Identification of Two GDP-6-deoxy-D-lyxo-4-hexulose Reductases Synthesizing GDP-β-rhamnose in Aneurinibacillus thermoaerophilus L420-91T

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The glycan repeats of the surface layer glycoprotein of Aneurinibacillus thermoaerophilus L420-91T contain β-rhamnose and 3-acetamido-3,6-dideoxy-β-galactose, both of which are also constituents of lipopolysaccharides of Gram-negative plant and human pathogenic bacteria. The two genes required for biosynthesis of the nucleotide-activated precursor GDP-β-rhamnose, gmd and rmd, were cloned, sequenced, and overexpressed in Escherichia coli. The corresponding enzymes Gmd and Rmd were purified to homogeneity, and functional studies were performed. GDP-β-mannose dehydratase (Gmd) converted GDP-β-mannose to GDP-6-deoxy-β-lyxo-4-hexulose, with NADP⁺ as cofactor. The reductase Rmd catalyzed the second step in the pathway, namely the reduction of the keto-intermediate to the final product GDP-β-rhamnose using both NADH and NADPH as hydride donor. The elution behavior of the intermediate and end product was analyzed by high performance liquid chromatography. Nuclear magnetic resonance spectroscopy was used to identify the structure of the final product of the reaction sequence as GDP-α,β-rhamnose. This is the first characterization of a GDP-6-deoxy-β-lyxo-4-hexulose reductase. In addition, Gmd has been shown to be a bifunctional enzyme with both dehydratase and reductase activities. So far, no enzyme catalyzing these two types of reactions has been identified. Both Gmd and Rmd are members of the SDR (short chain dehydrogenase/reductase) protein family.

S-layers 1 are two-dimensional protein crystals that form the outermost cell surface component of many archaea and bacteria (1, 2). Frequently, these S-layer proteins are glycosylated (3, 4). The S-layer glycoprotein of the Gram-positive, thermophilic bacterium Aneurinibacillus thermoaerophilus L420-91T, a member of the Bacillus/Clostridium group, is composed of identical repeats of β-rhamnose and 3-acetamido-3,6-dideoxy-β-galactose units (5). The principal architecture of S-layer glycoproteins resembles that of the LPS of Gram-negative bacteria (6). Both glycoconjugates exhibit a tripartite structural organization, where usually conserved core regions connect a glycan chain, composed of repeating units, either with the S-layer polypeptide or the lipid A of LPS. It has been proposed that comparable pathways are used for the biosynthesis of these similar glycoconjugates (3). β-Rhamnose is a rare sugar that is also constituent of the LPS of plant pathogens like Xanthomonas campestris (7), Pseudomonas syringae (8) and of human pathogens, most importantly Pseudomonas aeruginosa (9), Burkholderia cepacia (10), Campylobacter fetus (11), and Helicobacter pylori (12). Recently, a biosynthetic pathway for the nucleotide-activated form of β-rhamnose has been proposed (13). According to this model, GDP-β-rhamnose is converted to GDP-6-deoxy-β-rhamnose in two reaction steps (Fig. 1). The first step is the dehydration of GDP-6-deoxy-β-rhamnose (Gmd), which leads to the unstable intermediate GDP-6-deoxy-β-lyxo-4-hexulose (14, 15). This keto-derivative is further converted to the final product GDP-β-rhamnose by the action of GDP-6-deoxy-β-lyxo-4-hexulose reductase (Rmd).

The first reaction, which is particularly important, because it is also part of the GDP-fucose biosynthetic pathway, has been recently described for Escherichia coli (16), Arabidopsis thaliana (17), and Homo sapiens (18). Gmd is a unique member of the short chain dehydrogenase/reductase protein family (19–21). This diverse protein family consists of enzymes that function not only as reductases or dehydrogenases but also as epimerases, isomerases, and dehydratases (22, 23). Among the sugar-modifying enzymes, the crystal structures of UDP-glucose 4-epimerase from E. coli (24) and H. sapiens (25), of GDP-4-keto-6-deoxy-β-mannose epimerase/reductase (26, 27), of Gmd (21), and of ADP-β-glycero-β-manno-heptose epimerase (28) have been reported, recently. Several other enzymes such as RmlB and RmlD (29) were added to this protein family, based on alignments of protein sequences. A strictly conserved residue throughout this family is Tyr157 (in Gmd of E. coli), the residue deprotonating the C-4 hydroxyl group of the sugar moiety (21). Another highly conserved residue, Lys161, lowers the pKₐ of Tyr157, making catalysis of the oxidation reaction possible. Together, these two residues form the YXXK motif typical, but not unique, for the SDR protein family (20). Two

