Structure and Function of Sedoheptulose-7-phosphate Isomerase, a Critical Enzyme for Lipopolysaccharide Biosynthesis and a Target for Antibiotic Adjuvants*§

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The barrier imposed by lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria presents a significant challenge in treatment of these organisms with otherwise effective hydrophobic antibiotics. The absence of L-\(\text{glycero-\(\_\)manno-}\)heptose in the LPS molecule is associated with a dramatically increased bacterial susceptibility to hydrophobic antibiotics and thus enzymes in the ADP-heptose biosynthesis pathway are of significant interest. GmhA catalyzes the isomerization of D-sedoheptulose 7-phosphate into D-glycero-D-manno-heptose 7-phosphate, the first committed step in the formation of ADP-heptose. Here we report structures of GmhA from Escherichia coli and Pseudomonas aeruginosa in apo, substrate, and product-bound forms, which together suggest that GmhA adopts two distinct conformations during isomerization through reorganization of quaternary structure. Biochemical characterization of GmhA mutants, combined with in vivo analysis of LPS biosynthesis and novobiocin susceptibility, identifies key catalytic residues. We postulate GmhA acts through an enediol-intermediate isomerase mechanism.

Lipopolysaccharide (LPS)§ is an essential component of the outer membrane in Gram-negative bacteria (1). LPS not only functions as a protective barrier preventing cell entry of hydrophobic molecules, including bile salts, detergents, and lipophilic antibiotics, but also helps maintain the structural integrity of the outer membrane. Thus, LPS is vital for bacterial virulence and antibiotic sensitivity in pathogenic Gram-negative bacteria.

Gram-negative pathogens are increasingly becoming a serious clinical threat. Multidrug-resistant hospital-acquired infections caused by enteric bacteria such as Escherichia coli and Klebsiella pneumoniae, and by emerging pathogens of environmental origin such as Acinetobacter baumannii and Pseudomonas aeruginosa, are the next big problem facing the infectious disease community. Furthermore, Gram-negative pathogens of animal origin such as E. coli O157–H7 are ongoing threats to agriculture and water quality. New chemotherapeutic strategies against Gram-negative bacteria are therefore required. LPS biosynthesis represents a unique Gram-negative target for new antimicrobial intervention.

LPS comprises lipid A, a core oligosaccharide, and in some bacteria, an O-specific polysaccharide chain. The core oligosaccharide has an inner core region consisting of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and one or more heptose units, and an outer core, consisting of additional sugar residues (Fig. 1A) (reviewed in Refs. 1–4).

Lipid A and Kdo are highly conserved in Gram-negative bacteria and essential for cell viability. The biosynthesis of these molecules is therefore a target for traditional antibiotic discovery efforts. Indeed, small molecule inhibitors of lipid A biosynthesis have been reported to have anti-Gram-negative activity (5).

Most Gram-negatives also contain one or more L-glycero-D-manno-heptose molecules attached to the Kdo. Mutants in

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4, Tables S1 and S2, and a movie.

The atomic coordinates and structure factors (code 212W and 3BJZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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heptose metabolism, which are viable in laboratory conditions, are avirulent and highly susceptible to antibiotics (reviewed in Ref. 6). Heptose biosynthesis is thus a non-traditional target for Gram-negative selective antimicrobial agents. Inhibitors of heptose biosynthesis could be used as anti-virulence drugs or could be co-administered with antibiotics that do not normally cross the outer membrane barrier (e.g. novobiocin and erythromycin) to sensitize bacteria to these agents. We have termed such molecules antibiotic adjuvants (7).

The outer core carbohydrates and the O-specific polysaccharide side chains, also known as O-antigens, comprise the remainder of the LPS polymer. These components vary significantly by organism (1). They are not essential for cell growth but do mediate host-microbe interactions and play a significant role in virulence. Inhibitors of outer core and O-antigen biosynthesis could, therefore, be strategically deployed as organism-specific anti-virulence compounds.

All levels of LPS biosynthesis represent underexploited targets for new anti-microbial agents. The heptose biosynthetic pathway in Gram-negative bacteria, in particular, is highly attractive being essential for virulence and antibiotic sensitivity. Heptoses targeted to the inner core LPS are synthesized within pathway in Gram-negative bacteria, in particular, is highly gets for new anti-microbial agents. The heptose biosynthetic specific anti-virulence compounds. The heptose biosynthesis could, therefore, be strategically deployed as organism-specific anti-virulence compounds.

FIGURE 1. LPS structure and activity of GmhA. A, general structure of LPS in Gram-negative bacteria. Kdo, 3-deoxy-D-manno-oct-2ulosonic acid; Hep, heptose; P, phosphate. B, schematic of the isomerase reaction catalyzed by GmhA, where D-sedoheptulose 7-phosphate is converted into D-glycero-D-manno-heptose 7-phosphate.

EXPERIMENTAL PROCEDURES

Purification of GmhA—Purification of E. coli GmhA followed a previously described protocol (19). An additional purification step was performed for GmhA protein used in crystallization and analytical ultracentrifugation studies. For these studies, GmhA was applied to a Q-Sepharose column (Amersham Bioscience) and eluted using a linear KCl gradient (GmhA buffer A: 20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT); GmhA Buffer B: 20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM DTT, 500 mM KCl). Fractions containing only GmhA were pooled and dialyzed extensively against 20 mM sodium HEPES pH 8.0 and 4 mM DTT.

