UDP-galactofuranose Precursor Required for Formation of the Lipopolysaccharide O Antigen of Klebsiella pneumoniae Serotype O1 Is Synthesized by the Product of the rfbD<sub>KPO1</sub> Gene*

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The O-side-chain polysaccharide in the lipopolysaccharide of Klebsiella pneumoniae O1 is based on a backbone structure of repeat units of [→3]-β-D-Galp-(1→3)-α-D-Galp-(1→); this structure is termed α-galactan I. The rfbD (O-antigen biosynthesis) gene cluster directs the synthesis of α-galactan I and consists of six genes termed rfbDA-E<sub>KPO1</sub>. In this paper we show that rfbD<sub>KPO1</sub> encodes a UDP-galactopyranose mutase (NAD(P)H-requiring) (EC 5.4.99.9), which forms uridine 5′-(trihydrogen diphosphate) P-α-D-galactofuranosyl ester (UDP-Galp), the biosynthetic precursor of galactofuranosyl residues. The deduced amino acid sequence of rfbD<sub>KPO1</sub> shows 85% and 37.5% identity to the rfbD<sub>KPO8</sub> gene of K. pneumoniae serotype O8 and the gif gene of Escherichia coli, respectively. The molecular mass of the purified RfbD<sub>KPO1</sub> enzyme is 45 kDa as determined by SDS-polyacrylamide gel electrophoresis, while gel filtration revealed a molecular mass of 92 kDa, suggesting a dimeric structure for the native protein. The rfbD<sub>KPO1</sub> gene product interconversts uridine 5′-(trihydrogen diphosphate) P-α-galactopyranosyl ester (UDP-Galp) and UDP-Galp. Unlike Gif, RfbD<sub>KPO1</sub> showed a requirement for NADH or NAPDH, which could not be replaced by NAD or NADP. RfbD<sub>KPO1</sub> was used to synthesize milligram quantities of UDP-Galp, allowing this compound to be purified and fully characterized in an intact form for the first time. The structure of UDP-Galp was proven by NMR spectroscopy.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria. In enteric bacteria, the LPS molecule comprises a hydrophobic lipid A portion, which forms the outer leaflet of the outer membrane, a core oligosaccharide, and an O-side-chain polysaccharide. The O polysaccharide varies in structure from strain to strain, giving rise to unique antigenic epitopes (O antigens). In the genus Klebsiella, there exists a family of structurally related galactose-containing O polysaccharides. These are based on a backbone structure consisting of a disaccharide O-repeat unit [→3]-β-D-Galp-(1→3)-α-D-Galp-(1→) known as α-galactan I (1). Variations in O antigens arise from addition of side-chain α-D-Galp (2) and O-acetyl (2, 3) residues, or by addition of domains of varying structure (1, 4–6). Galactofuranosyl residues are present in a growing number of LPS O antigens e.g. in strains of Serratia marcescens (7, 8), Shigella dysenteriae (9), Shigella boydii (10), Escherichia coli (11), Pasteurella hemolytica (12), Hemophilus pleuropneumoniae (13), and Actinobacillus pleuropneumoniae (14). The T1-antigen polysaccharide of Salmonella furnedanau (15) and a variety of capsular or extracellular polysaccharides from both Gram-negative and Gram-positive bacteria (e.g. Refs. 16–20) contain Galp. Galactofuranosyl residues are a central component in the mycolyl-arabinogalactan complex, which is characteristic of the cell walls of mycobacteria (21) and the related genera Nocardia and Rhodococcus (22), in the lipoglycan of Mycoplasma mycoides (23), in the paracrystalline S-layer glycoprotein of Clostridium thermosulfurophilum S102–70 (24), and in the cellulosomal glycoproteins of Clostridium thermocellum (25) and Bacteroides cellulosolvens (26). In eukaryotes, galactofuranosyl residues are found in the lipophosphoglycan of Leishmania donovani (27), Leishmania major (28), and Leishmania mexicana (29), in the N-linked glycoproteins of Crithidia spp. (30), and in the lipoproteinphosphoglycan of Trypanosoma cruzi (31). A variety of fungal cell surface glycan, glycolipids and glycoproteins contain Galp residues in Penicillium spp. (32–34), Aspergillus spp. (35), Neurospora crassa (36), and Histoplasma capsulatum (37). Many of these microorganisms are important pathogens, and, for some, current therapies are limited. This observation, together with the absence of Galp residues in human glycoconjugates has fueled interest in the potential of generating novel therapeutic compounds directed against reactions involved in the formation of Galp precursors (38).

