Recently the genes encoding the human and Escherichia coli GDP-mannose dehydratase and GDP-fucose synthetase (GFS) protein have been cloned and it has been shown that these two proteins alone are sufficient to convert GDP mannose to GDP fucose in vitro. GDP-fucose synthetase from E. coli is a novel dual function enzyme in that it catalyzes epimerizations and a reduction reaction at the same active site. This aspect separates fucose biosynthesis from that of other deoxy and dideoxy sugars in which the epimerase and reductase activities are present on separate enzymes encoded by separate genes. By NMR spectroscopy we have shown that GFS catalyzes the stereospecific hydride transfer of the Pro8 hydrogen from NADPH to carbon 4 of the mannose sugar. This is consistent with the stereospecificity observed for other members of the short chain dehydrogenase reductase family of enzymes of which GFS is a member. Additionally the enzyme is able to catalyze the epimerization reaction in the absence of NADP or NADPH. The kinetic mechanism of GFS as determined by product inhibition and fluorescence binding studies is consistent with a random mechanism. The dissociation constants determined from fluorescence studies indicate that the enzyme displays a 40-fold stronger affinity for the substrate NADPH as compared with the product NADP and utilizes NADPH preferentially as compared with NADH. This study on GFS, a unique member of the short chain dehydrogenase reductase family, coupled with that of its recently published crystal structure should aid in the development of anti-microbial or anti-inflammatory compounds that act by blocking selectin-mediated cell adhesion.

GDP fucose is synthesized mainly by a de novo pathway from GDP mannose in a three-step reaction involving two enzymes as shown in Fig. 1. In the first step, GDP-4-keto-6-deoxymannose is formed by oxidation at C-4 and subsequent reduction at C-6 of the mannose ring, catalyzed by an NADP-dependent enzyme GDP-mannose 4,6-dehydratase. In reactions catalyzed by GDP-fucose synthetase, this intermediate then undergoes an epimerization at C-3 and C-5 of the mannose ring followed by reduction of the keto group at C-4 to yield GDP fucose (1).

Fucose is found widely distributed in complex carbohydrates as a component of glycoconjugates such as glycoproteins and glycolipids in a wide variety of species from humans to bacteria. Fucose is added to these glycoconjugates by specific transferases that utilize GDP fucose as the sugar donor. In bacteria fucose is present as a component of the capsular polysaccharides and lipopolysaccharides which function as antigenic determinants. In several species defects in the genes encoding GFS1 have shed some light on the role played by fucose in these organisms. Inactivation of the nolK genes in Azorhizobium caulinodans results in an inability to introduce the 6-O-fucosyl branch on the lipooligosaccharides of the Nod factors which is an important modification for nodulation in some host plants (2). Recently it has been shown that the GFS gene which is responsible for biosynthesis of the O-antigen in Helicobacter pylori is induced at low pH and plays a role in the survival of the bacterium under acidic conditions and potentially in the virulence of the organism (3). In mammals fucose is present as a component of human blood group antigens and also is involved in regulation of the immune response. Humans deficient in the biosynthesis of GDP fucose suffer from the immune disorder leukocyte adhesion deficiency type II (4, 5). These patients fail to synthesize fucosylated blood groups and their leukocytes do not express fucose as a component of the selectin ligand, sialyl Lewis X (6, 7). The lack of fucose on different glycoconjugates does not seem to arise from a defect in fucosyltransferase activities but is related to the decrease in the intracellular production of GDP fucose, which serves as a substrate for these transferases. Conversely, increased fucosylation of glycoconjugates involved in metastasis has been observed in cancer patients (8). Understanding the mechanism of synthesis of GDP fucose should aid in the development of drugs having potential as anti-inflammatory or antimetastatic agents.