P. aeruginosa GmhA was overexpressed in E. coli BL21-Gold (DE3) (Stratagene), harboring an extra plasmid encoding three rare tRNAs (AGG and AGA for Arg and ATA for Ile). Cells were grown in auto-inducible media (20) for 4–5 h at 37 °C and 12–15 h at 20 °C. Cells were sonicated in binding buffer (5 mM imidazole, 5% glycerol, 50 mM sodium HEPES, pH 7.5, 0.5 mM NaCl), supplemented with 1 mM phenylmethylsulfonyl fluoride and benzamidine and 0.5% IGEPA (Sigma). Clarified lysate was passed in series through DE52 and nickel-nitritol-triacetic acid (Qiagen) columns. GmhA was dialyzed in 10 mM sodium HEPES, pH 7.5, 0.5 mM NaCl, and concentrated using a BioMax concentrator (Millipore). Selenomethionine (SeMet)-enriched protein was produced according to a previously
described procedure (21, 22). Tris(2-carboxyethyl)phosphine (0.5 mM) was added to all purification buffers.

Structure Determination of GmhA—All GmhA crystals were grown at 20 °C using the hanging drop/vapor diffusion method. *E. coli* GmhA (10 mg/ml) was mixed with an equal volume of crystallization solution (3% (w/v) polyethylene glycol (PEG)-8000, 0.1 M imidazole, pH 7.3, and 3% (v/v) ethylene glycol) and dehydrated against 1.5 M (NH₄)₂SO₄. For crystallization of substrate-bound GmhA, S7P (see below) was added at a final concentration of 1 mM, and ethylene glycol was replaced with 1,6-hexanediol. Prior to flash freezing in liquid nitrogen, apo- and substrate-bound GmhA crystals were soaked (∼30–60 s) in a cryo-protecting solution (10 mM HEPES, pH 7.3, 2 mM DTT, 1.5% PEG-8000, 50 mM imidazole, 3% ethylene glycol, 30% glycerol; or 0.5 mM S7P, 15.45 mM HEPES, pH 7.3, 3.1 mM DTT, 2.31% PEG-8000, 77.27 mM imidazole, 4.67% 1,6-hexanediol, 30% glycerol, respectively). *P. aeruginosa* apo-GmhA SeMet crystals grew in a solution of 25% PEG-3350, 0.1 M ammonium sulfate, and 0.1 M Bis-Tris, pH 5.5, and were cryoprotected with a mixture of 8% glycerol, 8% ethylene glycol, and 8% sucrose. Product-bound GmhA crystallized in a solution of 2.5 mM S7P (Sigma), 2 M ammonium sulfate, 0.2 M potassium/sodium tartrate and 0.1 M sodium citrate, pH 5.6, and were cryoprotected with 25% ethylene glycol. All x-ray diffraction data sets were collected at 100 K. *E. coli* apo and substrate-bound GmhA data were collected with an R-AXIS IV image-plate detector mounted on an RU300 rotating-anode x-ray generator (Rigaku/MSC Ltd.). Data sets were processed and scaled using d*TREK (23). An initial search model for molecular replacement using MOLREP (24) was generated from *V. cholerae* GmhA (25), PDB code 1X94. Substrate-bound GmhA was solved by molecular replacement using the refined *E. coli* apo-GmhA structure as a search model. *P. aeruginosa* SeMet GmhA single wavelength anomalous diffraction data were collected at the 19ID beamline of the Structural Biology Center, Advanced Photon Source, Argonne National Laboratory, whereas product-bound GmhA data were collected at the 17ID beamline of the Industrial Macromolecular Crystallography Association Collaborative Access Team. These data were processed with HKL2000 (26). Using SOLVE (27), all 20 expected selenium sites in the asymmetric unit were located. Resolve (28) was then used to build an initial model. To determine the structure of product-bound *P. aeruginosa* GmhA, the structure of the SeMet *P. aeruginosa* GmhA was used as a search model. Model building and refinement for all GmhA structures were carried out using O (29), Coot (30), REFMAC5 (31), or CNS (32), until R values and model geometry statistics fell within acceptable ranges (Table 1, under “Results”). Surface area calculations were performed using POPSCOMP (33). Structural illustrations were generated using PyMOL Molecular Graphics System (DeLano Scientific).

GmhA Mutagenesis—Site mutations in *E. coli* gmhA were generated in both pET28a(+)gmhA and pBAD30gmhA inserts using the QuickChange site-directed mutagenesis protocol (Stratagene). Sequences of mutagenic oligonucleotide primers are described in supplemental Table S1. Mutations were verified by DNA sequence analysis (MOBIX, McMaster University) using vector-specific sequencing primers (supplemental Table S1).

Sedimentation Equilibrium—*E. coli* GmhA and GmhAD94N molecular weights in solution were determined by sedimentation equilibrium analysis using a Beckman-Coulter XL-1 analytical ultracentrifuge (Palo Alto, CA). Protein concentrations corresponding to 0.1, 0.2, and 0.4 A₂₈₀nm values, respectively, were loaded into a six-channel epon-charcoal cell with a 1.2-cm path length. Equilibrium was allowed to develop for 12–14 h at rotor speeds of 20,000 and 25,000 rpm. The reference solvent contained 20 mM HEPES, pH 8.0, 150 mM KCl, 5 mM DTT (ρ = 1.006 g/ml). Absorbance data were collected at 280 nm and analyzed using the Beckman-Coulter Optima XL-1 Analytical Ultracentrifuge Origin Data Analysis Package (version 60-4) and Microcoor Origin 6.0. GmhA partial specific volume (0.739 ml/g), and solvent densities were determined using SEDNTERP, a public domain program developed by Hayes, Laue, and Philo. Resulting gradients were then fit to a self-association model using the above software. Due to the poor absorption of GmhA, high protein concentrations were required for detection, prohibiting accurate K₅ₐ determination.