In the prototype system in K. pneumoniae O1, α-galactan I biosynthesis is directed by enzymes encoded by six genes in the chromosomal rfb locus. The polymer is synthesized in the cytoplasm by RfbCDEF activities and is then transported across the plasma membrane by a process involving an ATP-binding cassette (ABC-2) transporter, where RfbA is the transmembrane component and RfbB contains the consensus ATP-binding motifs (39). The synthesis of α-galactan I is initiated on a “primer” comprising undecaprenyl pyrophosphoryl N-acetyl-glucosamine. The primer is formed by the activity of the Rf
protein which appears to be an UDP-GlcNAc:undecaprenylphosphate GlcNac-1-phosphate transferase (40). Ribf is a bifunctional galactosyl transferase, which forms the disaccharide \([-3)-\beta-D-Galp-(1-\beta-D-Dgalp-1\rightarrow\) on the primer (41). The details of the subsequent polymerization reaction(s) have not yet been resolved.

The precursor for Gal biosynthesis is UDP-Gal (42), formed by the action of UDP-galactose 4-epimerase, encoded by the galE gene in the galactose operon (43). Early studies on the biosynthesis of the T1 antigen in S. enterica serovar Typhimurium (44, 45) suggested that Gal precursor proteins were derived from Galp at the level of UDP-linked sugars. The Galp precursor for a bifunctional galactosyl transferase, which forms the disaccharide \([-3)-\beta-D-Galp-(1-\beta-D-Dgalp-1\rightarrow\) on the primer (41). The details of the subsequent polymerization reaction(s) have not yet been resolved.

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For the purification of larger quantities of UDP-Galf, a CarboPac PA1 column (9 × 250 mm) operated at a flow rate of 3 ml/min was used. Separated compounds were recovered by lyophilization.

NMR Analysis—The 1H, 13C, and 31P spectra were recorded on a Bruker AMX-600 spectrometer, at 285 K using a 5-mm broadband tunable probe. The sample was prepared from 0.5 mg of UDP-Galf in ammonium acetate buffer, lyophilized, and redissolved in 0.6 ml of 10 mM potassium phosphate buffer (pH 7.2), containing 0.5 mM EDTA. The pH of the sample dropped below 6; therefore, additional K2HPO4 was immediately added to adjust the pH to 6.8. The sample was then lyophilized and redissolved in 0.6 ml of D2O and transferred to a 5-mm NMR tube. Acetone was added to the sample to provide the internal proton chemical shift reference at 2.225 ppm. The methyl resonance of an external acetone in D2O, set at 31.07 ppm, was used for the 13C chemical shift reference. The chemical shift reference for 31P was that of external phosphoric acid (25%) in D2O set at 0.0 ppm. All of the experiments were carried out without sample spinning and with the standard software and pulse programs provided by Bruker. The J(H,H) and J(P,H) coupling constants were measured directly from the one-dimensional 1H or 31P spectra processed with a digital resolution of 0.2 Hz/point.

The 1H spectrum of 256 scans was recorded with presaturation of the HOD resonance at 4.945 ppm. The 13C spectrum with proton decoupling was recorded overnight with 50,000 scans. The 31P spectrum of 4000 scans was recorded without proton decoupling. The two-dimensional homonuclear magnitude COSY, phase-sensitive TOCSY, 1H-31P HMQC, 1H-13C HMQC experiments were recorded and processed as described previously (56). The one-dimensional TOCSY z-filtered spectra of 2000 scans was performed as described previously (57).