The NADH/NADPH-dependent short chain dehydrogenase reductase family consists of enzymes which comprise a wide variety of activities and include alcohol and polyol dehydrogenases, steroid dehydrogenases, prostaglandin dehydrogenases, carbonyl reductases, dihydropteridine reductases, and ketoacyl reductases (9, 10). These enzymes are present in bacteria, yeast, plants, and animals indicating that these enzymes are evolutionarily important in several metabolic pathways. Based on sequence and structural alignment studies, the short chain dehydrogenase family has also been shown to be distantly related to the family of steroid dehydrogenases which include enzymes like 3-β-hydroxy-5-ene steroid dehydrogenase, UDP-galactose-4-epimerase (GalE), and some other proteins which in sum comprise five EC classes, dehydrogenases, reductases, dehydratases, epimerases, and isomerases. Most of the members of the SDR family show residue conservations of only about 15–30% with only 9 residues being conserved in more than 90% of the enzymes. These include the Ser-Tyr-Lys catalytic triad and the glycine residues involved in coenzyme binding.

Analysis of the amino acid sequence of GFS indicates that it
contains a conserved Ser-Tyr-Lys catalytic triad, suggesting that it belongs to the family of short chain dehydrogenase reductases (11, 12). However, in contrast to UDP-galactose-4-epimerase and other enzymes of the SDR family, GFS catalyzes two distinct reactions, epimerizations at C-3 and C-5 of the mannos ring and the subsequent NADPH-dependent reduction at C-4 (1, 12–14). In this paper we show that like other SDR’s (15–24) hydride transfer catalyzed by GFS is stereospecific and occurs at the ProS position of the nicotinamide ring. Furthermore, we present evidence that the epimerizations at C-3 and C-5 differ from that catalyzed by other members of the SDR family in that they do not involve the transient reduction and oxidation of an NAD or NADP cofactor and that the epimerization occurs in the absence of its cofactor NADPH. Our investigations also included various inhibition and fluorescence binding studies that indicated that GFS follows a random bi-bi mechanism. Our results show that the enzyme displays a much higher affinity for its cosubstrate NADPH, as compared with the product NADP, consistent with the catalytic mechanism in which NADPH binds to the enzyme transfers the hydride and is released as NADP.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Escherichia coli GFS**—The *E. coli* GFS gene was polymerase chain reaction amplified from pSEWCAG (1) using the following two oligonucleotides: GGCGCATATGAGTAAACAGFS gene was polymerase chain reaction amplified from pSEWCAG (1) and ligated into the pNdeI and ligated into the NdeI and HindIII sites of the *E. coli* expression vector pSETB (Invitrogen) to yield pT7WCAG. This resulted in isopropyl-1-thio-β-D-galactopyranoside inducible expression of GFS having its native sequence, driven by a T7 promoter. *E. coli* strain BL21(DE3/pLysS), containing pT7WCAG, was grown in DM4 minimal salts with 0.5% glucose, trace metals, and supplemented with 100 mM NADP, 500 mM NADPH, 600 μM GDP-4-keto-6-deoxymannose, and 300 μM GDP-4-keto-6-deoxymannose. A 96-well plate was incubated at 37 °C for 10 min and the reaction was initiated by the addition of 0.25 μg of GFS. Initial rates were determined by following the decrease in absorbance at 340 nm due to the conversion of NADPH. Typical reaction conditions were 100 mM MOPS, pH 7.0, 100 mM NaCl, 5 mM EDTA, 10 mM DTT, 150 μM GDP, 100 μM NADP, and 100 μM NADPH at 37 °C for 3–6 h. GDP-mannose dehydratase (0.1 mg/ml), which had been previously incubated with NADP at 37 °C, was added to initiate the reaction. The reactions were allowed to proceed to completion as judged by high performance liquid chromatography and the protein was removed by ultrafiltration using a Centricon 10. The remaining mixture was loaded onto a Sephadex G-10 desalting column eluted with water and the peak for GDP-4-keto-6-deoxymannose was determined by following the absorbance at 260 nm (ε260Å = 11.8 mmol−1 cm−1). GDP-4-keto-6-deoxymannose was then lyophilized and stored at −20 °C as a powder. GDP-4-keto-6-deoxymannose pregnenolone this procedure is ~100% pure as checked by NMR and mass spectroscopy.

