The X-ray Structure of dTDP-4-Keto-6-deoxy-d-glucose-3,4-ketoisomerase*

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The repeating unit of the glycan chain in the S-layer of the bacterium Aneurinibacillus thermoaerophilus L420-91T is composed of four α-D-rhamnose molecules and two 3-acetamido-3,6-dideoxy-α-D-galactose moieties (abbreviated as Fucp3NAc). Formation of the glycan layer requires nucleotide-activated sugars as the donor molecules. Whereas the enzymes involved in the synthesis of GDP-rhamnose have been well characterized, less is known regarding the structures and enzymatic mechanisms of the enzymes required for the production of dTDP-Fucp3NAc. One of the enzymes involved in the biosynthesis of dTDP-Fucp3NAc is a 3,4-ketoisomerase, hereafter referred to as FdtA. Here we describe the first three-dimensional structure of this sugar isomerase complexed with dTDP and solved to 1.5 Å resolution. The FdtA dimer assumes an almost jellyfish-like appearance with the sole sugar isomerase complexed with dTDP and solved to 1.5 Å resolution. The FdtA dimer assumes an almost jellyfish-like appearance with the sole sugar isomerase complexed with dTDP and solved to 1.5 Å resolution.

Many Gram-positive and Gram-negative bacteria, as well as some Archaea, synthesize cell surface envelopes referred to as surface or S-layers. These outermost cell layers are composed of proteins or glycoproteins that self-assemble into two-dimensional arrays. Depending upon the organism, these arrays can form hexagonal, square, trimeric, or oblique crystalline lattices (1). For those bacteria with an S-layer, it has been estimated that 20% of the total cellular protein synthesis is devoted to its production (2). With respect to the level of protein glycosylation, it varies from 2 to 10% (w/w) depending upon the organism (2).

Although the function of the S-layer is still not well understood, it has been postulated to be involved in bacterial virulence by facilitating invasion of host tissue and by protecting the pathogen against host defense mechanisms (3, 4). Interestingly, the S-layer glycans can be lost after prolonged growth of bacteria in rich media, and thus it has been speculated that the carbohydrate components of the S-layer confer a selective advantage on the bacteria in their natural habitat (2).

In recent years, the bacteria Aneurinibacillus thermoaerophilus strains L420-91T and DSM 10155/G+ and Geobacillus steaothermophilus strain NRS2004/3a have served as model systems for probing the nature of the S-layer glycans (5). In A. thermoaerophilus L420-91T, the S-layer is composed of identical 109-kDa glycoprotein subunits arranged in a square lattice (6). The repeating unit of the glycan chain is a hexasaccharide composed of four α-D-rhamnose units and two 3-acetamido-3,6-dideoxy-α-D-galactose residues linked together as indicated in Scheme 1. Formation of the glycan chains occurs in the cytoplasm and requires nucleotide-activated sugars as the donor molecules (7). For S-layer production in A. thermoaerophilus, both GDP-rhamnose and dTDP-3-acetamido-3,6-dideoxy-α-D-galactose (abbreviated as dTDP-Fucp3NAc) are required.

The biosynthetic pathway for the production of dTDP-Fucp3NAc in A. thermoaerophilus was elucidated in 2003 and is indicated in Scheme 2. Like most of the pathways for the synthesis of 3,6-dideoxyhexoses, the formation of this unusual sugar begins with the attachment of α-D-glucose 1-phosphate to dTMP via the action of glucose-1-phosphate thymidyltransferase. In the next step, the 6-hydroxyl group is removed, and the 4-hydroxyl group is oxidized to a keto-functionality yielding dTDP-4-keto-6-deoxyglucose. This reaction is catalyzed by dTDP-glucose 4,6-dehydratase. Both the thymidyltransferase and the dehydratase have been well characterized with respect to structure and function (8, 9).

Three additional enzymes are ultimately required for the synthesis of dTDP-Fucp3NAc, namely an isomerase, an aminotransferase, and an acetylase (Scheme 2). These enzymes are encoded by the fdtA, fdtB, and fdtC genes, respectively (10). The isomerase, hereafter referred to as FdtA, is especially intriguing in that it catalyzes the conversion of a 4-keto substrate into a 3-keto product with accompanying epimerization about C-4 of the hexose ring. From previous biochemical studies, it appears that FdtA does not require cofactors for activity (11). Thus it can be speculated that the reaction catalyzed by FdtA proceeds via a concerted acid-base mechanism similar to that proposed...
for triose-phosphate isomerase, the well known enzyme of the glycolytic pathway.

