and it is still able to bind substrate and catalyze deprotonation events. Cys109 can still act as a functional base ($B_{1'}$ in Figure 2) and is able to generate enolate intermediates, however, the absence of a functional acid (His179 or $B_{2'}$ in Figure 2) prevents epimerization and conversion to product. The fact that label is lost from the C-3″ position much more rapidly than from the C-5″ position is consistent with the notion that C-3″ is epimerized first in the normal reaction mechanism.

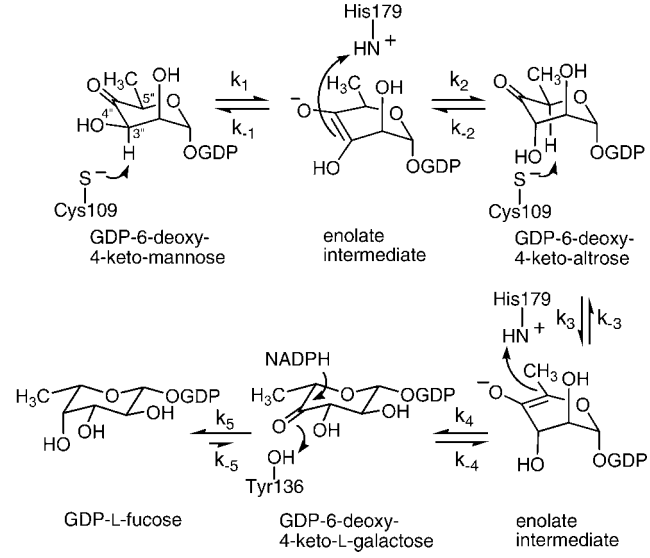

Figure 8. Proposed mechanism for the reaction catalyzed by GDP-L-fucose synthase.

Discussion

The results of this study support a mechanism for the GDP-fucose synthase reaction involving an ordered sequence of epimerizations catalyzed by two active site acid/base residues (Figure 8). In the first epimerization, Cys109 serves as a base to deprotonate the substrate at C-3″ and His179 serves as an acid to protonate the opposite face. In the second epimerization, both residues play analogous roles in inverting the stereochemistry at the C-5″ position. For the same residues to play the role of either base or acid in two consecutive catalytic steps, there must be a rapid shuttling of protons to and from the active site of the enzyme during the lifetime of the GDP-6-deoxy-4-keto-altrose intermediate in order to regenerate the appropriate protonation states. Alternatively, the residues may directly exchange protons within the active site in order to achieve these states. In the final step of the reaction, a reduction at C-4″ is catalyzed by Tyr136 of the conserved catalytic triad found in all SDR enzymes. With the wild type enzyme, the C-4″ carbonyl is only reduced once both epimerizations have occurred, and GDP-fucose is the only product detected. Although the studies in this manuscript do not directly address the reason behind this selectivity, an attractive explanation is that the reduction will only occur on a hexulose that preferentially adopts the $^1C_4$ conformation as would be expected for the GDP-6-deoxy-4-keto-L-galactose intermediate (Figure 8).

The fact that the Cys109Ser mutant generates GDP-6-deoxy-altrose as the major product supports the notion that C-3″ is epimerized first and that Cys109 is involved in the second epimerization step. According to the proposed mechanism, the mutation will slow down both deprotonation steps in the forward

reaction direction (k_1, k_{-1} and k_3, k_{-3} in Figure 8 will all decrease). GDP-6-deoxy-4-keto-mannose will bind to the mutant enzyme but is not reduced as it solely adopts the $^4C_1$ conformation. Instead, a slow epimerization at C-3″ (involving the Ser109 residue as a base) generates the GDP-6-deoxy-4-keto-altrose intermediate. This intermediate may partition forward in the second epimerization step and give GDP-fucose, or it may be prematurely reduced to generate GDP-6-deoxy-altrose. Since Cys109 is involved in the second epimerization step, reduction of this intermediate becomes competitive with GDP-fucose production. It is likely that the GDP-6-deoxy-4-keto-altrose intermediate preferentially adopts a $^4C_1$ conformation while bound in the active site and is not reduced in reactions with the wild type enzyme. However, in the case of the Cys109Ser mutant the lifetime of this intermediate is sufficient for premature reduction of a minor $^1C_4$ conformation to become competitive with turnover to GDP-fucose.