**Determination of Stereospecificity of Hydride Transfer—**[4-2H]NADPH was prepared by a previously published procedure for its synthesis from NADP, reduced glutathione, DTT, triethanolamine, and glutathione reductase in H2O (25, 26). Protiated NADPH was also prepared in a similar manner using H2O instead of D2O. 4-2H]NADPH was also prepared by a previously published procedure using NADP, acetaldehyde-D4, DTT, and aldehyde dehydrogenase (25, 26). The protinated and deuterated NADPH/D generated by the method above were then purified by chromatography on a Mono Q column followed by elution using a 0–1 M KCl gradient in 20 mM triethanolamine buffer (pH 7.0). 200 μM 4-[2H]NADPH was reacted with 400 μM GDP-4-keto-6-deoxymannose in 10 mM phosphate buffer, pH 6.8. The enzyme was added and the mixture was incubated at 37 °C. The reaction was allowed to go to completion determined by following the decrease in 340. After the reaction was over the mixture was lyophilized and exchanged with 1 ml of D2O twice. The mixture was finally resuspended in 0.7 ml of D2O and 1H NMR spectroscopy was performed on a 600 MHz Varian Unity Plus NMR spectrometer.

**Paper Chromatography—**[14C]-Labeled GDP-4-keto-6-deoxymannose was synthesized beginning with 14C-labeled GDP mannos by the same procedure as stated above. The 14C-labeled GDP-4-keto-6-deoxymannose was then incubated with GFS and NADPH, GFS and NADP, and GFS alone. The reaction products were then washed with 0.2 mM NaBH4 for 15 min to reduce the GDP-4-keto-6-deoxymannose and then cleaved from the sugar by incubating in 1 M trichloroacetic acid for 10 min in boiling water. The resulting mixture was chromatographed on Whatman 5MM paper in descending mode using a solvent system.
Fig. 2. Analysis of the stereospecificity of hydride transfer by NMR spectroscopy. 200 μM 4S- or 4R-[2H]NADPH was reacted with 400 μM GDP-4-keto-6 deoxymannose in 10 mM phosphate buffer, pH 6.8. The enzyme was added and the mixture was incubated at 37 °C. The reaction was allowed to go to completion determined by following the decrease in 340. After the reaction was over the mixture was lyophilized and exchanged with 1 ml of D₂O twice. The mixture was finally resuspended in 0.7 ml of D₂O and ¹H NMR spectroscopy was performed. A, NADP⁺; B, reaction of GFS with 4S-[2H]NADPH; C, reaction of GFS with 4R-[2H]NADPH.

Panel A  NADP

Panel B  Reaction using 4S[2H]-NADPH

Panel C  Reaction using 4R[2H]-NADPH

Determinations of Kinetic Parameters—Assays were carried out as described above at varying concentrations of NADPH and GDP-4-keto-6 deoxymannose (3.6:1.0:1.15) for 7 h. Free sugar standards were localized by AgNO₃ staining in acetone followed by NaOH in methanol. Radioactivity was detected by cutting the paper into strips of 1 cm × 1 cm and counting on a scintillation counter.
6-deoxymannose. Since the order of substrate binding was not known, initial velocities were measured at various concentrations of NADPH (10, 15, 20, 30, 40, 50, 60, and 70 μM) and GDP-4-keto-6-deoxymannose (5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 μM) at varying concentrations of NADP (5 to 100 μM) and GDP fucose (20 to 140 μM) at unsaturating concentrations of either NADPH or GDP-4-keto-6-deoxymannose. The data were fit to double reciprocal plots of 1/NADPH and 1/GDP-4-keto-6-deoxymannose versus 1/initial velocity (V_s). We also explored the effect of GDP at varying concentrations of GDP-4-keto-6-deoxymannose on the reaction and the data were analyzed by the same procedure as above.

**pH Dependence and Isotope Effect**—Purified proteins were assayed in 100 mM MES, pH 5.0–6.0, and 25 mM HEPES, pH 6.5–8.0, buffer that contained 100 mM NaCl, 5 mM EDTA, 10 mM DTT, and 150 μM NADPH. GDP-4-keto-6-deoxymannose was varied from 20 to 300 μM. A 96-well plate was incubated at 37 °C for 10 min and the reaction was initiated by the addition of 0.25 μM of GFS. Initial rates were determined by following the decrease in absorbance at 340 nm due to the conversion of NADPH (ε₃₄₀ = 6.22 mM⁻¹ cm⁻¹) to NADP. The deuterium isotope effect was determined using the spectrophotometric assay as described above.