To date, at least 65 ORFs3 demonstrating homology to FdtA in the NCBI data base have been identified, but until now, no three-dimensional structures have been reported (11). These ORFs are found only in bacteria and are typically associated with additional genes thought to be involved in the production of outer membrane polysaccharides (11).

Here we report the first x-ray structure of FdtA, complexed with dTDP and determined to a nominal resolution of 1.5 Å. Each subunit of the dimeric enzyme adopts a β-barrel motif with the dTDP ligand positioned near the opening of the barrel. Inspection of the active site region reveals a cluster of the following histidine residues: His49, His51, and His95. Both His49 and His51 are strictly conserved among the amino acid sequences presently reported. To probe the biological role of these two residues, each was changed to an asparagine via site-directed mutagenesis, and the mutant proteins were assayed for activity. In addition, a double mutant protein was prepared whereby both histidines were substituted with asparagines. Whereas the H51N mutant protein retained limited activity, the double mutant H49N/H51N was catalytically inactive. X-ray crystallographic analyses of these mutant proteins revealed no significant structural perturbations of their active site regions. On the basis of both biochemical and x-ray crystallographic data, we propose that His49 functions as an active site base to abstract the hydrogen from C-3 of the sugar and to deliver it to C-4, whereas His51 serves to shuttle protons between the C-3 and C-4 sugar oxygens. Details concerning the overall structure and function of FdtA are presented.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, all chemicals and reagents were purchased from Sigma. Platinum Pfx DNA polymerase was obtained from Invitrogen. Reagents for DNA purifications were manufactured by Qiagen.

Isolation of Genomic DNA—A. thermoaerophilus L420-91T was obtained from ATCC (700303). The freeze-dried pellet was reconstituted in sterile SVIII media (10) and subsequently used to inoculate a small volume of SVIII media. Cells were grown overnight at 55 °C with shaking and then harvested by centrifugation. Genomic DNA was isolated according to standard protocols (12).

Cloning of the fdtA Gene—The gene encoding the isomerase was PCR-amplified from genomic DNA. Forward and reverse primers containing the restriction sites for NdeI and NotI, respectively, were used to amplify the gene. The gene was subsequently ligated into a modified pET28b(+) vector (Novagen) in which the thrombin cleavage site was replaced with the recognition sequence for TEV protease. Proteins expressed with this modified vector possess an N-terminal hexahistidine tag that can be released by cleavage with TEV protease. Plasmids were tested for successful ligation by digestion with NdeI and NotI.

Protein Expression and Purification—The recombinant pET28-fdtA plasmid was utilized for the transformation of Escherichia coli Rosetta(DE3) cells (Novagen). A single colony was picked to inoculate an overnight starter culture of LB media. Subsequently, the starter culture was used to inoculate several large scale cultures of TB media. Cells were grown at 37 °C until an absorbance of ~1.0 at 600 nm was reached. Flasks were transferred to a shaker held at 22 °C, and cells were allowed to grow for 18 h. Protein expression was induced with isopropyl β-D-thiogalactopyranoside. Cells were harvested by centrifugation.

For protein purification, cells were suspended in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 10 mM imidazole) and maintained at 4 °C for all subsequent steps. Cells were lysed by sonication, and insoluble debris was removed by centrifugation. The clarified lysate was then loaded onto a Ni2+-nitrilotriacetic acid-agarose column (Qiagen). The recombinant His16-tagged protein was eluted with a linear gradient of 10–250 mM imidazole in lysis buffer. On the basis of SDS-PAGE analysis, fractions containing FdtA were pooled and dialyzed overnight against an excess of lysis buffer. The tag was removed with TEV protease, and cleaved FdtA was separated from the protease and uncleaved protein by running the digestion mixture over a Ni2+-nitrilotriacetic acid column. The protein was concentrated to 17 mg/ml (using an extinction coefficient of 0.91 (mg/ml)−1·cm−1 as calculated with Protein (DNAStar)) and dialyzed overnight against 1.0 liter of storage buffer (20 mM HEPES, pH 8.0, and 300 mM NaCl). Dialyzed protein was frozen in liquid nitrogen and stored at −80 °C.

Construction of Site-directed Mutant Proteins—Using the pET28-fdtA construct, mutations were introduced into the gene using a QuikChange XL site-directed mutagenesis kit (Stratagene). Three mutated genes were generated as follows: H49N, H51N, and H49N/H51N. Prior to expression of the proteins, the genes were sequenced to ensure no additional muta-

3 The abbreviations used are: ORF, open reading frame; HPLC, high pressure liquid chromatography; TEV, tobacco etch virus; MOPS, 4-morpholinepropanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.
tions had been introduced during the mutagenesis process. The mutant proteins were expressed and purified as described above for the wild-type enzyme.