The assignment of Cys109 as the base involved in the first deprotonation step is strongly supported by the measurement of kinetic isotope effects. With the wild type enzyme, no KIEs were observed with either [5″-^{2}H]-GDP-6-deoxy-4-keto-mannose or [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose indicating that the deprotonations (k_1 and k_3 in Figure 8) are not rate-limiting steps in catalysis. This is in agreement with previous studies showing that reactions with deuterated NADPH are slowed by a kinetic isotope effect and therefore reduction of the ketone is a rate-limiting step.[17] In the reaction catalyzed by the Cys109Ser mutant, GDP-6-deoxy-D-altrose is the major product and GDP-L-fucose is the minor product, but in forming either of them a deprotonation at the C-3″ position is required. The observation of a kinetic isotope effect when [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose is used as a substrate with Cys109Ser indicates that the mutation of this residue has raised the barrier to removal of the C-3″ proton and this step has become partially rate-limiting (k_1 in Figure 8 has decreased). Thus, Cys109 can be assigned as the base responsible for the first deprotonation step. A similar approach of using Cys-to-Ser mutants combined with kinetic isotope effect measurements has previously been used to identify the roles of cysteine residues in the reactions catalyzed by glutamate racemase and diaminopimelate epimerase.[32,33]

The final experiment described with the Cys109Ser mutant involved the wash-in of solvent-derived deuterium into GDP-fucose in the presence of $NADP^+$. This experiment supports the ordering of the epimerization steps and the proposed role of Cys109 as a base in the second epimerization step (forward reaction direction) (Figure 8). With wild type enzyme the label is readily incorporated into both the C-3″ and C-5″ positions due to the freely reversible nature of the reaction. However, with the Cys109Ser mutant deuterium is selectively incorporated into the C-5″ position. This is consistent with the proposed order of the inversions since C-5″ would be expected to be deprotonated first in the reverse reaction direction. The oxidation of GDP-fucose to give the intermediate GDP-6-deoxy-4-keto-L-galactose is facile since Cys109 is not involved in the redox step of the reaction. His179 will readily deprotonate the C-5″ position of this intermediate, however, an inversion of stereochemistry does not occur since Cys109 (which would act as an acid in the reverse reaction direction) has been mutated. Instead, the histidinium moiety may exchange its proton with solvent-derived deuterium during the lifetime of the enol(ate) intermediate, and subsequently transfer this deuterium to back to the C-5″ position.

Studies with His179Gln showed that while this mutant is essentially inactive toward GDP-fucose formation, it readily catalyzes the exchange of the C-3″ deuterium in [3″-^{2}H]-GDP-6-deoxy-4-keto-mannose with a solvent-derived proton. This indicates that the mutant is still properly folded and capable of catalyzing partial steps of catalysis. Since Cys109 is still functional, it may readily abstract the deuteron from C-3″ and generate an enol(ate) intermediate. No inversion of stereochemistry takes place since the acidic His179 residue has been mutated, but exchange of the deuteron with a solvent-derived proton can occur during the lifetime of the intermediate. Delivery of this proton back to the C-3″ position results in the observed isotopic exchange. This observation provides further support for the ordered epimerization in the GFS reaction and the proposed role of His179 in acting as an acid in the first epimerization step.