To minimize errors arising from slight variations in enzyme activity or assay conditions, data were collected at varying 4S-[2H]NADPH and 4S-[2H]NADPH on the same day.

**Fluorescence Spectroscopy**—All fluorescence measurements were carried out at 25 °C at pH 7.0 on a Photon Technology International Fluorimeter with 0.55 and 5.5 μM GFS. Binding of NADPH and NADP to GFS was followed by monitoring the quenching of enzyme fluorescence intensity induced by their binding to the apoenzyme. The apoenzyme was prepared by a previously published procedure (27) followed by dialyzing the purified protein against 2 liters of potassium phosphate buffer, pH 8.0, containing 5 mM EDTA and the buffer was changed periodically over a period of 2 days. The excitation wavelength was set at 280 nm and the quenching of the fluorescence emission of the protein at 339 nm was followed. The excitation and emission slits were 3 and 9 nm. The same procedure as above, quenching of fluorescence of apoenzyme, was used to determine the affinity of GFS for GDP fucose and GDP-4-keto-6-deoxymannose. The quenching of GFS fluorescence was analyzed using a previously published nonlinear least squares procedure (27) that fit the average number of moles (v) of bound substrate per mole of protein and the free ligand [L] to the Adair-Klotz equation. v was calculated by the same procedure using the relationship v = n[I − (I_a − I_s)]/L, where n = number of binding sites, I_a = fluorescence of apoenzyme, I = measured fluorescence intensity at a particular concentration of substrate, I_s = fluorescence of holoenzyme with its n of binding sites saturated with substrate. [L] was determined by using the relation [L] = [L] − n[E], where L and E represent total ligand and total enzyme concentration, respectively. 90% loss in fluorescence intensity was observed when enzyme was completely saturated with NADP and NADPH. Only 5% of the original fluorescence intensity of apoenzyme remained on complete saturation, of GFS, with GDP fucose, GDP and GDP-4-keto-6-deoxymannose.

**Stereospecificity of Hydride Transfer**—The ¹H NMR spectrum of GDP-fucose synthetase generated at the end of the reaction was analyzed for the retention of deuterium at the 4 position of the pyridine ring (Fig. 2) (28). A ProS specific enzyme removes deuterium from 4S-[2H]NADPH and the NADP formed as an end product reaction shows a doublet at 8.8 ppm of proton at the 4 position of the pyridine ring. Analogously when 4R-[2H]NADPH is used no doublet should be observed since it is the ProS proton that is transferred and the deuteron remains...
attached to the C-4 position of the pyridine ring. When NADPH deuterated at the 4S position was reacted with GFS the proton left over at the C-4 position exhibited a H1 splitting at about 8.8 ppm. When the experiment was repeated with NADPH deuterated at the 4R position no H1 splitting at position C-4 was observed, indicating deuterium was still attached at that position. This confirms that like all other enzymes belonging to the SDR class the hydride transfer catalyzed by GFS is stereospecific and occurs at the 4S position of NADPH.

NADPH Is Not Required for the Epimerization Reaction—To characterize the products of the epimerization and reduction reaction and to investigate the requirement for NADP in the epimerization reaction catalyzed by GFS we incubated the enzyme with 14C-labeled GDP-4-keto-6-deoxymannose under different conditions and the products of the reaction were identified by descending paper chromatography. Our data indicate that incubation of GDP-4-keto-6-deoxymannose with GFS alone or with GFS plus NADP converts GDP-4-keto-6-deoxymannose to GDP-4-keto-6-deoxyglucose. The expected monosaccharides resulting from reduction of GDP-4-keto-6-deoxyglucose by borohydride and cleavage from the nucleotide by acid, 6-deoxyglucose, and fucose, comigrate with their unlabeled standards (Fig. 3). This confirms that GFS alone is sufficient to convert GDP-4-keto-6-deoxymannose to the epimerized product GDP-4-keto-6-deoxyglucose and that NADP is not required for the epimerization reaction. We confirmed that the product produced on incubation of GDP-4-keto-6-deoxymannose and NADPH with GFS was GDP fucose by co-chromatography with authentic GDP fucose (Fig. 3). Incubation of GDP-4-keto-6-deoxymannose alone under the same conditions as above followed by reduction and cleavage yielded rhamnose and 6-deoxytalose. 6-Deoxytalose is not observed as it runs off the chromatograph in this solvent system (data not shown).