RESULTS

The K. pneumoniae Serotype O1 rfbD<sub>KPO1</sub> Gene Encodes a Functional Homologue of Glf of E. coli K-12—The rfbD<sub>KPO1</sub> gene cluster that directs formation of β-galactan I (39, 42) consists of six genes termed rfbA–F. The rfbD<sub>KPO1</sub> open reading frame comprises 1152 nucleotides. No consensous ribosome binding site was found upstream of the ATG initiation codon of rfbD-KPO1. The TGA stop codon of rfbD<sub>KPO1</sub> overlaps with the ATG initiation codon of rfbE<sub>KPO1</sub>, suggesting translational coupling. The predicted translational product of rfbD<sub>KPO1</sub> is a 384-amino acid protein with a molecular weight of 44,454 and a theoretical pI of 6.06. The N-terminal region between Lys-5 and Asp-33 contains a signature for an ADP-binding bab-bfold involved in FAD or NAD binding (58). The sequence deviates only at Gly-19 from the fingerprint. Consequently, a BLAST search (59) revealed striking similarities of the N terminus of rfbD<sub>KPO1</sub> to flavin-containing oxidases and dehydrogenases. Across the entire predicted polypeptide, 85% and 37.5% identity were found to the rfbD<sub>KPO8</sub> gene of K. pneumoniae serotype O8 (also comprising d-galactan I (3, 60) and the glf (orf6) gene of E. coli K-12, respectively (Fig. 1).

Direct evidence that rfbD<sub>KPO1</sub> encodes a Glf homologue was obtained from complementation experiments. The rfbD<sub>KPO1</sub> gene cluster was cloned into pBBR1MCS resulting in pWQ71. Site-directed mutagenesis was used to generate plasmid pWQ70, which contains an in-frame deletion of 630 base pairs in the rfbD<sub>KPO1</sub> gene of the β-galactan I gene cluster. A physical map of the DNA fragments cloned into pWQ70 or pWQ71 is given in Fig. 2. The LPS phenotypes conferred by these plasmids were analyzed by SDS-PAGE in DH5α and a derivative of strain SØ874 (CWG287). The glf gene, located in the cryptic E. coli K-12 rfb region (47, 61), is deleted in E. coli SØ874. The LPS of DH5α or CWG287 both containing the complete cluster (pWQ71) showed a ladder of smooth LPS (S-LPS) (Fig. 3). Deletion of the internal region of rfbD<sub>KPO1</sub> in pWQ70 resulted in no phenotypic alterations in DH5α, while in CWG287 the synthesis of S-LPS was completely abolished (Fig. 3), suggesting that a gene of the cryptic rfb cluster of E. coli K-12, most likely glf, is functionally equivalent to rfbD<sub>KPO1</sub>.
An E. coli K-12 Δrfb Strain Overexpressing RfbDKPO1 Accumulates UDP-Galp and a Novel Galactose-containing UDP Derivative—The function of RfbDKPO1 was further addressed by analyzing the UDP-sugars synthesized in vivo. In order to achieve high level expression of RfbDKPO1, a NdeI and a HindIII site overlapping with the start and stop codon of rfbD_KPO1, respectively, were introduced by site-directed mutagenesis. The NdeI-HindIII fragment containing only the rfbD_KPO1 open reading frame was cloned into the T7 expression vector pET30a(1), resulting in pWQ66. The functionality of the rfbD_KPO1 gene was proven by complementation of CWG287 (pWQ70). When pWQ66 was transformed into CWG287 (pWQ70), the synthesis of S-LPS was restored, even without isopropyl-1-thio-β-D-galactopyranoside induction (Fig. 3). To avoid further metabolism of in vivo synthesized UDP-Galp or UDP-Galp, CWG287 (E. coli K-12 Δrfb ADE3 galE) was constructed as a galE derivative of CWG287. Additionally, sorbitol, which has been reported to suppress elevated levels of UDP-sugar hydrolase activity as a result of UDP-galactose accumulation (62), was added to the medium to avoid enzymatic degradation. Nucleotide sugars were extracted from CWG288 (pWQ66) and from the control strain CWG288 (pET30a(+) ), and extracts were analyzed by HPAEC. From Fig. 4 it can be seen that both strains accumulated UDP-Galp (retention time 34.5 min), while an additional peak (p37) with a retention time of 37.5 min was unique to extracts of CWG288 (pWQ66). In experiments using [1-14C]galactose, p37 was found to contain about 5% of the radioactivity, with the remaining 95% confined to the UDP-Galp peak (data not shown), suggesting that p37 is a novel galactose-containing sugar nucleotide formed by the activity of RfbD_KPO1.

