FlaA1 from the human pathogen *Helicobacter pylori* is an enzyme involved in saccharide biosynthesis that has been shown to be essential for pathogenicity. Here we present five crystal structures of FlaA1 in the presence of substrate, inhibitors, and bound cofactor, with resolutions ranging from 2.8 to 1.9 Å. These structures reveal that the enzyme is a novel member of the short-chain dehydrogenase/reductase superfamily. Additional electron microscopy studies show the enzyme to possess a hexameric doughnut-shaped quaternary structure. NMR analyses of “real time” enzyme-substrate reactions indicate that FlaA1 is a UDP-GlcNAc-inverting 4,6-dehydratase, suggesting that the enzyme catalyzes the first step in the biosynthetic pathway of a pseudaminic acid derivative, which is implicated in protein glycosylation. Guided by evidence from site-directed mutagenesis and computational simulations, a three-step reaction mechanism is proposed that involves Lys-133 functioning as both a catalytic acid and base.

*Helicobacter pylori* is a spiral shaped, motile, microaerophilic Gram-negative bacterium that resides in the gastric mucus layer or adheres to the epithelial lining of the stomach (1). It has been estimated that half to two-thirds of the world’s population is chronically infected with *H. pylori*, and prevalence among adults in developing countries is typically 80–90% (2). The bacterium can asymptptomatically remain in the human stomach for decades; however, infections can lead to gastric inflammation and ulceration. In addition to its well established etiological role in several gastroduodenal diseases, *H. pylori* infection has also been implicated in the development of gastric cancer (3, 4). The significance of the discovery that *H. pylori* plays a causal role in gastritis and ulceration was recently recognized through the awarding of the 2005 Nobel Prize in Physiology or Medicine to Drs. Marshall and Warren.

The pathogenicity of *H. pylori* can be attributed to numerous virulence factors that allow for host colonization, among these are urease, adhesins, flagella, and lipopolysaccharides (LPS) (5–7). For example, the flagella enable *H. pylori* to penetrate the gastric mucus layer and reach the epithelial cells, and LPS O antigens employ mimicry to Lewis blood group antigens to facilitate subsequent cell adhesion and colonization (8). Because of drug resistance and an increase in treatment failure, the biochemical pathways responsible for the synthesis and delivery of these virulence factors have received much attention as they contain potential targets for drug development (9, 10).

The flaA1 (HP0840) gene product has been shown to be involved in the synthesis of both flagella and LPS, and as such it plays a critical role in *H. pylori* pathogenesis and colonization. Specifically, disruption of the flaA1 gene results in bacteria devoid of flagella and with altered LPS that lacks most of the O antigen (9, 10). FlaA1 is a 37-kDa protein whose sequence suggests that it is a member of the short-chain dehydrogenase/reductase (SDR) superfamily. Several biochemical studies have shown that the substrate of FlaA1 is UDP-linked N-acetylgalcosamine (UDP-GlcNAc; UDP-2-acetamido-2-deoxy-α-D-glucose). However, these same studies present conflicting data on the reaction product produced by FlaA1, hence characterizing the enzyme as a 4,6-dehydratase (11), a 4,6-dehydratase-4-epimerase (10), or a 4,6-dehydratase-5-epimerase (12). Unfortunately, the sequence of FlaA1, even given the structural conservation within the SDR superfamily, provides no indications on the nature of the catalyzed reaction. In fact, it is unclear for any of the three suggested catalytic functions what a possible reaction mechanism could be. The resulting confusion impacts the identification of the pathway in which the enzyme

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1 Recipient of a studentship from the Canadian Cystic Fibrosis Foundation.

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3 Recipient of a Doctoral Research Award from the Canadian Institute of Health Research.