It is interesting to compare the mechanism of the reaction catalyzed by GDP-fucose synthase to that of GDP-mannose 3,5-epimerase (Figure 3). A notable difference between these two enzymes is that the order of epimerizations are opposite. In the case of GDP-mannose 3,5-epimerase, a product of the reaction is GDP-L-gulose indicating that the C-5″ position is inverted before the C-3″ position is inverted.[28,29] Crystallographic studies have provided structures of this enzyme complexed with GDP-D-mannose, GDP-L-galactose, and a mixture of GDP-L-gulose and GDP-4-keto-L-gulose.[28] These structures provide excellent insight into the conformational changes that accompany catalysis and on the position of the active site residues relative to the bound hexose moiety. The tyrosine of the conserved catalytic triad is positioned appropriately to act as the acid/base catalyst during the hydride transfer steps of the reaction. In all of the structures the active site cysteine that is homologous to Cys109 of GFS is positioned appropriately to act as the base responsible for proton abstraction at either the C-3″ or C-5″ positions (GDP-D-mannose to GDP-L-galactose direction). Similarly, an active site lysine that is in a homologous position to His179 in GFS, is implicated as the acid that protonates the enolate intermediates. Thus the structural studies on GDP-mannose 3,5-epimerase are in agreement with our conclusions regarding the roles of active site residues in the GFS reaction. The structural studies on GDP-mannose 3,5-epimerase also indicate that the bound GDP-L-gulose adopts a $^1C_4$ conformation in the active site and thus has undergone a ring flip subsequent to (or during) the first epimerization. It is difficult to speculate on how this relates to the GFS mechanism since the conformational preferences of the two intermediates will be different and may be controlled by active site architecture. It is also important to note that GDP-mannose 3,5-epimerase must be able to catalyze redox reactions at the C-4″ position of all three stereoisomers, and thus the conformational requirements for hydride transfer are less stringent with this enzyme.

Conclusions

GDP-fucose synthase catalyzes a notably complex reaction involving two epimerizations and a reduction. It does so with remarkable fidelity and only generates a single isomeric product. Our studies support a mechanism involving an initial epimerization at C-3″, followed by an epimerization at C-5″, and then a final reduction of the C-4″ carbonyl. The roles of the basic and acidic residues involved in both of the epimerization steps have been assigned to Cys109 and His179, respectively. Further

structural studies on GFS complexed with sugar nucleotides will serve to elucidate the conformational changes that accompany catalysis.

Experimental Section

Materials and General Methods. GDP-D-Mannose, NADPH, and alkaline phosphatase (from bovine calf intestine) were purchased from Sigma-Aldrich. D_2O (99.9%) was purchased from Cambridge Isotope Laboratories, Inc. GDP-mannose 3,5-epimerase was prepared by overexpression of the pEHISTEV-GME plasmid in *E. coli* Rosetta (DE3) cells and purified as described previously.[28] Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard.[37] ^{1}H NMR spectra were obtained on a Bruker AV300 spectrometer at a field strength of 300 MHz. Proton decoupled ^{31}P spectra were recorded on the same spectrometer at 121.5 MHz. Mass spectra were aquired at the Mass Spectrometry Centre at the University of British Columbia (UBC) by electrospray ionization (ESI-MS) using a Bruker Esquire∼LC ion trap mass spectrometer. Oligonucleotides were synthesized by the Nucleic Acid Protein Service (NAPS) facility at UBC.

Cloning of the GMD and GFS Genes from *E. coli.* The GMD gene was PCR amplified from *E. coli* K12 genomic DNA using Platinum Taq polymerase (Invitrogen) and the following two primers: 5′-GGTATTGAGGGTCGCATGTCAAAAGTC-3′ (forward sequence) and 5′-AGAGGAGAGTTAGAGCCTTATGACTCCAGCG-3′ (reverse sequence). The amplified DNA was purified using a SpinPrep PCR Clean-up kit (Novagen) according to the manufacturer's directions. The PCR product was cloned into a pET-30 Xa/LIC vector (Novagen) according to the manufacturer's instructions to yield pT7GMD. The pT7GMD plasmid was amplified in NovaBlue GigaSingles competent *E. coli* cells (Novagen). This plasmid encodes for the expression of GMD bearing an N-terminal 43-residue peptide that terminates in a hexahistidine tag. An identical procedure was used to clone the *E. coli* GFS gene and produce pT7GFS. PCR amplification employed the following two primers: 5′-GGTATTGAGGGTCGCATGAGTAAACAACG-3′ (forward sequence) and 5′-AGAGGAGAGTTAGAGCCTTACCCCCGAAAG-3′ (reverse sequence).