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‡ The abbreviations used are: S-layer, surface-layer; LPS, lipopolysaccharide; Gmd, GDP-mannose 4,6-dehydratase; Rmd, GDP-6-deoxy-β-lyxo-4-hexulose reductase; ORF, open reading frame; PCR, polymerase chain reaction; His-Gmd, histidine-tagged Gmd; GST, glutathione S-transferase; GST-Gmd, GST-tagged Gmd; HPLC, high pressure liquid chromatography; COSY, correlation spectroscopy.
further residues, Thr133 and Glu135, complete the catalytic residues in the active center of E. coli Gmd. Another set of conserved residues, found near the N terminus in the Rossmann-fold (30), is responsible for the binding of NAD(P)⁺. Other residues important for substrate or cofactor binding are spread throughout the protein sequence.

For the second reaction step, the reduction of the hexulose, in P. aeruginosa an ORF directly upstream of the gmd gene has been proposed to code for the reductase Rmd. The corresponding ORF has been deleted, which results in mutants that lack A-band LPS, which is composed exclusively of D-rhamnose residues (31). So far, no functional studies of the enzyme have been performed. This second step in GDP-D-rhamnose biosynthesis seems to be a perfect target for enzyme inhibition to prevent the synthesis of D-rhamnose-containing LPS structures in pathogenic bacteria. Such an approach would lead to a promising therapeutic alternative to the careless use of antibiotics. Crystallographic analysis of the Rmd protein would help to corroborate the functional results and could lead to the design of enzyme inhibitors via molecular modeling. In this report we characterize for the first time the biosynthetic reactions involved in the conversion of GDP-mannose to GDP-D-rhamnose.

EXPERIMENTAL PROCEDURES

Materials—GDP-D-mannose, NAD⁺, NADH, NADPH, and dithiothreitol were obtained from Sigma. Glutathione (reduced form) was synthesized from mercaptoethanol (Sigma, St. Louis, MO). All standard DNA recombinant procedures were performed according to the methods described by Sambrook et al. (40). Other reagents were purchased from the corresponding manufacturer. PCR was carried out using a PCR Sprint thermocycler (Hybaid, Ashford, UK). DNA sequencing was performed either by MWG BIOTECH (Ebersberg, Germany) or AGOWA (Berlin, Germany).

Construction of a Phage Library and Identification of a gmd- and rmd-containing Clone—A genomic library of A. thermooerophilus strain L420-91 was prepared by cloning size fractionated, partially Sau3A-digested DNA that was partially filled in with dGTP and dCTP, into the phage λ vector λGEM-12. This vector was cut with XhoI and partially filled in with dTTP and dCTP. The presence of clones containing the gmd sequence was confirmed by PCR using the primer pair (5’-TTTC-CTGTAGTTTGGC-3’ and 5’-CAATATCCGATACTTCACAGG-3’) and phage lysate as template. A small, PCR-positive subpool was screened by plaque-lifting using a 32P-labeled gmd PCR fragment as probe. A clone (ΔEK1) containing gmd and about 10 kilobases of downstream DNA was converted into a plasmid suitable for sequencing by in vivo recombination in E. coli in 41. In brief, the vector pPGA1851, which contains 450 base pairs of DNA from the left arm of phage λ and 390 base pairs of its right arm, is linearized in between and cotransformed into yeast with a crude preparation of the respective phage λ using a simple lithium transformation protocol (42). About 10% of the yeast transformants contain the recombination product formed in vivo. The resulting (broad host range) plasmid pΔIK, containing the insert of the λ clone, was recovered from yeast, introduced into E. coli by electroporation, and used subsequently for sequencing.