4 Holds Canada Research Chair in Cystic Fibrosis and Microbial Glycobiology.

5 Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

6 The abbreviations used are: LPS, lipopolysaccharide; SDR, short-chain dehydrogenase/reductase; MES, 2-morpholineethanesulfonic acid; Pse, pseudaminic acid.
Structure and Mechanism of Inverting 4,6-Dehydratase FlaA1

Table 1: Data collection and refinement statistics

| Coenzyme   | NADPH | NADP⁺ | NADP⁺ | NADP⁺ | NADP⁺ |
|------------|-------|-------|-------|-------|-------|
| Substrate  | UDP-GlcNAc | UDP-GlcNAc | UDP | UDP-Glc | UDP-Gal |
| Space group| P6₃ | P6₃ | P6₁ | P6₁ | P6₁ |
| Cell dimensions a (Å), b (Å), c (Å) | 111.4, 110.8, 109.8 | 111.4, 110.8, 109.8 | 111.1, 107.8 | 111.1, 107.8 | 112.4, 107.3 |
| Wavelength (Å) | 1.100 | 1.100 | 0.9803 | 0.9795 | 1.100 |
| Resolution (Å) | 50-1.9 | 50-2.7 | 50-2.1 | 50-2.8 | 50-2.6 |
| Completeness (%) | 95.7 (80.5) | 97.6 (93.3) | 97.9 (93.2) | 96.8 (84.6) | 97.5 (92.0) |
| Rmerge(I) (%) | 4.9 (42.2) | 9.5 (29.5) | 6.9 (40.9) | 8.2 (26.4) | 8.3 (40.5) |
| I/σI | 16.0 (2.0) | 8.8 (2.8) | 11.3 (1.9) | 8.7 (3.3) | 10.0 (2.2) |
| Redundancy | 7.4 (4.8) | 4.4 (3.3) | 8.5 (7.5) | 6.5 (5.6) | 6.4 (6.4) |
| No. of reflections | 57,284 | 19,888 | 43,145 | 18,312 | 23,091 |
| No. of atoms/mean R-factor (%) | 19.7/22.9 | 20.4/26.9 | 20.9/24.5 | 22.0/30.7 | 19.6/25.0 |
| Resolution (Å) | 50-1.9 | 50-2.7 | 50-2.1 | 50-2.8 | 50-2.6 |
| Wavelength (Å) | 1.100 | 0.9803 | 1.100 | 0.9795 | 1.100 |
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| Resolution (Å) | 50-1.9 | 50-2.7 | 50-2.1 | 50-2.8 | 50-2.6 |
| Wavelength (Å) | 1.100 | 0.9803 | 1.100 | 0.9795 | 1.100 |
| Resolution (Å) | 50-1.9 | 50-2.7 | 50-2.1 | 50-2.8 | 50-2.6 |
| Completeness (%) | 95.7 (80.5) | 97.6 (93.3) | 97.9 (93.2) | 96.8 (84.6) | 97.5 (92.0) |
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| No. of reflections | 57,284 | 19,888 | 43,145 | 18,312 | 23,091 |
| No. of atoms/mean R-factor (%) | 19.7/22.9 | 20.4/26.9 | 20.9/24.5 | 22.0/30.7 | 19.6/25.0 |

* * *

**Electron Microscopy**—Purified FlaA1 was adsorbed to a carbon-coated Formvar film on a 400-mesh copper electron microscope grid, washed with double-distilled water, negatively stained with NANO-W™ (Nanoprobes, Yaphank, NY), blotted, and allowed to air-dry. Electron micrographs were taken on a Philips CM10 transmission electron microscope under low dose conditions at a nominal magnification of ×73,000 and recorded on Kodak Electron Microscope Film 4489. Micrographs were subsequently digitized at 4800 × 2400 dpi and analyzed using IMAGIC-V electron image processing software (18). Briefly, 592 smaller images comprising a single, defined FlaA1 complex were selected from micrographs and subjected to standard “single particle analysis” to define reproducible projection views (19).

**Site-directed Mutagenesis and Activity Assay**—Construction of mutants, protein production, and activity assays for FlaA1 K133E and K133M variants followed procedures described previously (20). Circular dichroism spectroscopy, employing a Jasco J-600 spectropolarimeter, was used to assess that mutations did not affect protein folding. For activity assays the production of product was measured by capillary electrophoresis analysis as described previously (11).