Site-Directed Mutagenesis. Plasmids encoding for the GFS mutants, Cys109Ser and His179Gln, were generated using the following two sets of oligonucleotides (nonmatching nucleotides are underlined): for Cys109Ser, 5′-CTCGGATCGTCCAGCATCTACCCGAAAC-3′ (forward sequence) and 5′-GTTTCGGGTAGATGCTGGACGATCCGAG-3′ (reverse sequence); for His179Gln, 5′-CACCCGAGTAATTCGCAGGTGATCCCAGCATTGC-3′ (forward sequence) and 5′-GCAATGATGGGATCACCTGCGAATTACTCGGGTG-3′ (reverse sequence). Mutations were introduced into the pT7GFS vector using the QuikChange Sited-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The GFS genes in the resulting plasmids were fully sequenced to ensure that only the desired mutations had been introduced during PCR amplification.

Purification of Recombinant Proteins. To generate GMD, the pT7GMD plasmid was transformed into chemically competent BL21 (DE3) *E. coli*. Cells were grown on Luria−Bertani (LB) agar plates containing kanamycin (30 mg/L) at 37 °C for 18 h. A single colony was used to inoculate 10 mL of LB medium containing 30 mg/L kanamycin and this was incubated for 12 h at 37 °C with shaking at 225 rpm. This culture was poured into 500 mL of LB medium containing 30 mg/L kanamycin and grown with shaking at 225 rpm and 37 °C until an OD_{600} between 6.0 and 7.0 was reached. At that point, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.3 mM and the cells were grown for an additional 4 h before harvesting by centrifugation (6000 rpm, 20 min at 4 °C). Cell pellets were flash frozen in liquid N_2 and stored at −80 °C. The cells were resuspended in 15 mL of lysis buffer (20 mM sodium phosphate, pH 8.0, containing 2 mM β-mercaptoethanol, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A) and lysed by passage through a French Pressure cell at 12 000 psi The lysate was clarified by spinning in a Sorvall SLA-1500 rotor at 6500 rpm for 1 h followed by filtration (0.2 μm pore, 33 mm syringe driven filter, Millipore). A column (20 mL) of Chelating Sepharose Fast Flow resin (GE Healthcare) was packed and flushed with distilled water. The column was charged with 2 column volumes (CV) of 100 mM $NiSO_4$, followed by washing with 2 CV of distilled water and 3 CV of start buffer (20 mM sodium phosphate, pH 8.0, containing 0.5 M NaCl, and 5 mM imidazole). The clarified cell lysate was loaded at a rate of 2 mL/min, and start buffer was passed through the column at a rate of 3 mL/min until no more flow-through protein eluted as determined by monitoring A_{280}. To remove nonspecifically bound proteins, a mixture of 80% start buffer and 20% elution buffer (20 mM sodium phosphate, pH 8.0, containing 0.5 M NaCl, 10% v/v glycerol, and 500 mM imidazole) was applied until no more protein eluted. Finally the histidine-tagged GMD was eluted with 100% elution buffer. The protein solution was then concentrated using Amicon Ultra centrifugal filter devices (Millipore). The concentrated enzyme solution was then divided into aliquots and flash frozen in liquid N_2. Enzyme could be stored at −80 °C for at least 2 months without significant loss in activity. Typically 50−60 mg of enzyme was purified from 500 mL culture. GFS (from pT7GFS) and the Cys109Ser and His179Gln mutants were generated and purified in an identical manner with the exception that the cell cultures were incubated for a total of 12 h at 25 °C following induction by IPTG. GFS could be stored at −80 °C for at least 3 months without significant loss in activity. Typical protein yields obtained from a 500 mL culture were 110 (GFS), 80 (Cys109Ser), and 60 mg (His179Gln).

Enzymatic Synthesis of GDP-6-Deoxy-4-keto-D-mannose. To a mixture of 20 mg GDP-D-mannose, 0.3 mg $NADP^+$, and 1 μL β-mercaptoethanol in 2 mL of sodium phosphate buffer (10 mM, pH 7.0, containing 10 mM NaCl) was added 20 mg of GMD (previously exchanged into 800 μL of the same buffer using Amicon Ultra centrifugal filter devices (Millipore)). The reaction mixture was incubated for 12 h at rt and monitored by negative electrospray ionization mass spectrometry (-ESI MS, GDP-mannose m/z = 604, GDP-6-Deoxy-4-keto-D-mannose m/z = 586) to ensure the reaction reached completion. Protein was removed using Amicon Ultra centrifugal filter devises (Millipore), and the filtrate was lyophilized to yield GDP-6-deoxy-4-keto-D-mannose in a mixture with phosphate buffer salt. This material was used directly in all experiments.