Activity Assays for Wild-type Protein and Mutant Forms of FdtA—The wild-type and mutant forms of FdtA were qualitatively assayed for activity via HPLC. Reaction mixtures contained 0.5 μmol of dTDP-glucose, 10 nmol RmlB (Scheme 2), 10 mM MOPS, pH 7.5, and 10 nmol of FdtA. A reaction containing no isomerase was used as a negative control. For the reactions containing mutated FdtA, the wild-type reaction served as a positive control. Reactions were incubated at 37 °C for 1 h and then boiled to denature the protein. Protein was removed by centrifugation, and the supernatant was run over a Resource Q column (Amersham Biosciences). Each sample was eluted with a linear gradient of ammonium acetate at pH 4.0. ESI mass spectrometry was used to identify the reaction products.

To verify the results of these assays, 10 nmol of the second enzyme in the N-acetylfucosamine pathway, FdtB (Scheme 2), was added to each reaction mixture. Each sample was eluted with a linear gradient of ammonium bicarbonate at pH 8.5. As before, ESI mass spectrometry was employed to analyze the eluted compounds.

### TABLE 1

| Protein                                                                 | Resolution | Independent | Completeness | Redundancy | Average I/average σ(I) | Rsym* |
|------------------------------------------------------------------------|------------|-------------|--------------|------------|------------------------|-------|
| Wild-type                                                              | 50.0-1.50  | 62,658 (5,740) | 98.7 (92.3) | 7.1 (3.9) | 48.3 (1.9)             | 6.4 (52.4)    |
| Selenomethionine-labeled (peak)                                        | 50.0-2.20  | 69,240 (6,080) | 100.0 (90.0) | 10.0 (4.0) | 58.9 (28.6)             | 6.9 (11.2)    |
| Inflection                                                             | 50.0-2.20  | 62,514 (5,740) | 98.7 (92.3) | 7.1 (3.9) | 48.3 (1.9)             | 6.4 (52.4)    |
| Remote                                                                 | 50.0-2.20  | 69,240 (6,080) | 100.0 (90.0) | 10.0 (4.0) | 58.9 (28.6)             | 6.9 (11.2)    |
| H49N                                                                   | 30.0-2.50  | 20,500 (1,990) | 98.7 (92.3) | 7.1 (3.9) | 48.3 (1.9)             | 6.4 (52.4)    |
| H51N                                                                   | 30.0-2.40  | 21,387 (1,990) | 98.7 (92.3) | 7.1 (3.9) | 48.3 (1.9)             | 6.4 (52.4)    |
| H49N/H51N                                                              | 30.0-2.50  | 20,500 (1,990) | 98.7 (92.3) | 7.1 (3.9) | 48.3 (1.9)             | 6.4 (52.4)    |

* Rsym = (Σ|Fo|−|Fc|)/Σ|Fo| × 100.

**Statistics for the highest resolution bin shown in parentheses.

### TABLE 2

| Protein | Wild-type | H49N | H51N | H49N/H51N |
|---------|-----------|------|------|-----------|
| R-factor (overall) %/no. of reflections | 19.5/62,623 | 21.2/15,280 | 20.8/13,985 | 20.1/12,145 |
| R-factor (working) %/no. of reflections | 19.4/62,263 | 21.1/15,280 | 20.8/13,985 | 20.1/12,145 |
| R-factor (free) %/no. of reflections | 24.5/63,700 | 29.1/13,629 | 28.2/12,595 | 28.1/12,319 |
| No. of protein atoms | 2203 | 2188 | 2180 | 2177 |
| Weighted root-mean-square deviations from ideality | 0.009 | 0.011 | 0.011 | 0.010 |
| Bond lengths (Å) | 0.009 | 0.011 | 0.011 | 0.010 |
| Bond angles (degree) | 2.0 | 2.4 | 2.1 | 2.2 |
| Trigonal planes (Å) | 0.006 | 0.005 | 0.005 | 0.007 |
| General planes (Å) | 0.010 | 0.008 | 0.008 | 0.007 |
| Torsional angles (degree) | 18.5 | 20.3 | 20.3 | 20.4 |

* R-factor = (Σ|Fo|−|Fc|)/Σ|Fo| × 100, where |Fo| is the observed structure-factor amplitude, and |Fc| is the calculated structure-factor amplitude.

**These include multiple conformations for residues Lys5, Ser64, Cys65, and Met107 in subunit 1 and Cys65, His65, and Met107 in subunit 2.