**Purification and Characterization of the RfbD KPO1 Protein**—In order to unequivocally determine the function of RfbDKPO1 and the nature of p37, the RfbD KPO1 protein was purified to homogeneity from CWG288 (pWQ66) by ammonium sulfate precipitation, dye-ligand affinity chromatography, and anion exchange chromatography as described in detail under “Experimental Procedures.” The protein was homogeneous as judged by SDS-PAGE (Fig. 5). The molecular mass of 45,000 Da of the denatured polypeptide determined by SDS-PAGE is in good agreement with the predicted molecular weight of 44,454. However, gel filtration on Superose 12 revealed a molecular weight of 92,000, suggesting a dimeric structure for the native protein (data not shown).

An in vitro assay was used to gain more information on the RfbD KPO1-mediated reaction. The enzyme reaction was carried out in a total volume of 100 μl, and the reaction products obtained after incubation at 37 °C for 30 min were analyzed by HPAEC on a CarboPac PA1. The enzyme showed an absolute requirement for NADH or NADPH (data not shown). Under these reaction conditions, a peak well separated from UDP-Galp or UDP-Glc with the same retention time as p37 was obtained (Fig. 6B). In all experiments about 5% of UDP-Galp was converted to p37. No reaction could be seen in assays lacking a cofactor or in assays with NAD or NADP (data not shown). Other than the cofactor requirements, these data are in agreement with the data de-
scribed for Glf of *E. coli* (48) and further support the conclusion that p37 indeed is UDP-Galf.

**NMR Analysis of UDP-Galf**—In order to unequivocally prove that p37 is UDP-Galf, about 1.5 mg of p37 was pooled from several HPLC runs on a semipreparative CarboPac PA1 (9 × 250 mm). After lyophilization, a small amount was redissolved in 100 mM ammonium acetate, pH 7.0, and analyzed by HPAEC. The material was found to be >95% pure (Fig. 6D) as judged by peak integration. During preparation of the sample for NMR analysis, the pH of the sample dropped below pH 6.0, which resulted in partial degradation of the nucleotide sugar. However, a complete assignment by two-dimensional methods of all proton, carbon, and phosphorous NMR resonances permitted the identification of intact p37 and of all the major components of the sample. By integration of the 1H spectrum, the sample was found to be a mixture of the following major components: 26% UDP-Galp, 53% 5′-UMP, and 21% Galf-1,2-P.

The 31P spectrum showed the presence of various phosphorylated compounds, one of which contained a pyrophosphate group that has characteristic shifts at 211.2 ppm and 212.4 ppm and a J(P@,P?) coupling constant of 21 Hz (63). A 1H-31P HMQC spectrum indicated the proton resonances, which were coupled to the pyrophosphate group. The proton resonances at 5.629 ppm and 4.152 ppm, which were coupled to the 31P resonance at 212.4 ppm had J(P,H) coupling constants of 5.5 Hz and 2.3 Hz, respectively. The complete coupled spin system for these proton resonances was identified by COSY, TOCSY, and one-dimensional TOCSY experiments. The proton-proton coupling constants (J(H,H)) could be obtained from the one-dimensional TOCSY spectrum. A 1H-13C HMQC permitted the assignment of all the carbon atoms directly bonded to these protons (C-H). The proton chemical shifts, J(H,H) coupling constants, 13C chemical shifts (64), and 31P chemical shifts were all characteristic of a terminal α-Galf residue bonded to a pyrophosphate group.

From a similar analysis, the resonances at 4.24 and 4.21 ppm, which bonded to the pyrophosphate resonance at −11.2 ppm, were found to belong the H5′ and H5″ resonances of the ribose moiety of UDP-Galf. The proton chemical shifts, J(H,H) coupling constants, and J(P,H) coupling constants of the UDP moiety were found to be characteristic for those found for UDP sugars (65).

5′-UMP—In the 1H-31P HMQC spectrum, the 31P resonances
at 3.5 ppm bonded to the proton resonances at 4.032 ppm and 3.970 ppm were found to belong to 5'-UMP by a complete assignment of the 1H and 13C spectra. The proton chemical shifts, \(J_{(H,H)}\) coupling constants, and \(J_{(P,H)}\) coupling constants of the 5'-UMP were found to be similar to those previously reported for this compound (66).