Enzyme Kinetics. Kinetic constants for the GFS reaction were obtained using a continuous spectroscopic assay that directly monitors the conversion of NADPH to $NADP^+$ by following absorbance changes at 340 nm. Assay mixtures contained 50 mM sodium phosphate buffer, pH 8.0, 100 μM NADPH (saturating), and 0.25−10 μM GDP-6-deoxy-4-keto-D-mannose in a total final volume of 1.0 mL. The concentration of GDP-6-deoxy-4-keto-D-mannose stock solutions was determined by measuring A_{260} and using an ε_{260} of 11.8 mM^{-1} cm^{-1}. Reactions were initiated by addition of 10 μL of enzyme solution (containing 0.1 μg of WT-GFS or 60 μg of C109S-GFS) and the decrease in A_{340} was monitored at 25 °C. Initial velocities were calculated using an ε_{340} of 6 220 M^{-1} cm^{-1}. Kinetic parameters were determined from fitting the initial velocities to the Hill equation using the program GraFit.

Enzymatic Synthesis of GDP-L-Fucose. A sample containing 20 mg of the lyophilized GDP-6-deoxy-4-keto-D-mannose was redissolved in 2.0 mL of distilled water, and 30 mg of NADPH and 20 mg of GFS (in 1.0 mL of storage buffer) were added. The reaction was incubated at 28 °C for 2 h and monitored by negative electrospray ionization mass spectrometry (-ESI MS, GDP-fucose m/z = 588, GDP-6-deoxy-4-keto-D-mannose m/z = 586) to ensure

(37) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.

it reached completion. The protein was removed using Amicon Ultra centrifugal filter devices (Millipore) and the filtrate was loaded onto a Dowex DE52 anion exchange resin column (1.5 cm diameter × 28 cm length). Product was eluted with a linear gradient of 0−0.3 M triethylammonium bicarbonate buffer, pH 7.5. Eluent fractions were monitored at A_{260} and GDP-L-fucose eluted at 0.18 M buffer as a mixture with $NADP^+$. NADPH eluted immediately afterward. All the NADPH-free fractions were pooled and lyophilized. This mixture of $NADP^+$ and GDP-L-fucose was redissolved in 10 mL of 20 mM Trien-HCl buffer, pH 8.0, and 80 units of alkaline phosphatase was added. The resulting solution was incubated for 12 h at 30 °C and then lyophilized. A second anion exchange column was run using identical conditions, and the fractions containing GDP-L-fucose (well separated from those containing NAD^+) were pooled and lyophilized. The residue was redissolved in 15 mL of distilled water and lyophilized again to GDP-L-fucose (typical overall yield was 18−20 mg, ∼75%) as a bis-triethylammonium salt. GDP-L-fucose prepared in this fashion showed identical -ESI MS, ^{31}P and ^1H spectra to those previously reported in the literature.[34]