Sedoheptulose 7-Phosphate Synthesis—S7P was synthesized enzymatically from D-serine and ribose 5-phosphate based on the protocol by Lee and colleagues, with minor modifications (34). *E. coli* transketolase was purified as previously described (19). Porcine D-amino acid oxidase (gift of V. Massey) was purified from *E. coli* BL21(DE3)/pET28a(+)DAO cells by anion exchange using a Q-Sepharose column. Purified protein was analyzed using 12% SDS-PAGE, and activity was confirmed using a lactate dehydrogenase-coupled enzyme assay (35). D-amino acid oxidase was stored in the presence of 5 mM FAD. S7P synthesis and purity was determined using liquid chromatography/electrospray mass spectrometry and ¹H, ¹³C, and ³¹P NMR.

*E. coli* GmhA Steady-state Kinetic Analysis—GmhA activity was monitored by coupling product formation to HldE and GmhB and monitoring Pₐ release, as previously described (19) with the following modifications. The reaction mixture consisted of 20 mM HEPES, pH 8.0, 10 mM MgCl, 10 mM KCl, 6 mM ATP, 0.4% Tween 20, 0.214 nmol of GmhA, 0.375 nmol of GmhB, 0.094 nmol of HldE, and 0.2 unit of pyrophosphatase in a total volume of 90 μl. Reactions were initiated with 10 μl of S7P for final concentrations ranging from 0 to 2 mM. Initial rates were fit to Equation 1 describing Michaelis-Menten kinetics using Grafit 4 software (Erithacus Software, Staines, UK).

\[
\nu = k_{cat}[S]/(K_m + [S]) \quad (Eq. 1)
\]

GmhA in Vivo Complementation Studies—pBAD30gmhA wild-type and mutant vectors were used to transform *E. coli* BW25113ΔgmhA cells (36) to create complement strains. Positive and negative control strains were created by transforming the pBAD30 vector into *E. coli* BW25113 and *E. coli* BW25113ΔgmhA cells, respectively. Cells were cultured overnight at 37 °C, 250 rpm in M9 minimal media, 0.2% arabinose, 100 μg/ml ampicillin. To confirm GmhA expression, 1 ml of overnight culture was harvested, resuspended in 50 μl of 10 mM Tris, pH 7.5, 1 mM EDTA buffer, 50 μl of 2X SDS loading dye. Cells were lysed by boiling 30 min and analyzed by 15% SDS-PAGE. For immunoblot analysis, gel contents were transferred...
to a polyvinylidene fluoride membrane. GmhA was detected using mouse IgG anti-histidine primary antibody (Amersham Biosciences) and peroxidase-conjugated Affini-pure donkey anti-mouse IgG secondary antibody (Jackson Immuno-Research). PerkinElmer Life Sciences Western lighting chemiluminescence reagent was used in detection. Minimal inhibitory concentrations (MIC) of novobiocin were determined as follows: Overnight cultures, as described above, were diluted to $A_{600 \text{ nm}} 0.11$ and further diluted 1 in 200. Strains were grown at 37 °C in 96-well plates in the presence of varying concentrations of Me$_2$SO-dissolved novobiocin (2–10$^2$/10$^3$ g/ml). $A_{600 \text{ nm}}$ was measured after 20 h to assay growth. MIC was determined as the concentration of novobiocin required to reduce the $A_{600 \text{ nm}}$ of each strain to 90% of the $A_{600 \text{ nm}}$ in the absence of drug.

**RESULTS**

**Structure-function of GmhA**

GmhA was detected using mouse IgG anti-histidine primary antibody (Amersham Biosciences) and peroxidase-conjugated Affini-pure donkey anti-mouse IgG secondary antibody (Jackson Immuno-Research). PerkinElmer Life Sciences Western lighting chemiluminescence reagent was used in detection. Minimal inhibitory concentrations (MIC) of novobiocin were determined as follows: Overnight cultures, as described above, were diluted to $A_{600 \text{ nm}} 0.11$ and further diluted 1 in 200. Strains were grown at 37 °C in 96-well plates in the presence of varying concentrations of Me$_2$SO-dissolved novobiocin (2–10$^2$/10$^3$ g/ml). $A_{600 \text{ nm}}$ was measured after 20 h to assay growth. MIC was determined as the concentration of novobiocin required to reduce the

**LPS Analysis—E. coli BW25113 ΔgmhA/pBAD30gmhA** wild-type and mutant strains were grown at 37 °C for 24 h on M9 minimal media, 0.2% arabinose, 100 µg/ml ampicillin agar plates. LPS was extracted from these cells as previously described (37). LPS samples were analyzed by 10% SDS-PAGE in the Tricine buffer system and detected by silver staining (37, 38). Gels were fixed overnight in 250 ml of fixing solution (60% MeOH, 10% acetic acid). Gels were washed, in order, using 200 ml of 7.5% acetic acid for 30 min, 200 ml of 0.7% periodic acid for 30 min, milliQ H$_2$O for 3/11003 15 min, 200 ml of staining solution (42 ml of 0.36% NaOH, 2.8 ml of concentrated NH$_4$O H , 8 ml of 19.4% silver nitrate, 148 ml of H$_2$O) for 25 min, milliQ H$_2$O for 2 × 15 min, and 200 ml of developing solution (42 ml of 0.36% NaOH, 2.8 ml of concentrated NH$_4$O H , 8 ml