***Heteroatoms include 2 dTDP molecules and 157 waters.

****Heteroatoms include 2 dTDP molecules and 121 waters.

#### SCHEME 2

X-ray Structure of a 3,4-Ketoisomerase

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Preparation of Selenomethionine-labeled Protein—A starter culture of E. coli Rosetta(DE3) cells harboring the pET28-fdtA plasmid was grown overnight at 37 °C in M9 minimal media. Subsequently, the overnight culture was used to inoculate several large scale cultures of M9 minimal media supplemented with 5 mg/liter thiamine. Cultures were grown at 37 °C to an absorbance of ~0.9 at 600 nm and then cooled on ice. Flasks were transferred to a shaker held at 16 °C, and expression of selenomethionine-labeled protein was induced as described previously (13). Cells were harvested by centrifugation, and selenomethionine-labeled protein was purified to homogeneity as described for the wild-type protein.

Crystallization of Wild-type and Selenomethionine-labeled FdtA—An “in-house” sparse matrix screen consisting of 144 unique conditions was used to identify crystallization conditions. The hanging-drop method of vapor diffusion was employed, and crystallization trials were conducted at both 25 and 4 °C. Preliminary crystals of the cleaved protein in complex with 10 mM dTDP and 20 mM d-fucose grew at 25 °C from poly(ethylene glycol) 3400 and KCl solutions at pH 7.5. Crystallization conditions were subsequently optimized to 20–21% poly(ethylene glycol) 3400, 150–200 mM KCl, and 100 mM HEPES, pH 7.5. Crystals routinely grew in 1–2 days to maximum dimensions of ~0.4 × 0.4 × 1.0 mm. They belonged to the space group P4₁2₁2₁ with unit cell dimensions of a = b = 62.7 Å and c = 201.3 Å and one dimer per asymmetric unit.

Crystals of the selenomethionine-labeled protein in complex with dTDP grew out of conditions similar to those for the unlabeled protein (22% poly(ethylene glycol) 3400, 200 mM KCl, and 100 mM HEPES, pH 7.5). These crystals belonged to the same space group and had similar unit cell dimensions.

Crystallization of the Mutant Proteins—All crystals of the mutated proteins were grown in the pres-
ence of 10 mM dTDP and 20 mM fucose and achieved similar dimensions as the wild-type protein crystals within several days. Crystals of the H51N and H49N/H51N protein grew out of 18% poly(ethylene glycol) 3400, 150 mM KCl, 200 mM NaCl, 10 mM dTDP, 20 mM fucose, and 100 mM HEPES, pH 7.5. They were subsequently frozen after transfer into a cryoprotectant solution composed of 25% poly(ethylene glycol) 8000 with 100 mM HEPPS, pH 8.5, and 100 mM KCl. The space groups for all of the mutant protein crystals were the same as that for the wild-type protein, and the unit cell dimensions were very similar.

**X-ray Data Collection and Processing**—Crystals of both the selenomethionine-labeled and wild-type protein were stabilized for x-ray data collection by harvesting them into a synthetic mother liquor composed of 17% poly(ethylene glycol) 3400, 150 mM KCl, 200 mM NaCl, 10 mM dTDP, 20 mM fucose, and 100 mM HEPES, pH 7.5. They were subsequently frozen after transfer into a cryoprotectant solution composed of 25% poly(ethylene glycol) 3400, 225 mM KCl, 400 mM NaCl, 10 mM dTDP, 20 mM fucose, 15% ethylene glycol, and 100 mM HEPES, pH 7.5. X-ray data from both the selenomethionine-labeled and wild-type protein crystals were collected on a CCD detector at SBC Beamline 19-BM (Advanced Photon Source, Argonne, IL). These data were processed and scaled with HKL2000 (14).

Crystals of the mutant proteins were stabilized for x-ray data collection in a similar manner, and x-ray data sets from these crystals were collected at 100 K with a Bruker AXS Platinum 135 CCD detector controlled with the Proteinum software suite (Bruker (2004), PROTEUM, Bruker AXS Inc., Madison, WI). The x-ray source was CuKα radiation from a Rigaku RU200 x-ray generator equipped with moncel optics and operated at 50 kV and 90 mA. These data were processed with SAINT (version V7.06A, Bruker AXS Inc., Madison, WI) and internally scaled with SADABs (version 2005/1, Bruker AXS Inc., Madison, WI). Relevant x-ray data collection statistics are listed in Table 1.