Plasmid Construction—Oligonucleotide primers for the amplification of DNA fragments containing either the gmd or rmd gene were designed with attB1 or attB2 sites for the insertion into the GATEWAY donor vector pDONR201 (Life Technologies, Inc.) by homologous recombination. Primers with the following sequences were synthesized by Life Technologies, Inc.: GMD1α (forward), 5’-attB1-GCGAGAATCTCTTCTTCAACGATGAAAAAGTTATACCC-3’; GMD2α (reverse), 5’-attB2-CTCGAATCCGATGTTATTAGG-3’; RMD1α (forward), 5’-attB1-GCGAGAATCTCTTCTTCAACGATGAAAAAGTTATACCC-3’; and RMD2α (reverse), 5’-attB2-CTCGAATCCGATGTTATTAGG-3’. attB1 and attB2 are the minimal 25-base pair sequences required for efficient homologous recombination. The PCR products were cloned into pDONR201, and the resulting plasmids gGMDD1 and gRMD1 were used to transfer the gene sequences into pDEST15 (GST fusion) or pDEST17 (His fusion) via homologous recombination. The corresponding plasmids gGMDD2 (GST fusion), gGMDD3 (His fusion) and gRMD3 (His fusion) were used for overexpression of the fusion proteins in E. coli BL21-S1.

Enzyme Purifications—Cells carrying gGMDD3 were grown at 37 °C to an optical density at 600 nm of 0.5 in 1 liter of culture volume, and expression was performed for 3 h. Cells harboring the GST fusion plasmid gGMDD2 were grown at 30 °C to an optical density at 600 nm of 0.5 in 1.4 liter culture volume, and expression was carried out overnight. A 1.4-liter culture harboring gGMDD2 was grown at 37 °C to an optical density of 0.5, and expression was done for 4 h. Production of the fusion proteins was induced by the addition of NaCl to a final concentration of 0.3 M. The cells were disrupted on ice by ultrasonication, cell debris was pelleted by centrifugation at 31,000 × g, and membrane fractions were removed by ultracentrifugation at 331,000 × g. Purifications using Hitrap Chelating and GSTrap columns were performed as recommended by the manufacturer. A HiPrep Desalting column was used for buffer exchange. Anion exchange chromatography on a MonoQ column was carried out in 20 mM Tris-HCl buffer, pH 7.7, using 1 M KCl as eluant. Dithiotreitol (0.5 mM) was added to the buffers when using GSTrap, HiPrep Desalting, and MonoQ columns. Purification of histidine-tagged Gmd was performed with Hitrap Chelating, and imidazole was removed using a HiPrep Desalting column (20 mM Tris-HCl buffer, pH 7.7, 50 mM NaCl). Glutathione S-transferase-tagged Gmd was purified using GSTrap and MonoQ (elution at 300 mM KCl) columns.

DNA Manipulations, Polymerase Chain Reaction, and DNA Sequencing—All standard DNA recombinant procedures were performed according to the methods described by Sambrook et al. (40) or as recommended by the corresponding manufacturer. PCR was carried out using a PCR Sprint thermocycler (Hybaid, Ashford, UK). DNA sequencing was performed either by MWG BIOTECH (Ebersberg, Germany) or AGOWA (Berlin, Germany).

Sequence Analysis—BLAST basic logic alignment search tool; Ref. 31; and MultAlin (38) were used to analyze nucleotide and protein sequences. For protein family analysis, the Prosit data base was used (39).

**FIG. 1.** Schematic representation of the biosynthesis pathway for GDP-D-rhamnose.
GDP-\(\delta\)-rhamnose Biosynthesis

Rmd His-tagged fusion protein was purified by fast protein liquid chromatography using HiTrap Chelating and MonoQ (elution at 330 mm KCl) columns. The proteins were stored at 4 °C or after stabilization with 50% glycerol at −20 °C. The purity of the enzymes was checked by SDS-polyacrylamide gel electrophoresis analysis (4% stacking gel and 12% separating gel).

Enzyme Assays—20 nmol of GDP-\(\delta\)-mannose or GDP-6-deoxy-\(\delta\)-lyxo-4-hexulose were used for enzyme assays. His-Gmd was removed by ultrafiltration after in situ preparation of GDP-6-deoxy-\(\delta\)-lyxo-4-hexulose. The assay buffer contained 50 mm KH_{2}PO_{4} (pH 7.0) and 10 mm MgCl_{2}. Reactions were performed in 100 μl of reaction volume at 37 °C for 30 min. The samples were analyzed by HPLC on a CarboPac PA-1 column.