**Nuclear Magnetic Resonance Spectroscopy**—As the product of FlaA1 may be labile, the FlaA1 reaction was monitored with NMR, and the reaction product was identified directly in aqueous reaction buffer without purification. FlaA1 protein (90 µg) was suspended in 200 µL of 90% H₂O, 10% D₂O sodium phosphate buffer (25 mM NaPO₄, 100 mM NaCl, pH 7.2) and placed in a 3-mL[sacp]m NMR tube. To begin the reaction, 5 mM UDP-GlcNAc was added. The enzymatic reaction was monitored using ¹H NMR analysis (25 °C) at 500 MHz with a Varian Inova spectrometer (Varian, Palo Alto) equipped with a Varian Z-gradient 3-mL[sacp]m triple resonance (¹H, 13C, 31P) probe. The reaction was stopped after 3 h when the concentrations of starting material and product were equal by removing the FlaA1 protein. The structure of the product was then examined using standard homo- and heteronuclear correlated NMR pulse sequences (from Varian) and selective one-dimensional
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The three-dimensional structure of FlaA1 is bilobal in shape (Fig. 1A). The larger, predominantly N-terminal, lobe consists of residues 1–174, 208–234, and 265–317 and contains a 10-stranded mostly parallel β-sheet flanked on either side by eight helices. The smaller, predominantly C-terminal, lobe is composed of residues 175–207, 235–264, and 318–333 and possesses three α-helices and two short two-stranded β-sheets. There are a total of five crossover points between the two lobes, resulting in an intertwined connectivity. The fold of FlaA1 confirms that this enzyme is a member of the SDR superfamily. However, FlaA1 is unique in that within the larger lobe the six-stranded parallel β-sheet of the Rossmann fold has been extended by four additional strands.

Biophysical studies for FlaA1 have suggested that under physiological conditions the enzyme is organized in a higher order structure; gel filtration experiments suggest that the enzyme is a dimer (11), whereas dynamic light scattering studies of purified samples predict at least a tetrameric arrangement (data not shown). Analysis of crystal packing interactions also suggests that FlaA1 does not exist as a monomer under physiological conditions. The FlaA1 protomer possesses two extended buried interfaces within the crystal lattice, both located on the larger lobe of the molecule, positioned as a wedge. The first interface buries ~3300 Å² of surface area and is predominantly hydrophobic in nature. The second interface buries ~2700 Å² of surface area and incorporates 4–6 salt bridges. Because of the combination of crystallographic and noncrystallographic symmetry, six FlaA1 protomers form a compact doughnut in which ~20% of the total surface area is buried (Fig. 1B). To examine the physiological relevance of this hexameric arrangement, electron microscopy was performed using a diluted FlaA1 sample. Doughnut-shaped protein particles were observed in negatively stained micrographs. Subsequent single-particle analysis revealed FlaA1 oligomers that are in excellent agreement with the hexameric structure derived from crystallographic analysis, confirming that the enzyme exists as a hexamer in solution (Fig. 1, C–F).

Overall Structure and Oligomeric Arrangement of FlaA1—The three-dimensional structure of FlaA1 is bilobal in shape (Fig. 1A). The larger, predominantly N-terminal, lobe consists of residues 1–174, 208–234, and 265–317 and contains a 10-stranded mostly parallel β-sheet flanked on either side by eight helices. The smaller, predominantly C-terminal, lobe is composed of residues 175–207, 235–264, and 318–333 and possesses three α-helices and two short two-stranded β-sheets. There are a total of five crossover points between the two lobes, resulting in an intertwined connectivity. The fold of FlaA1 confirms that this enzyme is a member of the SDR superfamily. However, FlaA1 is unique in that within the larger lobe the six-stranded parallel β-sheet of the Rossmann fold has been extended by four additional strands.

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Active Site Architecture and Catalytic Residues—The FlaA1 crystal structures reveal the cofactor and substrate-binding sites to be located in the larger and smaller lobes, respectively. The cofactor for FlaA1 is identified as NADP⁺/NADPH, which binds in a canonical manner to the Rossmann fold portion of the predominantly N-terminal lobe. Most intriguingly, exogenous NADP⁺ was not included as part of the crystallization conditions, indicating that NADP⁺ was co-purified and thus must bind tightly to the enzyme. In fact, supplementing the crystallization conditions with excess NAD⁺ did not dislodge the NADP⁺. This is in agreement with data from biophysical studies where addition of exogenous cofactor had no effect on the catalytic activity of FlaA1 (11).