Galf-1,2-P—The \(^{31}P\) resonance at 17.1 ppm is characteristic of a five-membered cyclic ester (63). In the \(1H^{31}P\) HMQC spectrum, this \(^{31}P\) resonance was coupled to the proton resonances at 6.000 ppm and 4.813 ppm, with \(J_{(P,H)}\) coupling con-
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TABLE II
Proton chemical shifts

| Compound            | Moiety | H1  | H2  | H3  | H4  | H5  | H5' | H6  | H6' |
|---------------------|--------|-----|-----|-----|-----|-----|-----|-----|-----|
| UDP-Galf'           | Gal'   | 5.629 | 4.152 | 4.238 | 3.823 | 3.76 | 3.629 | 3.711 |
|                    | Ribose | 5.992 | 4.38 | 4.38 | 4.289 | 4.24 | 4.21 | 7.972 |
|                    | Ura    | 6.002 | 4.423 | 4.351 | 4.259 | 4.032 | 3.970 | 8.141 |
| 5'-UMP              | Ribose' | 6.002 | 4.813 | 4.404 | 3.975 | 3.894 | 3.745 | 3.639 |
| 5'-UMP              | Ura'   | 6.002 | 4.813 | 4.404 | 3.975 | 3.894 | 3.745 | 3.639 |

TABLE III
31P chemical shifts in ppm

| Compound            | Moiety | C1  | C2  | C3  | C4  | C5  | C6  | C7  | C8  |
|---------------------|--------|-----|-----|-----|-----|-----|-----|-----|-----|
| UDP-Galf'           | Gal'   | 98.6 | 77.4 | 74.3 | 82.6 | 72.9 | 61.9 |     |     |
|                    | Ribose | 89.1 | 74.6 | 70.5 | 84.1 | 65.7 |     |     |     |
|                    | Ura    | 103.5 | 124.4 |     |     |     |     |     |     |
| 5'-UMP              | Ribose' | 89.1 | 74.7 | 70.9 | 84.9 | 64.0 |     |     |     |
| 5'-UMP              | Ura'   | 103.5 | 142.9 |     |     |     |     |     |     |
| Galf-1,2-P          |       | 102.9 | 85.6 | 75.9 | 86.6 | 71.6 | 63.0 |     |     |

TABLE IV
31P chemical shifts

| Compound            | Chemical shift |
|---------------------|----------------|
| UDP-Galf'           | 11.2(1), -12.4(3) |
| 5'-UMP              | 3.5            |
| Galf-1,2-P          | 17.1           |
| (O,P)β'            | 1.7            |
| (O,P(PO₂)γ')      | -7.9           |

TABLE V
Spin-spin coupling constants (J) for 31P

| Compound            | Spins | J   | Spins | J   |
|---------------------|-------|-----|-------|-----|
| UDP-Galf'           | Pγ, H5' | 6  | Pγ, H5'' | 5  |
|                    | Pγ, H1  | 5.5 | Pγ, H2  | 2.3 |
|                    | Pγ, Pγ  | 21 |     |     |
| Galf-1,2-P          | P, H1   | 14 | P, H2  | 6.8 |

TABLE VI
J N, α coupling constants in Hz for the Galf moiety

| H1, H2, H3, H4, H5, H6, H5', H6' |
|-------------------------------|
| UDP-Galf' | 4.3 | 8.6 | 7.6 | 5.2 | 4.3 | 7.2 | -11.9 |
| Galf-1,2-P | 4.5 | 2.2 | 4.0 | 7.3 | 3.9 | 6.2 | -11.9 |