Synthesis of GDP-\(\delta\)-rhamnose—20 μmol of GDP-\(\delta\)-mannose were converted quantitatively to GDP-6-deoxy-\(\delta\)-lyxo-4-hexulose, as judged by HPLC analysis, using appropriate amounts of GST-Gmd in 20 mm Tris-HCl buffer, pH 7.7, 0.3 M KCl, and 0.5 mm dithiothreitol. The dehydratase was removed by ultrafiltration using Ultrafree-MC 10000 ultrafiltration cartridges. For the second reaction step Rmd and NADPH were added, and after complete conversion to GDP-\(\delta\)-rhamnose, NADP\(^{+}\) and NADPH were removed by HPLC. Ammonium acetate was removed by a desalting step using Sephadex G-10, and lyophilization resulted in pure GDP-\(\delta\)-rhamnose in 18.5% yield.

No ultrafiltration step was performed when synthesizing GDP-\(\delta\)-rhamnose with GST-Gmd only. Additional enzyme was added after the first reaction step, but inactivation of the enzyme resulted in incomplete reduction of the keto-intermediate and the yield of pure GDP-\(\delta\)-rhamnose was only 2%.

NMR Spectroscopy—The lyophilized material of the first preparation (2.0 mg) was dissolved in 99.95% D_{2}O (0.5 ml). The solution of the second sample (0.2 mg) contained 10 mg of NaCl, which was added for resolution enhancement. Spectra for both solutions (\(\delta = 5\)) were recorded at 300 K at 300.13 MHz for \(^{1}H\), at 75.47 MHz for \(^{13}C\), and 121.49 MHz for \(^{31}P\), with a Bruker AVANCE 300 spectrometer equipped with a 5 mm quadruple nuclear inverse probehead (QNP). External calibration was performed for \(^{1}H\) spectra with 2,2-dimethyl-2-silapentane-5-sulfonic acid (\(\delta = 0\)). For \(^{13}C\) spectra using 1,4-dioxane (\(\delta = 67.40\)), and for \(^{31}P\) spectra with H_{2}PO_{4} (\(\delta = 0\)). Measurement of correlation spectroscopy (COSY), heteronuclear multiple bond correlation, heteronuclear multiple quantum correlation, and H,P-correlated spectra was performed with standard Bruker software.

RESULTS

Cloning of the \(\delta\)-Rhamnose Operon—The genes encoding enzymes for the biosynthesis of a nucleotide sugar precursor are usually clustered together within the corresponding gene cluster for the particular bacterial polysaccharide (43). An alignment of 18 Gmd and putative Gmd sequences found in the NCBI data base was carried out using MultiAlign (36). Within the amino acid sequence there are several highly conserved regions. One of these regions, GILFNHES, was found to be identical in 16 of 18 data base entries, and the seven-amino acid stretch GILFNHE was used for the design of the degenerate oligonucleotide probe 5'-GGI ATH YTI TTY AAY CAY GA-3' (where I is inosine, H is A/C/T, and Y is C/T). This digoxigenin-labeled probe was used for Southern hybridization experiments to get highly specific signals with completely digested chromosomal DNA of A. thermoaerophilus A240-91\(^{T}\). Hybridization of EcoRI-digested DNA at 36.5 °C resulted in one specific band at 3 kilobases that was isolated and cloned into the plasmid pBCKS. The insert of the corresponding plasmid pRMD38 was sequenced and contained part of the gmd gene besides one hypothetical ORF (Fig. 2). The amino acid sequence GILFNHES is also part of the new gmd gene. The downstream part of gmd was sequenced as described previously (44) to yield further sequence information completing the gmd gene and part of a second gene, putatively coding for the reductase Rmd. Complete sequence of the second gene was obtained by sequencing of plasmid pBK1 (Fig. 2), which is derived from a λgem-12 clone. The \(\delta\)-rhamnose operon seems to consist only of the two ORFs coding for Gmd and Rmd, because there are no other ORFs on the upstream and downstream sequences, respectively.