Crystal structures of FlaA1 were obtained with the substrate UDP-GlcNAc (with reduced and oxidized cofactor) and with...
UDP, UDP-glucose (UDP-Glc), and UDP-galactose (UDP-Gal). In all five structures well defined density was observed for the UDP moiety whose uracil group forms an H-bond to the carbonyl oxygen of Pro-197 and whose diphosphate group is stabilized by two arginine residues (205 and 258) and a helix dipole moment. However, density for the saccharide moiety was only seen in the FlaA1-NADPH-UDP-GlcNAc and FaA1-NADP+–UDP-Gal ternary complexes (Fig. 2). It is not uncommon that saccharide density is lacking or ill defined in SDR enzymes that bind UDP-hexoses as this moiety must be inherently mobile for catalysis to proceed (16, 28, 29). However, the FlaA1-NADPH-UDP-GlcNAc structure provides insight on the average location of the hexose moiety of the substrate in FlaA1, which is in close proximity to the nicotinamide group of the cofactor, with both rings being parallel to each other. Surrounding the GlcNAc moiety are several residues that most likely play a role in catalysis, specifically Thr-131 and Tyr-141 of the (S/T)YK triad commonly found in SDR enzymes, and Asp-132 and Lys-133 (Fig. 2A). Previously, the importance of Tyr-141 for catalysis was illustrated through site-directed mutagen-

FIGURE 2. **Active site architecture.** A, the active site structure (green) in the FlaA1-NADPH-UDP-GlcNAc abortive ternary complex contains UDP-GlcNAc in a catalytically competent orientation. The $F_o - F_c$ SA-omit map (blue mesh) of UDP-GlcNAc is contoured at 2.0σ. B, the active site structure (orange) in the FlaA1-NADP+–UDP-Gal ternary complex contains UDP-Gal in an inhibitory orientation. The $F_o - F_c$ SA-omit map (purple mesh) of UDP-Gal is contoured at 2.0σ. NADPH and NADP+ are shown in gray.

FIGURE 3. Reaction mechanism for FlaA1 inverting 4,6-dehydratase activity. The proposed reaction mechanism for FlaA1 consists of three sequential steps as follows: oxidation, dehydration, and reduction. Computed pKₐ values for the active site residues Asp-132, Lys-133, and Tyr-141 are tabulated for each of these steps, and the appropriate protonation states are reflected in the mechanism.
Structure and Mechanism of Inverting 4,6-Dehydratase FlaA1

Six biosynthetic enzymes are involved in the production of CMP-Pse5Ac7Ac in *H. pylori*. Six biosynthetic enzymes are involved in the production of CMP-Pse5Ac7Ac from UDP-GlcNAc: FlaA1, an inverting 4,6-dehydratase; HP0366, a pyridoxal phosphate (PLP) and Glu-dependent aminotransferase; HP0327, an acetyl-CoA (AcCoA) utilizing aminotransferase; HP0326b, a hydrolase; HP0178, a synthase that catalyzes the condensation of phosphoenolpyruvate (PEP); and HP0326a, a synthetase that uses CTP to activate Pse5Ac7Ac.

Neither UDP-Glc nor UDP-Gal is a substrate for FlaA1. However, unlike UDP-Glc, UDP-Gal is able to completely inhibit the enzyme (11). The crystal structure of FlaA1-NADP+·UDP-Gal shows that unlike in the complex with UDP-Glc, the hexose ring is ordered but positioned away from the nicotinamide ring, allowing it to make specific interactions with the enzyme (Fig. 2B). These increased points of interaction between UDP-Gal and FlaA1 are sufficiently to result in effective inhibition of the enzyme.

Reaction Catalyzed by FlaA1—FlaA1 has been characterized previously as a 4,6-dehydratase (11), a bifunctional 4,6-dehydratase-4-reductase (10, 11), and a bifunctional 4,6-dehydratase-5-epimerase (12). The structural data presented here are incompatible with FlaA1 being a 4,6-dehydratase-4-reductase, which would convert UDP-GlcNAc to UDP-N-acetyl-α-D-quinovosamine. The reason is that such a conversion cannot be accomplished in a manner that regenerates the NADP+ cofactor, mandating replenishment. However, we have shown that the NADP+/NADPH is tightly bound to FlaA1 and cannot be readily dislodged. Yet, it remains possible that FlaA1 is either a 4,6-dehydratase or a 4,6-dehydratase-5-epimerase. To address the disparity and contradictions on the reaction catalyzed, we used NMR spectroscopy to elucidate the chemical structure of the product of FlaA1 catalysis. Because intermediates of UDP-saccharide biosynthetic pathways are known to be unstable during reaction product purification (31), the reaction catalyzed by FlaA1 was monitored directly in an NMR tube so that the product could be analyzed in a temporal fashion.