Enzymatic Synthesis of GDP-6-deoxy-D-mannose. To a mixture of 25 mg GDP-D-mannose, 0.38 mg $NADP^+$, and 1.3 μL β-mercaptoethanol in 2.5 mL of sodium phosphate buffer (10 mM, pH 7.0, containing 10 mM NaCl) was added 20 mg of GMD (previously exchanged into 800 μL of the same buffer using Amicon Ultra centrifugal filter devices (Millipore)). The reaction mixture was incubated for 12 h at rt and monitored by negative electrospray ionization mass spectrometry (-ESI MS, GDP-mannose $m/z = 604$, GDP-6-deoxy-4-keto-D-mannose $m/z = 586$) to ensure the reaction reached completion. To this mixture was added 35 mg of NADPH and the reaction was incubated for a further 12 h at rt. The progress of the reaction was judged to be complete by the consumption of GDP-6-deoxy-4-keto-D-mannose (m/z of 586) and the production of GDP-6-deoxy-D-mannose (m/z of 588) as monitored by -ESI MS. The enzyme was removed and product was purified as described for GDP-L-fucose synthesis. The final product was isolated as a bis-triethylammonium salt with an overall yield of 60% (∼20 mg). ^1H (400 MHz, D_2O, as triethylammonium salt, pH ∼7.4): δ (ppm) 8.13 (s, 1H, 8-H), 5.95 (d, $J_{1',2'} = 6$ Hz, 1H, 1′-H), 5.46 (br, d, $J_{1'',P} = 6.8$ Hz, 1H, 1″-H), 4.80 (dd, $J_{2',1'} = 6$ Hz, $J_{2',3'} =$ 4.8 Hz, 1H, 2′-H), 4.54 (dd, $J_{3',2'} = 4.8$ Hz, $J_{3',4'} = 3.6$ Hz, 1H, 3′H), 4.37 (m, 1H, 4′-H), 4.23 (m, 2H, 5a′-H, 5b′-H), 4.06 (br, m, $J_{2'',3''} = 3.2$ Hz, 1H, 2″-H), 3.94 (dd, $J_{3'',2''} = 3.2$ Hz, $J_{3'',4''} = 10$ Hz, 1H, 3″-H), 3.90 (dd, $J_{5'',4''} = 10$ Hz, $J_{5'',6''} = 3.2$ Hz, 1H, 5″-H), 3.31 (t, $J_{4'',3''} = 10$ Hz, $J_{4'',5''} = 10$ Hz, 1H, 4″-H), 1.25 (d, $J_{6'',5''} = 3.2$ Hz, 3H, 6″-H); ^{31}P {^1H} NMR (121.5 MHz, D_2O, as triethylammonium salt, pH ∼7.4): δ (ppm) −10.88 (d, $J_{p,p} = 20$ Hz, P_α), −13.31 (d, $J_{p,p} = 20$ Hz, P_β). MS (ESI^-) for $C_{16}H_{25}N_5O_{15}P_2$ (free acid, 589.3) = m/z 588.2 [M − H].

Enzymatic Synthesis of [5″-^{2}H]-GDP-6-Deoxy-4-keto-D-mannose. The labeled substrate was prepared in an identical fashion to the unlabeled compound except that the GMD reaction was carried out in buffered D_2O. A deuterated sodium phosphate buffer ((10 mM, pD 7.4, containing 10 mM NaCl) was prepared by lyophilizing a sample of nondeuterated buffer and redissolving it in an equal volume of D_2O. This was repeated three times to ensure complete isotopic exchange. The GFS reaction was run as described above (with previous exchange of GFS into the same deuterated buffer) and negative electrospray ionization mass spectrometry indicated that the reaction proceeded to completion and that >97% of the product contained a single deuterium (-ESI MS, GDP-mannose $m/z = 604$, [5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose $m/z = 587$).

Enzymatic Synthesis of [3″-^{2}H]-GDP-6-Deoxy-4-keto-D-mannose and [3, 5″-^{2}H]-GDP-6-Deoxy-4-keto-D-mannose. Both the [3″-^{2}H]- and the [3, 5″-^{2}H]-labeled substrates were prepared from a mixture containing [3, 5″-^{2}H]-GDP-mannose (80%), [3, 5″-^{2}H]-GDP-L-gulose (5%) and [3, 5″-^{2}H]-GDP-L-galactose (15%) that was generated using GDP-mannose 3,5-epimerase in buffered D_2O. A sample of Trien-DCl buffer in D_2O (20 mM, pD 7.4) was prepared by repeated (3×) lyophilization of a Trien-HCl buffer (20 mM, pH 7.0) and dissolution in an equal volume of D_2O. To 2.0 mL of this buffer was added 25 mg of GDP-mannose, 1.0 μL of β-mercaptoethanol, and 25 mg of GDP-mannose 3,5-epimerase (previously exchanged into 800 μL of the same buffer using Amicon Ultra centrifugal filter devices (Millipore)). This reaction mixture was incubated for 12 h at 37 °C and monitored by negative electrospray ionization mass spectrometry to ensure that two atoms of deuterium were incorporated into >95% of the material ($m/z =$ 606). The protein was then removed using Amicon Ultra centrifugal filter devices (Millipore). The filtrate was lyophilized, redissolved in 1.5 mL of distilled water, and loaded onto a size exclusion chromatography column (Biogel P-2, 200−400 mesh, 2.5 cm diameter × 70 cm length). The mixture of dideuterated sugar nucleotides was eluted with distilled water and fractions absorbing at 260 nm were pooled and lyophilized. Nondeuterated control samples for use in KIE determinations were also prepared in buffered H_2O under otherwise identical conditions.