Sequence Analysis—The protein sequence of Gmd from A. thermoaerophilus A240-91\(^{T}\) was aligned with 27 Gmd sequences obtained from data bases. These sequences include Gmd proteins from bacteria as well as from archaea and eukarya. The alignment showed a high level of overall identity in all sequences used. The highest homology to the Gmd sequence of A. thermoaerophilus A240-91\(^{T}\) was obtained with the following sequences: Caulobacter crescentus (GenBank\(^{TM}\) accession number AAC38668), P. aeruginosa (AAAG08828), and Aquifex aeolicus (D70393). Recently, the crystal structure of Gmd from E. coli was reported, and several conserved amino acid residues were proposed to be important for substrate and cofactor binding and catalysis (21). All these residues were found in Gmd of A. thermoaerophilus A240-91\(^{T}\) except Thr\(^{135}\), which was replaced by Ser (Fig. 3). According to these results, gmd from A. thermoaerophilus A240-91\(^{T}\) is unequivocally identified as the gene encoding the GDP-\(\delta\)-mannose 4,6-dehydratase.

An NCBI BLAST search was performed using the newly determined protein sequence of the second open reading frame in this operon, which putatively codes for Rmd. Two highly homologous proteins were found in the data base, one Mycobacterium tuberculosis putative dehydrogenase (GenBank\(^{TM}\) accession number C70840, 35% identity, 53% similarity) and a P. aeruginosa protein (AAAG08839, 33% identity, 54% similarity) described as Rmd, without any functional characterization. Further comparison of Rmd from A. thermoaerophilus A240-91\(^{T}\) with Gmd from A. thermoaerophilus A240-91\(^{T}\) and E. coli showed considerable similarity (Fig. 3). Especially two important motifs GXGXXG, the cofactor-binding Wierenga motif, and YXXK are conserved. The catalytic residues Tyr and Lys are completed by a Ser residue to form the catalytic triade, which is, together with the Wierenga motif, a characteristic feature of the SDR protein family (19). Because of the functional difference between Gmd and Rmd, some of the residues described to be important for binding of NADP\(^{+}\) and GDP-\(\delta\)-mannose in Gmd are not conserved (Fig. 3). Even one of the catalytic residues, Glu\(^{135}\) (Gmd of E. coli), is not conserved throughout the Rmd sequences. However, this residue is proposed to abstract a hydrogen atom from the hydroxyl group at C-5 of the sugar (21), and Rmd functions by reducing the keto group at C-4.

Expression and Purification of Gmd and Rmd—Because of the intrinsic instability of the GDP-\(\delta\)-mannose dehydratase, it was not possible to obtain sufficient active, native protein, even
after only one purification step. His-Gmd was also partially inactivated during purification with the HiTrap Chelating column. However, GST-Gmd revealed higher stability. GST-Gmd was purified using GSTrap and MonoQ columns and proved to be stable at 4 °C and at −20 °C. GDP-6-deoxy-D-lyxo-4-hexulose reductase was expressed as a histidine-tagged fusion protein and purified using HiTrap Chelating and MonoQ columns. Rmd was stable at 4 °C and at −20 °C. His-Gmd, GST-Gmd, as well as histidine-tagged Rmd were used for the functional characterization of the GDP-β-rhamnose biosynthetic pathway. The molecular masses of the denatured proteins, determined by SDS-polyacrylamide gel electrophoresis analysis, were in good agreement with the calculated molecular masses (His-Gmd, 40.1 kDa; GST-Gmd, 65.2 kDa; His-Rmd, 38.1 kDa; Fig. 4).

Gmd Catalyzes the Dehydration of GDP-β-mannose—GDP-β-mannose was converted quantitatively to GDP-6-deoxy-D-lyxo-4-hexulose, as judged by anion exchange HPLC analysis ($t_r$ = 33.0 min; Fig. 5). His-Gmd required the addition of NADP$^+$ as cofactor. NAD$^+$ addition resulted in less than 10% conversion, and this was also observed when no cofactor was added. GST-Gmd converted the substrate without the addition of any cofactor. The integrity of GDP-6-deoxy-D-lyxo-4-hexulose was investigated by chemical reduction of the keto group with NaBH$_4$. The reduction products were analyzed by HPLC (data not shown). Two products were formed in a 1:2 ratio. The minor peak comigrated with GDP-D-rhamnose ($t_r$ = 26.3 min), when synthesized with Rmd (see below). The major peak showed a retention time of 26.7 min, being slower than GDP-β-rhamnose but faster than GDP-β-mannose ($t_r$ = 27.0 min). This product is supposed to be GDP-6-deoxy-D-talose. Because of the lack of an appropriate standard for nucleotide-activated 6-deoxytalose, no further proof of this assumption was possible. Attempts to isolate the intermediate product failed because of the instability of the D-lyxo product. GDP-6-deoxy-D-lyxo-4-hexulose was decomposed to form GMP and GDP, as judged by HPLC analysis, even upon storage on ice and after desalting on a Sephadex G-10 column.