**FIGURE 2. Structure of the E. coli GmhA apoprotein.** A, GmhA monomer. β-Strand and α-helix in red and blue, respectively; B, GmhA tetramer. Dashed lines in subunits B and D represent disordered regions of GmhA not observed in the final model.

| Data collection | Apo$_{EC}$ | Sub-bound$_{EC}$ | Apo$_{PA}$ | Product-bound$_{PA}$ |
|-----------------|------------|------------------|------------|----------------------|
| Space group     | P2$_2$1,2$_1$ | P2$_1$         | P2$_2$,2 | P6$_2$,2 |
| Cell parameters | a,b,c (Å)   | 83.9, 89.6, 106.9 | 73.0, 76.5, 78.3 | 123.8, 131.6, 48.8 |
| β, γ (°)        | 90, 90, 90 | 90, 106.1, 90  | 90, 90, 90 | 90, 90, 120 |
| Molecules in ASU | 4          | 4                | 4          | 2 |
| Resolution (Å)  | 45.93-1.95 (2.02-1.95) | 47.39-2.79 (2.89-2.79) | 40.0-2.40 (2.46-2.40) | 40.0-2.30 (2.38-2.30) |
| Unique reflections | 59,294 | 20,533            | 30,927    | 24,669 |
| Redundancy (%)  | 4.27 (4.18) | 2.63 (2.68) | 7.6 (6.8) | 12.4 (12.6) |
| Completeness (%)| 99.8 (100.0) | 98.6 (99.4) | 99.9 (100.0) | 99.9 (100.0) |
| I/σ(I) (%)      | 10.9 (3.4)  | 6.7 (2.6)       | 29.1 (4.2) | 25.0 (6.2) |
| Rmerge (%)      | 7.5 (39.8)  | 11.1 (36.0)     | 7.0 (40.0) | 12.5 (40.4) |

* Statistics for the highest resolution shell are shown in parentheses.

**TABLE 1**

GmhA data collection and model refinement statistics

**FIGURE 2. Structure of the E. coli GmhA apoprotein.** A, GmhA monomer. β-Strand and α-helix in red and blue, respectively; B, GmhA tetramer. Dashed lines in subunits B and D represent disordered regions of GmhA not observed in the final model.

**TABLE 1**

GmhA data collection and model refinement statistics
space group \(P2_12_12_1\) with four molecules of GmhA in each asymmetric unit as shown in Fig. 2 (subunits A, B, C, and D). Analysis of oligomerization using the program PISA (39) strongly suggested that GmhA would exist in solution as a tetramer. This was further verified by sedimentation equilibrium studies (supplemental Fig. S1). No electron density was observed for residues 83–97 in chains B or D, and therefore these regions are represented as dotted lines in Fig. 2. Three intersubunit Cys–Cys disulfide linkages were observed in the asymmetric unit, one at Cys-90 linking chain A–C, and two others linking Chains A–B and C–D at Cys-57, respectively. These linkages are likely artifacts, because addition of a reducing agent or substitution of Cys to Ser resulted in increased GmhA activity (discussed below). The A–D and B–C dimer interfaces are extensive (2680 Å²), each resulting from reciprocal interactions between helices H1, H3, and H6. A–B and C–D interfaces are less extensive (1515 Å²) and are formed primarily through H4 and reciprocal interactions with loop regions joining H3–β2 or β2–H4. The final model was refined to \(R\) and \(R_{free}\) values of 19.2 and 22.4, respectively. Apo-GmhA from \(P.\ aeruginosa\) was crystallized in space group \(P2_12_12_1\), and the structure was determined to 2.4 Å using SeMet-substituted protein and single-wavelength anomalous diffraction. The final model was refined to \(R\) and \(R_{free}\) values of 19.7 and 27.4, respectively. As with the apo-GmhA structure from \(E.\ coli\), a tetramer of GmhA was observed in the asymmetric unit and residues 83–96 in each chain were disordered. The Ca traces of these monomers could be superimposed with an r.m.s.d. of 1.1 Å (supplemental Fig. S2).

Each GmhA monomer consists of a central five-stranded parallel β-sheet, flanked by five alpha helices (Fig. 2A), forming a three-layered HβH sandwich. Helical layers are composed of H2, H3, and H6 on one side and H4 and H5 on the opposing side of the central β-sheet with topology β2, 1, 3, 4, and 5. The overall fold is quite similar to the flavodoxin-type nucleotide-binding motif and is essentially identical to GmhA structures from \(V.\ cholerae\) (PDB 1X94) and \(Campylobacter jejuni\) (PDB 1TK9) (25).