In order to obtain [3″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose, the mixture of dideuterated epimeric GDP-hexoses was treated with GMD in buffered H_2O as described for the synthesis of GDP-6-deoxy-4-keto-D-mannose. The unlabeled control sample for use in KIE studies was prepared in a similar fashion. In order to obtain [3,5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose, the mixture of dideuterated epimeric GDP-hexoses was treated with GMD in buffered D_2O as described for the synthesis of [5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose. The reactions were monitored by -ESI-MS and observed to stop after approximately 80% of the total material had been consumed, indicating that only GDP-mannose served as a substrate for the GMD reaction. Mass analysis of the mono- and dideuterated products indicated that >90% of the material contained one and two atoms of deuterium, respectively (-ESI MS, [3″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose $m/z = 587$, [3″,5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose $m/z = 588$).

Product Analysis with the Cys109Ser Mutant. Lyophilized GDP-6-deoxy-4-keto-D-mannose (20 mg, prepared as described above) and 30 mg of NADPH were dissolved in 2.0 mL of water. To this solution was added 20 mg of Cys109Ser (in 1.0 mL of sodium phosphate buffer) and the reaction was incubated at 28 °C for 4 h. The progress of the reaction was monitored by -ESI MS and judged to be complete by the disappearance of the GDP-6-deoxy-4-keto-D-mannose signal ($m/z = 586$). The GDP-hexose products were purified as described for the enzymatic synthesis of GDP-L-fucose. The final product mixture was isolated as bis-triethylammonium salts with an overall yield of 65% (16 mg). The reactions of deuterated substrates were carried out under analogous conditions and monitored by -ESI-MS.

Deuterium Wash-in Experiments with GDP-L-Fucose. Deuterium wash-in experiments were performed using GFS-WT, Cys109Ser, and His179Gln. A sample of deuterated phosphate buffer (10 mM, pD 7.4) was prepared by repeated (3×) lyophilization of a phosphate buffer (10 mM, pH 7.0) and dissolution in an equal volume of D_2O. Three samples containing 2.0 mg GDP-fucose, 0.1 mg $NADP^+$, and enzyme (3 mg of GFS-WT, 7 mg of Cys109Ser, or 10 mg of His179Gln, previously exchanged into deuterated buffer using Amicon Ultra centrifugal filter devices (Millipore)) were prepared in the deuterated buffer (final volume 800 μL). The samples were incubated at 28 °C and the progress of deuterium wash-in was monitored using ^1H spectroscopy. The mass of the final product was confirmed by -ESI-MS upon completion of the deuterium wash-in process.

Deuterium Wash-out Experiments with His179Gln. Samples containing 5 mg of substrate ([3″, 5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose, [3″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose, or [5″-^{2}H]-GDP-6-deoxy-4-keto-D-mannose), 4.5 mg of His179Gln (previously exchanged into the same buffer using Amicon Ultra centrifugal filter devices (Millipore)), and 5 mg NADPH were prepared in 10 mM sodium phosphate buffer (pH 7.0, 10 mM, 750 μL final volume).

The samples were initiated by the addition of enzyme, incubated at rt, and monitored by -ESI-MS in 15 min intervals.

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Supporting Information Available: Kinetic traces for the reaction of the wild type GFS and the KIE determination with the Cys109Ser mutant. This material is available free of charge via the Internet at http://pubs.acs.org/.

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