Analysis of the NMR data obtained for the FlaA1 product was inconsistent with it being UDP-N-acetyl-α-D-quinovosamine. Instead, the compound was identified as UDP-2-acetamido-2,6-dideoxy-β-L-arabinose-hexose-4,4-diol (Supplemental Material). By taking into consideration the aqueous conditions, this gem-diol compound is in equilibrium with UDP-2-acetamido-2,6-dideoxy-β-L-arabinose-hex-4-ulose. Therefore, we have identified FlaA1 to be a UDP-GlcNAc 4,6-dehydratase that additionally inverts the chirality at the C-5 position. Henceforth we will describe the activity of FlaA1 as an inverting 4,6-dehydratase activity, to distinguish it from other 4,6-dehydratases that do not affect the chirality at C-5. Note that the reaction catalyzed by FlaA1 can be accomplished in a manner that regenerates the cofactor during catalysis, consistent with tight binding of NADP+/NADPH.

**DISCUSSION**

Our structural studies have shown that FlaA1 is a hexameric enzyme belonging to the SDR superfamily, possessing inverting 4,6-dehydratase activity. To our knowledge FlaA1 is the first SDR superfamily member with a hexameric quaternary structure and the first inverting 4,6-dehydratase that is structurally characterized. However, three-dimensional structures of several 4,6-dehydratases that retain the chirality of the C-5 atom have been determined. Specifically, structural studies have been reported for dTDP-Glc 4,6-dehydratases (29, 32, 33), GDP-Man 4,6-dehydratases (34, 35), and CDP-Glc 4,6-dehydratases (36, 37). These enzymes share 18–21% sequence identity and possess a common structural core of ~270 residues. In comparison, FlaA1 shares only 12–16% sequence identity with these enzymes, and the structural common core is limited to ~220 residues. In fact, FlaA1 is as similar, if not more so, to 4-epimerases such as WbpP as it is to 4,6-dehydratases (11).

Among the different retaining 4,6-dehydratases the dTDP-Glc 4,6-dehydratase RmlB has become the archetype. A detailed reaction mechanism has been proposed for RmlB, which consists of three sequential steps (29, 38, 39). In the first step, the C-4 hydroxyl is oxidized to form a 4-keto group. This step employs the conserved (S/T)YK triad, and its mechanism is identical to what has been described previously for 4-epimerases (40). In the second step dehydration occurs across the
bond between C-5 and C-6, thus forming a 4-keto-5,6-ene-hexose. Here the C-5 proton is abstracted by a catalytic base (Glu-135), whereas the C-6 hydroxyl is simultaneously protonated by a catalytic acid (Asp-134), effecting a concerted elimination of water. In the concluding reduction step the hydride from the nicotinamide cofactor is transferred to C-6, whereas C-5 is re-protonated, resulting in the formation of a methyl group. The catalytic acid for this final step has been proposed to be the Tyr of the (S/T)YK triad or alternatively Glu-135. We propose that the inverting 4,6-dehydratase reaction mechanism for FlaA1 is analogous to that described for RmlB: a three-step sequential mechanism composed of oxidation, intramolecular dehydration, and reduction, in which the inversion of C-5 chirality is achieved in the concluding reduction step. Intriguingly, although much of the FlaA1 reaction mirrors that of RmlB, the enzyme does not have an analogous constellation of active site residues; specifically, the structural analog of the catalytic base in the dehydration step is Lys-133 in FlaA1, whereas it is Glu-135 in RmlB. Note that the FlaA1 K133E mutant that mimics the RmlB active site constellation is catalytically inactive.