In addition to apo-structures of GmhA, we also determined structures of GmhA in the presence of substrate and product. The structure of \(E.\ coli\) GmhA in complex with S7P was determined to 2.79 Å (PDB 2I22). This complex crystallized in a different space group \(P2_1_2\) compared with apo-protein. The final model was refined to \(R\) and \(R_{free}\) values of 20.3 and 25.7, respectively. The major difference observed between the apoprotein and substrate-bound complex, aside from the presence of S7P, centers on the loop connecting β2 and H4, which becomes disordered in the presence of substrate (Fig. 3A). Because wild-type GmhA isomerase was used to generate these crystals, and crystals took several days to grow, a mixture of product and substrate is expected to have been present during crystal formation. Clear additional electron density was observed at only one of the four potential active sites within the GmhA tetramer. As shown in Fig. 4A, this density is consistent with the presence of substrate; however, given the relatively low resolution to which this structure was determined, further structural and functional analysis was required to fully characterize the active site of GmhA.

The product-bound structure of GmhA from \(P.\ aeruginosa\) was determined in space group \(P6_5_22\) to 2.3 Å (PDB 1X92). These crystals were generated following incubation of GmhA with substrate (see “Experimental Procedures”). In this case, clear electron density corresponding to product was observed in each active site region (Fig. 4B). In contrast to the apo and substrate-bound structures of \(E.\ coli\) GmhA, the product-bound protein crystallized as a dimer in the asymmetric unit (Fig. 3A). However, by combining two dimers from crystallographic related symmetry mates, a tetramer could be generated (Fig. 3B).

Because wild-type GmhA was used in all of these studies, it would appear that crystallization conditions (as opposed to an inactive GmhA) were responsible for selecting distinct conformations of GmhA capable of binding either substrate or product.

To date, six structures of GmhA have been determined: apo and substrate-bound \(E.\ coli\) GmhA, apo and product-bound \(P.\ aeruginosa\) GmhA, \(V.\ cholerae\) GmhA, and \(C.\ jejuni\) GmhA. As shown in Fig. 5, these structures can be categorized into two distinct conformations, designated “open” and “closed.” The \(E.\ coli\) structures as well as the apo \(P.\ aeruginosa\) and the \(V.\ cholerae\) structures adopt an open conformation, whereas the \(P.\ aeruginosa\) and \(C.\ jejuni\) exist in the closed state. Three major differences between the open and closed conformations are apparent. First, a new helix \(H3'\) (in the product-bound structure is present in place of the disordered loop located between β2–H4 in the apo and substrate-bound structures (Fig. 3A, purple arrow). A second difference is the overall positioning of the loop joining H3 and β2. In the closed state this loop is rotated inward toward the opposing subunit by ~20 Å relative to the open conformation (Fig. 3A, red arrow), with the exception of apo \(P.\ aeruginosa\), where the H3–β2 loop is in line with the closed conformation rather than the open. Finally, compared with the open conformation structures, the tetramer formed in the closed conformation structures is more compact and bury substantially more dimer–dimer surface area (2500 versus 1250 Å²) due to the packing of \(H3'\). Fig. 3 (B and C) illustrates the difference between these two tetrameric forms, highlighting a large reorganization of the dimer–dimer interface. Bringing dimers of product-bound GmhA together involves a corkscrew-like movement with concerted translational (5 Å) and rotational (25°) movements between A–D and B–C dimers (see the supplemental movie illustrating the structural transition between open and closed conformations, as illustrated in Fig. 3B). As shown in Fig. 3D, the formation of \(H3'\) in the closed conformation of GmhA is responsible for repositioning the H3–β2 loop due to steric hindrance.

Both substrate and product are found at the interface formed between subunits A and D. As discussed above, only one of the four active sites within GmhA contained substrate, whereas the structure of product-bound GmhA contained fully occupied active sites. In the S7P-bound structure numerous contacts were observed between substrate and the following amino acid
side chains (supplemental Table S2): Ser-55, Thr-120, Asp-169, and Gln-172 of chain D, and His-61, Glu-65, and His-180 of chain A. In general, the active sites observed for both the substrate and product-bound structures are comparable (Fig. 4). Several residues from both structures remain unchanged, in particular: Ser-55 (Ser-54), Ser-119, Thr-120, Ser-121, Ser-124, and His-180 (His-182) (residues in parentheses correspond to product-bound P. aeruginosa GmhA). Although not perfectly superimposible, side chains from residues Glu-65 (Glu-64) and Gln-172 (Gln-174) did not differ significantly in their overall position between the two structures. In contrast, residues His-61 (His-60) and Arg-69 (Arg-68) adopt different positions largely due to the dramatic change of position in the H3–H9 loop. The most striking difference, however, occurs in the product-bound form, by additional contacts made with residues Asn-93 and Asp-94 of chain B (Fig. 4B). The finding that residues from three chains (A, B, and D) are involved in binding product suggests that assembly of a GmhA tetramer may be required for function.

Rationale for Site-directed Mutagenesis—Potential GmhA active site residues were chosen for analysis based on crystallographic active site data of both the substrate-bound E. coli enzyme and product-bound P. aeruginosa enzyme (Fig. 4). A total of eight residues was selected for analysis and is described by E. coli residue number. Equivalent residues of Glu-65, Thr-120, and Gln-172 show direct contact in both substrate and product bound crystal structures. The highly conserved Ser-55, Ser-119, Thr-120, Ser-121, and Ser-124 pocket was hypothesized to bind the S7P phosphate, rather than play a direct role in catalysis. As such, only one residue from this pocket, Thr-120, was chosen for mutagenesis. His-61, Asp-169, and His-180 contacts are unique to the E. coli substrate-bound structure, whereas Arg-69 and
Asp-94 contacts are unique to the *P. aeruginosa* product-bound structure. As shown in Fig. 6, all mutated residues are conserved across Gram-negative species, with the exception of Asp-169. Asp-169 was examined even without conservation due to its prominent position depicted in the *E. coli* active site.