Steady State Mechanism of GDP-fucose Synthetase

**Steady State Mechanism of GDP-fucose Synthetase**

**Fig. 4. Lineweaver-Burk plots of the effect of the products GDP fucose and NADP+ on the initial velocities of the GFS-catalyzed reaction.** When NADPH was varied GDP-4-keto-6-deoxymannose was held at 30 μM. When GDP-4-keto-6-deoxymannose was varied NADPH was held at 20 μM. A, initial velocities of the oxidation of NADPH at a fixed concentration of GDP-4-keto-6-deoxymannose at varying concentrations of NADPH and several fixed concentrations of GDP fucose. The GDP fucose concentrations were 0, 40, 60, 80, and 120 μM. B, initial velocities of the oxidation of NADPH at a fixed concentration of NADPH at varying concentrations of GDP-4-keto-6-deoxymannose and several fixed concentrations of GDP fucose. The GDP fucose concentrations were 0, 40, 60, 80, and 120 μM. C, initial velocities of the oxidation of NADPH at a fixed concentration of GDP-4-keto-6-deoxymannose at varying concentrations of NADPH and several fixed concentrations of NADP. The NADP concentrations were 0, 40, 60, 80, and 120 μM. D, initial velocities of the oxidation of NADPH at a fixed concentration of NADPH at varying concentrations of GDP-4-keto-6-deoxymannose and several fixed concentrations of NADP. The NADP concentrations were 0, 40, 60, 80, and 120 μM.
the decrease in absorbance at 340 nm due to the oxidation of NADPH at varying concentrations of NADPH and GDP-keto-6-deoxymannose (Table I). Based on initial velocity studies, GFS displayed a much lower affinity for NADH (K_m = 108.8 μM and V_max = 6.6 μmol min^{-1} mg^{-1}) as compared with NADPH. Product inhibition studies were carried out in order to elucidate the suspected order of substrate binding of GDP fucose synthetase. GDP fucose was a competitive inhibitor with respect to NADPH and GDP-4-keto-6-deoxymannose since the double-reciprocal plots of 1/V versus 1/S at unsaturating concentrations of GDP-4-keto-6-deoxy mannose and NADPH intersect on the 1/V axis (Fig. 4, A and B). NADP, the other product of the reaction, was also a competitive inhibitor when varied at different concentrations of GDP-4-keto-6-deoxymannose and NADPH (Fig. 4, C and D). Thus the steady state kinetic results are consistent with a random bi-bi mechanism in which either the cofactor or substrate can bind to the enzyme in the absence of the other. GDP was a competitive inhibitor with respect to GDP-4-keto-6-deoxymannose with a K_i of 60.5 ± 6.8 μM.

**pH Profile and Isotope Effects**—A number of buffers were screened to find a system where the rate of the reaction would be independent of buffer concentration. We found that MES over pH values 5.0, 5.5, and 6.0 and HEPES over pH values 6.5, 7.0, 7.5, and 8.0 were suitable for assays over the pH range 5.0–8.0. The effect of pH on log V_max/K_m indicates that there exists at least one catalytic group on the enzyme with a pK_a between 6 and 6.5 (Fig. 5). An isotope effect of 1.4 ± 0.2 was observed on ΔV arising from deuterium substitution at the pro-S hydrogen at C-4 of NADPH. This effect is to small to be significant, hence hydride transfer may only be partially rate-limiting in the mechanism.

_Determination of Dissociation Constants for NADPH and NADP_—When excited at 280 nm the emission spectrum of GFS has two maxima, a primary maxima at 339 nm which is about twice as intense as the secondary maxima at 450 nm. The dissociation constants were determined by monitoring the increase and decrease in signal intensity at 450 nm and 338 nm induced by the binding of NADPH and NADP to the apoenzyme. The K_f for NADPH was determined to be 1.29 ± 0.07 μM assuming a ratio of 1 between concentration of binding sites and protein concentration. The dissociation constant for NADP was determined by monitoring the decrease in fluorescence intensity at 339 nm. The enzyme displayed a much weaker affinity for its product NADP as compared with NADPH and had a K_f of 74.2 ± 6.9 μM with the data best fitting the Adair-Klotz equation assuming n = 1 per monomer. This agrees with the crystal structure studies, which also indicates a 1:1, stoichiometry between NADPH/NADP and binding site per subunit (11, 12).