Rmd Catalyzes the Reduction of GDP-6-deoxy-D-lyxo-4-hexulose—Because of the instability of GDP-6-deoxy-D-lyxo-4-hexulose, this intermediate product had to be prepared in situ for the characterization of the second reaction step in the GDP-β-rhamnose biosynthetic pathway. GDP-β-rhamnose was converted quantitatively to the hexulose, and Gmd was removed from the reaction mixture by ultrafiltration. A new product, migrating faster than GDP-β-rhamnose, as derived from HPLC analysis, appeared when the intermediate product was converted by the Rmd protein (Fig. 5). Under the applied assay conditions, NADH and NADPH worked equally well as hydride donor. Because of lack of a commercially available authentic standard for GDP-β-rhamnose, this new product had to be further characterized. The final product was hydrolyzed with trifluoroacetic acid, and the released monosaccharides were analyzed by isocratic anion exchange chromatography on a CarboPac PA-1 column ( Dionex system). The product comigrated with an authentic rhamnose standard (Fig. 6). Ribose could also be detected and was released from guanosine during hydrolysis. NMR analyses were performed to elucidate the
reaction time, and, again, a product comigrating with GDP-D-mannose, converted with Gmd and NADH.

was observed, with either NAD or NADP as cofactor. Gmd and Rmd had the same characteristics concerning the dehydration and the reduction reactions, the specificity of Rmd is higher and the extension of the time scale for conversion of larger amounts of GDP-6-deoxy-D-lyxo-4-hexulose by Gmd resulted in an inactivation of Gmd and incomplete conversion of the intermediate product.

**NMR Analysis of GDP-α-D-rhamnose**—All connectivities of the proton and carbon signals were unambiguously assigned using H,H COSY, heteronuclear multiple quantum correlation, and heteronuclear multiple bond correlation measurements (Table I and Fig. 7). The signal at 1.25 ppm corresponds to the 6-deoxy group, and the values of the coupling constants of the connected ring proton signals are in full accordance with the presence of a manno-configured system, thus establishing the presence of a rhamnopyranosyl unit. The value of the heteronuclear coupling constant J_C1,H1 (174.9 Hz) is consistent with the α-anomeric configuration of the rhamnopyranosyl unit. In addition, all proton and carbon signals of the guanosine unit could be assigned (Table I). In the proton-decoupled 31P spectrum, two doublets with chemical shifts being characteristic of diphosphodiester units were observed at $-10.87$ and $-13.30$ ppm, which were then correlated to the H5/H5’ protons of the ribose unit and the anomeric proton of the rhamnose residue, respectively (Fig. 7). The structural identity of the GDP-α-D-rhamnose samples, resulting from either the Gmd/Rmd incubation or the Gmd treatment alone, was established by comparison of their proton spectra.