**In Vitro Mutational Analysis**—The ability of purified *E. coli* GmhA wild-type and mutant proteins to convert enzymatically synthesized S7P into product was assessed using a coupled assay monitoring *P*₁ release. Initial studies, using wild-type GmhA, determined that synthesized S7P was indeed a substrate of GmhA, and that the reaction was linear for at least 10 min in the presence of 0.214 nmol of protein. Purified mutant GmhA proteins were then assayed for activity against S7P (Table 2). Of the eight mutants tested, only H61Q and R69Q demonstrated measurable *in vitro* activity. R69Q turnover (0.45 ± 0.1 s⁻¹) was equal to that of wild-type GmhA (0.44 ± 0.07 s⁻¹), whereas the turnover of H61Q (0.23 ± 0.07 s⁻¹) was roughly half that of wild type. The remaining GmhA mutants, E65N/Q, D94N, T120A, D169N, Q172E, and H180Q, showed undetectable *in vitro* activity (limit of detection, 0.003 s⁻¹ at 2 mM S7P).

**In Vivo Mutational Analysis**—To further explore the role of each residue in the active site, *in vivo* complementation studies were performed using *E. coli* BW25113 Δ*gmhA/pBAD30gmhA* wild-type and mutant-expressing strains. A positive control strain, *E. coli* BW25113/pBAD30 (wild-type plus vector only), and negative control strain, *E. coli* BW25113Δ*gmhA/pBAD30 (gmhA deletion plus vector only), were also generated. Equivalent amounts of GmhA expression, and therefore complementation to the chromosomal deletion, were confirmed in each mutant strain by anti-histidine immunoblot (supplemental Fig. S3).

The growth of each *gmhA*-expressing strain was analyzed to ensure the overexpression of *gmhA* did not have adverse effects. For the first 20 h, growth of all strains was consistent, as measured by *A*₁₆₀₅₀ nm. After 20-h growth, the *A*₁₆₀₅₀ nm of E65N-, E65Q-, Q172E-, H180Q-, and D94N-expressing strains, as well as the negative control strain, reached a maximum of ~0.6, and actually began to decrease with time. Conversely, the remaining strains continued to increase in *A*₁₆₀₅₀ nm after 18 h growth. By 48 h, however, the *A*₁₆₀₅₀ nm of all strains reached a consistent...
level, suggesting the presence or absence of GmhA, whether present in basal or overexpressed levels, has little effect on the growth of *E. coli* cells.

The susceptibility of these mutant gmhA complemented strains to the antibiotic novobiocin was then examined (Fig. 7A). Under normal wild-type conditions with an intact LPS, *E. coli* is insensitive to novobiocin. In contrast, *E. coli* exhibiting a deep-rough phenotype, where only lipid A and Kdo are synthesized, display increased sensitivity to novobiocin (40). This property was confirmed in the control strains, with the positive control exhibiting a novobiocin MIC of 1024 µg/ml, compared with an MIC of 64 µg/ml determined for the negative control (gmhA deletion, heptoseless) strain. The H61Q, R69Q, T120A, and D169N complement strains were able to completely

FIGURE 6. Sequence alignment of GmhA from various bacterial pathogens. Boxed residues are completely conserved between organisms. Colors indicate properties of conserved residues: Yellow, partial conservation; red, acidic; dark blue, basic; pale blue, Gly/Pro; pink, Asn/Gln; green, His; brown, Cys; and purple, Ser/Thr.

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TABLE 2
Summary of kinetic parameters of wild type and mutant E. coli GmhA purified proteins

GmhA activity was determined using the malachite green phosphate detection assay by coupling product formation to HldE and GmhB and monitoring the release of inorganic phosphate spectrophotometrically at 660 nm. Reactions were initiated with S7P to final concentrations of 0–2 mM. A detection limit of 0.003 s⁻¹ was determined for this assay.

| GmhA mutant | kcat | km | kcat/km |
|-------------|------|----|---------|
| Wild type   | 0.44 ± 0.7 | 0.9 ± 0.3 | 0.5 |
| H61Q        | <0.003 | 1.2 ± 0.7 | 0.2 |
| E65N        | <0.003 | 0.45 ± 0.1 | 0.9 |
| E65Q        | <0.003 | 0.5 ± 0.3 | 0.9 |
| R69Q        | <0.003 | 0.003 | 0.003 |
| D94N        | <0.003 | 0.003 | 0.003 |
| T120A       | <0.003 | 0.003 | 0.003 |
| D169N       | <0.003 | 0.003 | 0.003 |
| H180Q       | <0.003 | 0.003 | 0.003 |

FIGURE 7. Novobiocin MIC and LPS analysis of GmhA mutants. A, minimum concentration of novobiocin required to inhibit the growth of E. coli BW25113:pBAD30 (+ve control), BW25113ΔgmhA pBAD30 (-ve control), and BW25113ΔgmhA pBAD30ΔgmhA mutant strains as described, to 90% of standard growth in the absence of drug (MIC). Strains were grown in M9 minimal media supplemented with 0.2% arabinose and 0–1024 μg/ml novobiocin. B, LPS analysis of E. coli BW25113ΔgmhA pBAD30ΔgmhA mutants by silver-stained 10% SDS-PAGE. LPS was extracted from cultures of the above strains, grown overnight in M9 minimal media plus 0.2% arabinose.