DISCUSSION

Galactofuranosyl residues are found in a growing number of surface glycoconjugates from both Gram-negative and Gram-positive bacteria, as well as protozoa and fungi. These include well documented pathogens of humans and livestock. In bacteria (this work, and Refs. 44, 45, and 48) and fungi (46), the biosynthetic precursor for Galf residues is believed to be UDP-Galf. Galactofuranosyl-containing glycoconjugates have not been reported in humans so far, which makes UDP-Galf formation an interesting target for novel therapeutic compounds (38). However, development of strategies for inhibitors has been limited by the lack of fundamental information regarding the biosynthesis of galactofuranosyl residues. Galectocarolose, a β-D-(1→5)-linked polygalactofuranosid produced by *P. charlesii*, was the first polysaccharide found to contain galactofuranosyl residues (34, 67). Trejo et al. (46) isolated a nucleotide sugar in cell-free enzyme preparations of *P. charlesii*, which was capable of acting as a donor of galactofuranosyl residues in the biosynthesis of a galactofuranosyl-containing polymer. Based on chemical analysis of the reaction products obtained by acid and alkaline hydrolysis as well as periodate oxidation, Trejo et al. (46) predicted that the sugar nucleotide is UDP-Galf. More recently, Nassau et al. (48) cloned the glf gene of *E. coli* and showed that it encodes UDP-galactopyranosyl mutase (EC 5.4.99.9). Nassau et al. (48) concluded that the reaction product of the UDP-galactopyranosyl mutase reaction is UDP-Galf based on HPLC analysis of the sugar 1-phosphate obtained after phosphodiesterase treatment of the reaction product. The modification of an HPABE procedure, to give a method that separates UDP-Galf from UDP-Gal, allowed us for the first time to purify milligram quantities of UDP-Galf and to unequivocally prove by NMR the structure to be uridine 5′-(trihydrogen diphosphate) P′-α-D-galactofuranosyl ester. This will provide an essential reagent for biochemical analyses of synthetic systems for galactofuranosyl-containing glycoconjugates.

The data presented here establish that RibDₖₙₚ₁ catalyzes the interconversion of UDP-Galp and UDP-Galf. The LPS profiles of *E. coli* K-12 strains containing pWQ70 and pWQ71 clearly demonstrated that Glf (48) of *E. coli* can complement the ribDₙₚ₁ deletion in pWQ70. Glf and RibDₙₚ₁ are therefore functionally equivalent. Although the *K. pneumoniae* RibDₙₚ₁ protein shows 37.5% identity to Glf of *E. coli*, the two proteins do exhibit remarkable differences. Glf has been reported to be relatively unstable, resulting in complete loss of activity after storage of the purified protein at 4°C for >24 h (48). In contrast, RibDₙₚ₁ can be stored at 4°C for periods in excess of 1–2 weeks without significant loss of activity (data not shown). Moreover, no cofactor requirement other than FAD has been reported for Glf, while RibDₙₚ₁ has an absolute requirement for NADH or NADPH. These differences are difficult to explain. One possibility is that the binding constants for NADH or NADPH are different in both proteins. Alternatively, the differences may be a result of the purification procedure used. Cibacron Blue 3GA used in the purification procedure reported here is thought to bind to NAD binding sites. Consequently, binding of the enzyme to this resin may displace the essential cofactor. Analysis of the predicted sequence of both proteins revealed a ADP-binding βαβ-fold at its N terminus, which could be involved in binding FAD or NAD. The motif in *K. pneumoniae* differs at Gly-19 from the fingerprint. However, the known structure of p-hydroxybenzoate hydroxylase, which has a glycine at the same position (position 15 of the finger-
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print), demonstrates that peptides with a glycine at this
position can form a functional BbB unit (68). The presence of FAD in
the purified Gif protein has been reported (48). Although we
have not analyzed this cofactor in RbfD<sub>KP</sub>, the yellow color of
the pure protein is consistent with its containing FAD. Gel
filtration of the RbfD<sub>KP</sub> protein on Superoxide 12 revealed a
molecular weight of 92,000 for the native protein. The possible
dimerization of Gif has not been investigated.

Little is known about the mechanism of the UDP-galactopy-
ranose mutase (NAD(P)H-requiring) reaction. Stevenson et al.
(47) suggested that the reaction may proceed via a 2-keto
intermediate. Since in our hands the UDP-galactopyranose
mutase shows an absolute requirement for NADH or
NADP+ and was found to be inactive with NAD or NADP,
an alternative is that the first step of the reaction may be a
reduction step, although there is no net oxidation/reduction in
the interconversion of UDP-Gal and UDP-Galf. The stability of
the RbfD<sub>KP</sub> protein and the possibility to synthesize and
purify larger quantities of UDP-Galf will facilitate a detailed
analysis of the reaction mechanism and determination of the
protein structure.

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Note Added in Proof—While this manuscript was in press, a report
by others independently confirmed the nature and structure of UDP-
Galf as the product of the UDP-galactopyranose mutase reaction (Lee,
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