To assess details of the reaction mechanisms for FlaA1, the pKa values for the suggested catalytic residues Asp-132, Lys-133, and Tyr-141 were computed along the reaction coordinate (Fig. 3). These simulations are in full agreement for the role of Tyr-141 in the initial oxidation step. However, the calculations suggested that for the subsequent dehydration step, Asp-132 could not act as a catalytic acid nor could Lys-133 function as a catalytic base. This finding is in contrast to the roles of analogous residues in the reaction mechanism of RmlB. The computed increase in pKa for Asp-132 after the first oxidation step suggests that Asp-132 increases the nucleophilic character of the C-6 oxygen, favoring proton abstraction from Lys-133. In this scheme, Lys-133 acts sequentially as a catalytic acid protonating the C-6 hydroxyl group and a catalytic base abstracting the C-5 proton, thus resulting in water elimination. This dual function for Lys-133 is reminiscent of the proposed role for Lys-73 in RTEM-1 β-lactamase, which is suggested to first function as a base and then as an acid, effecting the transfer of a proton from a serine to the β-lactam antibiotic (41). As for the final reduction step, a hydride that is transferred from NADPH to C-5 and C-6 is protonated, resulting in the change of chirality at the C-5 center. Our computational analyses suggest that the catalytic acid for this step is neither Tyr-141 nor Lys-133, instead that the likely candidate is the water molecule generated in the previous step.

A time course analysis of the reaction clarifies some of the reasons for the previously erroneous characterization of FlaA1. We observe that after an hour a second product appears, UDP-2-acetamido-2,6-dideoxy-α-D-xylono-hexose-4,4-diol (see Supplemental Material). This compound is identical to the product of FlaA1, except for an inversion of chirality at C-5. This second product is most likely the result of racemization of the FlaA1 product through enolization, involving a double bond between C-4 and C-5. Whether this racemization occurs in the FlaA1 active site or in solution is unclear. However, given the large time delay for the appearance of the second product, it is doubtful that there is a physiologic relevance to the racemization reaction.

By having determined the catalytic role of FlaA1 to be an inverting 4,6-dehydratase, the next question to address would be to determine its role in H. pylori. Recently, several studies have addressed an intriguing link between the biosyntheses of several virulence factors and protein glycosylation in H. pylori (9). In particular, pseudaminic acid (Pse) derivatives such as 5,7-diacetamido-3,5,7,9-tetrae-deoxy-α-L-glycero-α-manno-nonulosonic acid (Pse5Ac7Ac) have been demonstrated to play an integral role in the protein glycosylation of H. pylori and closely related Campylobacter jejuni (10, 12, 42, 43). Pse5Ac7Ac is a nine-carbon saccharide that has been exclusively found in bacteria, and its activated form is as a CMP-saccharide in H. pylori (10). Based on the recent development in the studies of biosynthesis of sialic acid in bacteria (44, 45) and by anticipating the plausible catalytic steps to occur in such a pathway, the complete genome sequence of H. pylori (46, 47) was used to identify candidates in a likely pathway for the biosynthesis of CMP-Pse5Ac7Ac in H. pylori (Fig. 4). This pathway starts with UDP-GlcNAc, which is a common precursor in complex saccharide biosynthesis, and involves six enzymes. To date, biochemical data are available for the first two enzymes (FlaA1 and HP0366) (11, 12, 20). The assignment of the remaining enzymes involved in this proposed pathway was based on sequence similarity of these enzymes with other proteins possessing similar functions and by the involvement of their homologs in bacterial protein glycosylation (42, 48–50).

In conclusion, the structural studies of FlaA1 have unveiled the three-dimensional structure and reaction mechanism of an inverting 4,6-dehydratase involved in the CMP-Pse5Ac7Ac biosynthetic pathway that is critical for H. pylori pathogenicity. The fundamental understanding of the reaction mechanism of FlaA1 involving Lys-133, which plays the dual function during dehydration, will assist in future efforts to develop antimicrobial treatments targeting protein glycosylation in H. pylori and other related bacterial pathogens.

**Protein Data Bank Accession Codes**—The atomic coordinates and structure factors have been deposited with the following accession codes: 2GN4 (FlaA1-NADPH-UDP-GlcNAc), 2GN6 (FlaA1·NADP⁺-UDP-GlcNAc), 2GN8 (FlaA1·NADP⁺-UDP), 2GNN9 (FlaA1·NADP⁺-UDP-Glc), and 2GNA (FlaA1-NADP⁺-UDP-Gal).

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