The open form is characterized by an extended H3–H2 loop region, and a less well packed dimer-dimer interface. In the closed form, H3–H2 loop inward toward the active site cleft by ~20 Å but also permits more extensive dimer-dimer interactions resulting in a more compact tetramer. The open conformation is observed in structures of GmhA in apo and substrate-bound forms from E. coli and also in the apo structures from P. aeruginosa and V. cholerae. The closed conformation is observed in the apo and product-bound structures from C. jejuni and P. aeruginosa, respectively. The fact that only two conformations are observed despite structures having been determined in different space groups, from multiple organisms, and in three different states of ligand binding, suggests that GmhA is likely to exist in two distinct conformations. The open and closed conformations from full-length LPS based on relative migration distance, because truncated LPS migrates much faster through the gel. As depicted in Fig. 7B, H61Q, R69Q, T120A, and D169N GmhA replacements did not affect LPS production, because the gmhA-deleted strains containing these proteins produce full-length LPS. The remaining five GmhA constructs (E65N/Q, D94N, Q172E, and H180Q) resulted in at least partially truncated LPS. The complete truncation due to both Glu-65 mutant-expressing strains was consistent with MIC data, suggesting substantially reduced GmhA activity. Surprising, however, was the partial truncation due to Q172E and H180Q mutations. GmhA containing these mutations appear to be at least partially active in vivo. The partial truncation of D94N is also somewhat contradictory to MIC results, although the D94N-expressing strain demonstrated decreased novobiocin MIC compared with wild type. Based on these data, it appears that complete LPS truncation is not required to sensitize E. coli to novobiocin. Rather, LPS can be partially truncated, as observed in the D94N mutant without compromising the membrane to a threshold limit, beyond which the permeability barrier is breached, as with the Q172E and H180Q mutants.

DISCUSSION

Structural Analysis of GmhA—We have determined the three-dimensional structures of GmhA from E. coli and the opportunistic bacterial pathogen P. aeruginosa, in apo, substrate- and product-bound forms. These structures complement available structures from C. jejuni and V. cholerae that have recently emerged from structural genomics studies (25). The E. coli and C. jejuni proteins crystallize as tetramers in the asymmetric unit, whereas the P. aeruginosa and V. cholerae proteins crystallize as dimers; as is often the case, the contents of the crystallographic asymmetric unit do not necessarily reflect biological units of activity. Analytical ultracentrifugation studies performed in this work further suggest that the biologically active oligomeric state of GmhA is a tetramer. This interpretation is also supported by the extensive total surface areas buried in tetramer formation for apo (8400 Å²), substrate (7550 Å²), and product-bound (10380 Å²) structures.

Of particular note when comparing the available structures of GmhA, is that all six available structures can be classified into either of two very distinct forms: an open and a closed form. The open form is characterized by an extended H3–β2 loop, an unstructured H3’ region, and a less well packed dimer-dimer interface. In the closed form the H3’ region adopts a helical structure that in turn causes not only repositioning of the H3–β2 loop inward toward the active site cleft by ~20 Å but also permits more extensive dimer-dimer interactions resulting in a more compact tetramer. The open conformation is observed in structures of GmhA in apo and substrate-bound forms from E. coli and also in the apo structures from P. aeruginosa and V. cholerae. The closed conformation is observed in the apo and product-bound structures from C. jejuni and P. aeruginosa, respectively. The fact that only two conformations are observed despite structures having been determined in different space groups, from multiple organisms, and in three different states of ligand binding, suggests that GmhA is likely to exist in two distinct conformations. The open and closed conforma-
Structure-function of GmhA

FIGURE 8. Proposed mechanism of GmhA. The GmhA catalyzed conversion of D-sedoheptulose 7-phosphate into D-glycero-D-manno-heptose 7-phosphate is predicted to proceed through an enediol intermediate, where Glu-65 serves as the catalytic base and His-180 serves as the catalytic acid.

Determinations represent structures most suited for binding substrate and product, respectively. GmhA is an isomerase and should be able to readily catalyze both forward and reverse reactions, suggesting that both S7P and D-glycero-D-manno-heptose 7-phosphate are “substrates” of GmhA. With this in mind, it is not surprising that the structure of C. jejuni GmhA crystallized in the product bound, closed conformation even with no ligand bound.