### DISCUSSION

The GDP-α-D-rhamnose operon of *A. thermoaerophilus* L420-91T has been cloned by Southern hybridization experiments using a degenerate probe derived from an alignment of 18 Gmd sequences from the NCBI data base. It had been postulated that genes coding for enzymes involved in the synthesis of nucleotide sugar precursors are usually found in operons within the corresponding gene cluster for a particular bacterial polysaccharide (43). Indeed, on the genome of *A. thermoaerophilus* L420-91T the ORF of the *rmd* gene starts immediately after the end of the ORF of *gmd*. Gmd is highly conserved throughout the domains Archaea, Bacteria, and in higher eukaryotic organisms, such as plants and animals. Only two of 45 Gmd and putative Gmd protein sequences currently available in the NCBI data base deviate from the eight-amino acid stretch GILFNHES, namely those of *Streptomyces noursei* (AAC96486) and *Paramecium bursaria chlorella virus* I (AAC96486). The amino acid residues reported to be important for cofactor or GDP-α-D-rhamnose binding and catalysis are conserved in the newly described *gmd* gene from *A. thermoaerophilus* L420-91T. Only Thr<sup>133</sup> from the sequence in *E. coli* is replaced by a Ser residue in *A. thermoaerophilus* L420-91T. However, for UDP-glucose 4-epimerase, the exchange of a comparable Ser residue by Thr did not affect $k_{cat}$ and reduced $k_{cat}/K_m$ 3-fold (45). The influence of the exchange of Ser to Thr in Gmd in *A. thermoaerophilus* L420-91T is not known. The conservation of the complete Gmd protein in archaea, bacteria, plants, and animals and even the virus is remarkable. This fact may reflect the importance of the enzyme throughout evolution. A BLAST<sup>®</sup>-search with the second protein sequence of the GDP-α-D-rhamnose operon yielded two highly homologous proteins. The first one is a putative dehydrogenase of *M. tuberculosis* (C70840), which has also a putative Gmd sequence in its genome. These two enzymes, however, are not part of the same operon or cluster, and it is not known whether *M. tuberculosis* possesses α-D-rhamnose in one of its polysaccharide structures. The second highly homologous protein is the Rmd enzyme of *P. aeruginosa* (AAG08839). Recently, in a knock-out experiment,
the \textit{rdm} gene has been deleted to result in a \textit{P. aeruginosa} mutant, lacking any A-band LPS (31). However, this protein has not been expressed and purified, so far, to do functional characterization. In \textit{P. aeruginosa} both the \textit{gmd} and \textit{rdm} genes belong to the A-band gene cluster. Further comparison of the conserved residues among the \textit{Gmd} and the \textit{Rmd} proteins have been expressed and purified, so far, to do functional characterization. In \textit{P. aeruginosa} both the \textit{gmd} and \textit{rdm} genes belong to the A-band gene cluster. Further comparison of the conserved residues among the \textit{Gmd} and the \textit{Rmd} proteins have shown that at least the most prominent residues are found in both functionally different proteins. Especially the Wierenga motif GXGXGGX and the catalytic triade Ser-Tyr-Lys are present in both proteins. These sequence features are characteristic of the SDR protein family, a large and diverse group of dehydrogenases, reductases, dehydratases, isomerases, and epimerases (20). Rmd is a novel member of this protein family. A number of other residues important in Gmd catalysis are not found in Rmd. For example, Glu\textsuperscript{135} of the \textit{E. coli} enzyme is not conserved among the (putative) Rmd proteins, although it was identified in Rmd of \textit{A. thermoaerophilus} L420-91\textsuperscript{T}. Glu\textsuperscript{135} was reported to abstract an H atom from C-5 of the previously produced keto-intermediate (21). Subsequently, dehydration of mannose is completed. In the following reduction reaction by Rmd, the C-5 atom of GDP-6-deoxy-D-\textit{xylo}-4-hexulose is not involved.

The inherent instability of the \textit{gmd} gene product, which we experienced during our investigations, is in agreement with literature reports on porcine thyroid (46), \textit{E. coli} (16), and human (47) Gmd proteins. Thus, expression and characterization of the rather stable fusion proteins, His-Gmd and particularly GST-Gmd, was an optimal way to enable synthesis of GDP-\textit{d}-rhamnose. The reason for the widespread instability of these enzymes is not known to date. Another serious problem of this reaction cascade is the instability of GDP-\textit{d}-deoxy-\textit{d}-\textit{lyxo}-4-hexulose. Recently, groups working with \textit{Perinereis cultrifera} and \textit{A. thaliana} have also observed instability of GDP-\textit{d}-deoxy-\textit{d}-\textit{lyxo}-4-hexulose (14, 15), and the same has been reported for dTDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose (29, 48). Because of the instability of dTDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose, this substrate was generated \textit{in situ} for kinetic analysis of dTDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose reductase (29). \textit{In situ} generation of GDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose for kinetic measurements of Rmd is not possible because of the bifunctionality of Gmd from \textit{A. thermoaerophilus} L420-91\textsuperscript{T}. Rapid decomposition of isolated GDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose results in decreasing substrate concentrations, and, therefore, initial reaction velocities are underestimated. In previous kinetic studies of GDP-fucose synthetase, when using this substrate, it was not stated, however, whether this problem was considered (49, 50).

The dehydratase Gmd is dependent on addition of NAD\textsuperscript{+}, at least for the His-tagged fusion protein. When working with the GST-tagged protein, the independence of any added cofactor might be due to tight binding of the cofactor to the protein, even during purification over GSTrap and MonoQ columns. NAD\textsuperscript{+} dependence of the Gmd protein was also reported for the enzymes of \textit{Klebsiella pneumoniae} (51), \textit{E. coli} (16), and \textit{H. sapiens} (47). This fact can be explained with a conserved Arg residue, binding the 2'-phosphate of NAD\textsuperscript{+} (21). In porcine thyroid NAD\textsuperscript{+} is proposed to play this role (46).