**Determination of Dissociation Constants for GDP-4-keto-6-deoxymannose, GDP Fucose, and GDP**—The affinity of apoenzyme for GDP-4-keto-6-deoxymannose was determined by monitoring the quenching of enzyme fluorescence upon substrate binding at 338 nm. The K_f was determined to be 2.9 ± 0.10 μM with n = 1 per monomer. Thus like NADP and NADPH the enzyme has two substrate-binding sites per functional dimer. We also determined the affinity of GFS for its product GDP fucose by fluorescence spectroscopy and calorimetry. The dissociation constant obtained from fluorimetry was 47 ± 2.8 μM and was comparable to that obtained from calorimetry which was 51 ± 3.2 μM (Fig. 6). The heat of the reaction determined from calorimetry was -6696 cal/mol and the value of binding sites obtained was 1.01 ± 0.03 per monomer. The ratio of binding sites to protein concentration was also in agreement with that determined by fluorimetry, which was two binding sites per functional dimer as reported for GDP-4-keto-
6-deoxymannose. We tried to determine the dissociation constant for GDP-4-keto-6-deoxymannose by calorimetry but were unsuccessful in doing so due to the heat of reaction generated on account of epimerization of the substrate when it comes in contact with high concentrations of the enzyme. The dissociation constant for GDP was also ascertained by following the formation of a binary complex was further confirmed by monitoring the quenching of enzyme fluorescence at 339 nm. The data were fit to the Adair-Klotz equation and gave a \( K_d \) of 51.8 ± 5.9 \( \mu \text{M} \) assuming \( n = 1 \) per monomer. Inset, fluorescence emission spectra of apo-GFS and following titration with GDP. B, Lineweaver-Burk plot of the effect of GDP on the initial velocities of the GFS-catalyzed reaction. When GDP-4-keto-6-deoxymannose was varied \( NADPH \) was held at 20 \( \mu \text{M} \). The GDP concentrations were 0, 40, 80, and 120 \( \mu \text{M} \).

FIG. 7. A, effect of binding of GDP on the fluorescence emission spectrum of apo-GFS. The decrease in fluorescence intensity at 339 nm was used to analyze GDP binding of GFS. The apoenzyme concentration was 5.5 \( \mu \text{M} \) in 50 \( \text{mM} \) phosphate buffer, pH 7.0. The excitation and emission slits were set at 3 and 9 nm, respectively. The data were fit to the Adair-Klotz equation and gave a \( K_d \) of 51.8 ± 5.9 \( \mu \text{M} \) assuming \( n = 1 \) per monomer. Inset, fluorescence emission spectra of apo-GFS and following titration with GDP. B, Lineweaver-Burk plot of the effect of GDP on the initial velocities of the GFS-catalyzed reaction. When GDP-4-keto-6-deoxymannose was varied \( NADPH \) was held at 20 \( \mu \text{M} \). The GDP concentrations were 0, 40, 80, and 120 \( \mu \text{M} \).

DISCUSSION

GFS as isolated from \textit{E. coli} is a homodimer of 394 amino acids per subunit (11, 12). Analysis of its amino acid sequence with that of its recently published crystal structure reveals the presence of the conserved Ser-Lys-Tyr catalytic triad observed in other members of the SDR family. The ability of GFS to preferentially transfer the ProS hydrogen of NADPH is consistent with the chirality preference described for other members of the SDR family. The products resulting after incubation of GDP-4-keto-6-deoxymannose either with GDP alone or with GFS plus NADP ascertained from paper chromatography provides evidence that excludes a non-enzymatic mechanism for epimerization for GDP-4-keto-6-deoxymannose and shows that it is not mediated by the transient reduction and oxidation of a bound NADP or NAD cofactor. GFS preferentially utilizes NADPH as compared with NADH. This is consistent with the crystal structure of the enzyme wherein the side chain of Arg-36 makes hydrogen bonds with the 2′-phosphate groups of NADPH and is thus able to compensate for the negative charge of the phosphate groups.