Determination of GmhA Mechanism of Action—Results from both in vivo and in vitro analysis of GmhA mutational studies suggest the importance of residues Glu-65, Gln-172, and His-180 in enzyme activity. Amino acid substitutions at Glu-65, Asp-94, Thr-120, Asp-169, Gln-172, and His-180 residues result in no observable GmhA activity when analyzed in vitro using the phosphate detection assay. Diminished activity of these mutants was expected, because these residues are all located within the active site. However, it was difficult to elucidate the actual role of these residues in GmhA action from kinetic analysis alone. For this reason, in vivo studies were performed. Both in vitro and in vivo studies suggest that His-61 and Arg-69 play no significant role in GmhA activity, because wild-type activity was maintained when each was mutated. Conversely, Glu-65, Gln-172, and His-180 were deemed necessary in maintaining the LPS permeability barrier as illustrated in novobiocin MIC studies and supported by LPS analysis, suggesting these residues are important to GmhA function. The remaining mutated residues, Asp-94, Thr-120, and Asp-169, did not alter the permeability of E. coli to novobiocin. This does not imply, however, that Asp-94, Thr-120, and Asp-169 have no role in activity. There are a number of reasons why these mutations show diminished activity in vitro and not in vivo, specifically the nature of each assay. Kinetic studies were designed to assay the reaction over a limited time period. Furthermore, mutant proteins were overexpressed in vivo, which could help to restore protein activity based on the quantity of protein in the cell. With this consideration in mind, overexpression can be advantageous in demonstrating inactivity, as even with an abundance of protein, the activity of Glu-65, Gln-172, and His-180 could not be completely, or in the case of Glu-65, even partially restored. The role of Thr-120 was hypothesized to be involved in substrate-phosphate binding, as previously predicted by analogy to glucose-6-phosphate isomerase (6). It is quite possible that the role of Thr-120 could be partially compensated by surrounding residues, in particular Ser-55, Ser-119, Ser-121, and Ser-124, which are also predicted to function in phosphate binding. The Asp-94 residue was not observed in the active site of the substrate-bound structure; rather, this mutation was designed based on contacts observed in the product-bound structure, between GmhA-D94 and bound product. Asp-94 is unique among residues mutated within the active site and is of particular interest because it is donated from the opposing dimer (chain B). Other residues contributing to substrate or product binding originate from the A–D dimer. Sedimentation equilibrium analysis of both the wild-type GmhA and GmhA-D94N mutant indicate that the E. coli protein exists as a tetramer in solution. Structural and mutational data also support a tetrameric GmhA, because the E. coli protein crystallized as a tetramer in the asymmetric unit and mutation of Asp-94 shows at least partial inhibition of GmhA activity. Interestingly, Thr-120 (conserved phosphate binding pocket) and Asp-94 (H3 region) belong to regions of GmhA primarily involved in product binding. Results from mutational studies are therefore consistent with the possibility that product stabilization is an important mechanism used by GmhA to establish desired isomerization reaction kinetics. Given that Glu-65 and His-180 were identified as the most critical residues for GmhA activity, a mechanism of action of GmhA can be proposed by analogy to other known aldo-keto isomerases, with Glu-65 and His-180 acting as the catalytic residues. Histidine residues are often found involved in isomerase activity, acting most frequently as a catalytic base, facilitating the reaction through proton shuffling (41, 42). Specifically, the active site of the isomerase domain of glucosamine 6-phosphate (Gln6P) synthase has been shown to rely on His and Glu residues (43, 44). This enzyme shares the greatest structural similarity to GmhA among currently characterized isomerases (25). A structural comparison of the quaternary and active site structures of these two isomerase enzymes is provided in supplemental Fig. S4. As expected, a collection of four serine and threonine residues forms a structurally conserved phosphate-binding pocket. The catalytic Glu-65 residue identified in E. coli GmhA is also structurally conserved. Further similarity exists for His-180, which is replaced by a lysine (Lys-485) in Gln6P synthase, where the catalytic lysine (Lys-603) of Gln6P synthase has no homologous residue in GmhA. The catalytic histidine (His-504) of Gln6P is not conserved structurally; however, it does reside close to Asp-94 within GmhA. Unlike Gln6P synthase, though, mutational analysis does not suggest that GmhA requires a third residue for activity, as fulfilled by His-504 in Gln6P synthase, suggesting the S7P sugar ring opens non-enzymatically prior to catalysis. Interestingly, although Gln6P synthase exists as a dimer, each monomer contains two structurally
related domains (supplemental Fig. S4A). Each of these domains is homologous to a single monomer of GmhA. Thus, similar to GmhA, the isomerase portion of Gln6P synthase adopts an overall quaternary structure comprised of four structurally equivalent domains. This finding provides additional support for the biological significance of a GmhA tetramer.

A potential mechanism of action for GmhA, based on previous studies of the isomerase domain from Gln6P synthase, is outlined in Fig. 8. This mechanism proposes that either Glu-65 or His-180 could act as the catalytic base, abstracting a proton from C2 of the S7P substrate, while the other residue would act as a catalytic acid, donating a proton to C1 for stabilization. The reaction then proceeds through the resulting cis-enediol intermediate resulting in an aldo form, which then cycles non-enzymatically to give the final product, D-glycero-α,β-d-manno-heptose 7-phosphate. Although it is difficult to predict which residue, Glu-65 or His-180, performs each catalytic role, based on the complete absence of any activity when Glu-65 is mutated, it can be hypothesized that Glu-65 assumes the critical role of the catalytic base in this mechanism.

Together the crystallographic and mutational studies presented here offer new insight into the structure-function relationship of GmhA, an essential protein in maintaining the permeability barrier of Gram-negative bacteria. GmhA is highly conserved between pathogenic species, both in sequence and structure. As such, knowledge gained from the current studies of GmhA from E. coli and P. aeruginosa should be readily transferable to other pathogenic Gram-negative species. Inhibition of GmhA, in synergy with known Gram-positive antimicrobial agents, may aid in treatment of Gram-negative infection. An understanding of the structure and mechanism of GmhA are the first steps in exploiting the heptose biosynthetic pathway as a novel Gram-negative antimicrobial target.

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