The conversion of GDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose to GDP-\textit{d}-rhamnose by Rmd proceeds quantitatively, because no reverse reaction has been detected. These results imply that Rmd is a reductase, which has also been shown for dTDP-\textit{d}-deoxy-\textit{l}-\textit{lyxo}-4-hexulose reductase (RmdL), the enzyme catalyzing the equivalent reduction step in the biosynthetic pathway of dTDP-\textit{l}-rhamnose (29). Upon isolation of GDP-\textit{d}-rhamnose chemical and NMR analyses have confirmed integrity of this product.

Gmd from \textit{A. thermoaerophilus} L420-91\textsuperscript{T} catalyzes both the dehydration and the reduction step. This is the first report of a

\begin{table}[h]
\centering
\caption{Assignment of the 1H and 13C NMR resonances of GDP-\textit{d}-rhamnose (29).}
\begin{tabular}{llcccc}
 & Atom & H/C/P & \textsuperscript{1}H (ppm) & \textsuperscript{13}C (ppm) & J (Hz) \\
\hline
 & \alpha-Rha-1\textsuperscript{a} & \textsuperscript{1}H & 5.42 & 97.41 & 174.9 \\
 & & \textsuperscript{13}C & 87.84 & 71.36 & 70.69 \\
 & & J\textsubscript{C,H} (Hz) & & 73.06 & 70.62 \\
 & & J\textsubscript{P,P} (Hz) & & 17.78 & 12.50 \\
 & \beta-Rib-1\textsuperscript{a} & \textsuperscript{1}H & 5.93 & 97.41 & 71.36 \\
 & & \textsuperscript{13}C & 87.84 & 71.36 & 70.69 \\
 & & J\textsubscript{C,H} (Hz) & & 73.06 & 70.62 \\
 & & J\textsubscript{P,P} (Hz) & & 17.78 & 12.50 \\
 & \alpha-Rha-1-P\textsuperscript{a} & \textsuperscript{31}P & -13.30 & -13.30 & -13.30 \\
 & & J (Hz) & & 20.60 & 7.60 \\
 & & J\textsubscript{P,P} (Hz) & & 5.80 & 5.80 \\
 & & J\textsubscript{C,P} (Hz) & & 4.60 & 3.90 \\
 & P-5-Rib & \textsuperscript{1}H & 5.13 & 97.41 & 71.36 \\
 & & \textsuperscript{13}C & 87.84 & 71.36 & 70.69 \\
 & & J\textsubscript{C,H} (Hz) & & 73.06 & 70.62 \\
 & & J\textsubscript{P,P} (Hz) & & 17.78 & 12.50 \\
 & Guanine & \textsuperscript{1}H & 6.00 & 160.08 & 154.99 \\
 & & \textsuperscript{13}C & 3.90 & 152.89 & 117.38 \\
 & & J (Hz) & & 8.11 & 138.77
\end{tabular}
\end{table}
bifunctional enzyme displaying both dehydratase and reductase activities. The enzyme has been purified to homogeneity, and bifunctionality cannot be attributed to contaminating activities, because E. coli itself does not produce GDP-D-rhamnose. A BLAST search, using E. coli sequences currently available in the NCBI data base, with the A. thermaoerobius Rmd protein, yielded Gmd sequences from several strains, but no Rmd sequence was identified. A possible explanation of the observed bifunctionality may be mutation events affecting the gmd gene. We assume that the investigated A. thermaoerobius strain has no disadvantages regarding growth, when the enzyme involved in dehydration of GDP-D-mannose also catalyzes the reduction of the intermediate product to form GDP-D-rhamnose. Nevertheless, Rmd is far more efficient in catalyzing the reduction reaction. With Gmd alone, it might be that insufficient amounts of GDP-D-rhamnose are produced for complete S-layer protein glycosylation.

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Identification of Two GDP-6-deoxy-d-lyxo-4-hexulose Reductases Synthesizing GDP-d-rhamnose in Aneurinibacillus thermoaerophilus L420-91

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