Product inhibition and fluorescence binding studies provided evidence in support of a random mechanism. Inhibition by NADP-like GDP fucose was competitive with respect to both NADPH and GDP-4-keto-6-deoxymannose suggesting that either the cofactor or the enzyme can bind to the substrate first. A \( K_i \) of 55.3 ± 3.9 \( \mu \text{M} \) and 69.3 ± 5.9 \( \mu \text{M} \) for GDP fucose and NADP, respectively, obtained from product inhibition studies is in agreement with the dissociation constants obtained from fluorescence binding studies. The quenching of enzyme fluorescence observed on titrating the apoenzyme with GDP-4-keto-6-deoxymannose and GDP fucose demonstrated that a binary complex was possible in the absence of the pyridine nucleotide which function as cosubstrates in the reaction. The formation of a binary complex was further confirmed by monitoring the binding of GDP fucose by calorimetry. In contrast to earlier reports that the human enzyme exhibits half-sites reactivity (29), the fluorescence data presented here indicate that each subunit of the \textit{E. coli} enzyme contains one substrate and one cofactor-binding site. This is in agreement with the crystal structure data, which shows the appearance of electron density for two pyridine nucleotides per dimer (11, 12).

On the basis of the decrease in fluorescence intensity observed on binding of NADPH to the apoenzyme, GFS was found to have one binding site per monomer with a \( K_i \) of 1.3 ± 0.07 \( \mu \text{M} \). The value for the \( K_m \) of NADPH is 9 ± 0.8 \( \mu \text{M} \) which is not that much greater than the dissociation constant. Other enzymes of the SDR family which follow an ordered Theorrell-Chance mechanism like CDP-paratose synthase, flavonol 3-O-methyltransferase, and glutamic-γ-semialdehyde dehydrogenase have a much higher \( K_i/K_m \) ratio ranging from 25 to 5000 (30). The fact that both substrate and cofactor can bind to the enzyme alone, coupled with the product inhibition data seems to indicate that catalysis is unlikely to proceed by an ordered mechanism. The interpretation of the kinetic data is complicated, however, since all of our velocity studies monitor only the reduction reaction although the enzyme also catalyzes an epimerization reaction. Hence a special form of a random bi-bi reaction wherein one of the substrates adds in a rapid
Ser107, Tyr136, and Lys140 of GFS (11). However, as mentioned, triads in both Gal E and GFS seems to suggest similar roles for data that NADPH is released as NADP after GFS catalysis. The absence of this loop in GFS also results in NADPH binding in a more solvent-exposed manner which is consistent with the kinetic confirmation by interaction with the hydroxyl groups of the ribose sugar. This mechanism is supported by the structure of the ternary complexes of GalE with NADH and UDP sugars and mutagenesis experiments with GalE as well as the structure of ternary complexes of other SDR enzymes (11, 33). In GFS the Ser-Tyr-Lys catalytic triad is thus properly positioned to play an analogous role in the epimerization and NADPH-dependent reduction of the GDP-keto-6-deoxymannose at C-3/C-5 and C-4, respectively. However, in the absence of a complex structure for GFS with the GDP sugars, residues in the active site involved in the epimerization reaction cannot be confirmed.

In summary, our data indicates that GFS is truly a bifunctional enzyme that catalyzes both the epimerization and reduction reactions. The enzyme residues involved in the epimerization and reduction reaction should be confirmed by determining the structure of the complex of GFS with NADP and the GDP sugar and by site-directed mutagenesis experiments. This will help demonstrate if these reactions are typical of other SDR enzymes.

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In summary, our data indicates that GFS is truly a bifunctional enzyme that catalyzes both the epimerization and reduction reactions. The enzyme residues involved in the epimerization and reduction reaction should be confirmed by determining the structure of the complex of GFS with NADP and the GDP sugar and by site-directed mutagenesis experiments. This will help demonstrate if these reactions are typical of other SDR enzymes.