**X-ray Structural Analysis of FdtA**—The three-dimensional structure of FdtA was solved via MAD phasing. The program SOLVE was used to locate the positions of seven of the eight selenium atoms in the asymmetric unit and to calculate initial protein phases (15). Solvent flattening with RESOLVE produced a readily interpretable electron density map calculated to 2.2 Å resolution (16). The entire polypeptide chain for the FdtA subunit was traced, with the exception of residues Met1 and Gly139 in subunit 2. In addition, the N-terminal Gly-His residues resulting from the tag were not visible. The FdtA model was then employed as a search probe for molecular replacement with the software package EPMR (17) against the x-ray data set collected to 1.5 Å resolution from the wild-type crystal. Alternate cycles of manual model building and least squares refinement with the program TNT (18) reduced the R-factor to 19.5% for all measured x-ray data from 50.0 to 1.5 Å.

The three-dimensional structures of the site-directed mutant proteins were solved by molecular replacement with the program EPMR (17) using the wild-type protein model as a search probe. Relevant refinement statistics for all of the protein models are given in Table 2.

**RESULTS**

**Overall Three-dimensional Structure of FdtA**—Crystals of the wild-type protein employed for the structural analysis presented here diffracted to a nominal resolution of 1.5 Å and contained a complete dimer in the asymmetric unit. The electron densities for the polypeptide chain backbones were very well ordered for both subunits with electron density missing for only Met1, Lys137, Glu138, and Gly139 in subunit 1 and Met1, Glu136, Lys137, Glu138, and Gly139 in subunit 2. The quality of the model is excellent with 90.1% of the residues adopting φ, ψ angles in the “most favored” and 9.9% in the “additionally allowed” regions of the Ramachandran plot.

A ribbon representation of the dimer is presented in Fig. 1a. The dimer has overall dimensions of ~47 × 51 × 64 Å and assumes an almost jellyfish-like appearance with the sole α-helices representing the tentacles. The subunit-subunit interface is rather extensive with a total buried surface area of ~3100 Å². Formation of the FdtA dimer represents a classic example of domain swapping whereby β-strands 2 and 3 from one subunit form part of a β-sheet in the second subunit (19). The “hinge” loop required for the domain swapping is formed by a type II turn (Glu22 to Lys25) and a loop (Asn26 to Lys32) that together connect β-strands 3 and 4. This type of swapping phenomenon was first revealed in the dimeric structure of diptheria toxin (20) and has now been observed in at least 40 structurally and biologically diverse proteins (21). It has been postulated that some domain-swapped multimeric proteins may have arisen from monomeric proteins as a result of destabilizing mutations (20, 22).

The overall tertiary structure of each subunit is dominated by two layers of anti-parallel β-sheet that form a flattened barrel as can be seen in Fig. 1b. The two layers of sheet within the subunit each contain five β-strands. As indicated in the topology plot presented in Fig. 1c, one layer is composed of β-strands 1, 4, 6, 9, and 11, and the second layer contains β-strands 5, 7, 8, 10, and 12. Because of domain swapping, however, one layer of sheet contains an additional two anti-parallel β-strands contributed by the second subunit with β-strand 3 in one subunit abutting β-strand 4 in the second subunit. As a consequence, β-strands 3 from each subunit contribute significantly to the dimer interface. In addition to β-strand 3, the dimeric interface is also formed by the β-sheet composed of β-strands 1, 4, 6, 9 and 11, which projects primarily hydrophobic side chains into the interstitial space. This is illustrated in Fig. 2, a and b. Not surprisingly, given the hydrophobic character of the subunit-

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**FIGURE 1.** The three-dimensional structure of FdtA. A ribbon representation of the FdtA dimer is depicted in a with one subunit colored in gold and the other in green. The position of the local 2-fold rotational axis relating one subunit to the other is indicated by the black arrow. The dTDP molecules, drawn in stick representations, are separated by ~24 Å. A ribbon representation of subunit 2, in the same orientation as a is presented in b. The 12 β-strands are labeled. These figures, as well as Figs. 2, 3, and 5, were prepared with the software package PyMOL (30). A topology drawing for one subunit of FdtA is shown in c. It was generated from the TOPS data base of protein structural topology (31). The red triangles represent strands of β-sheet, the green sphere corresponds to a helical turn, and the yellow sphere denotes the single α-helix in the subunit. C-term, C terminus; N-term, N terminus.
The Active Site of FdtA—Electron density corresponding to the dTDP ligand in subunit 2 is presented in Fig. 3a, and as can be seen, the nucleotide is well ordered. Unlike that observed in subunit 2, however, the electron density for the ligand in subunit 1 is indicative of multiple conformations.

Consequently, for the sake of simplicity, the following discussion only refers to subunit 2 because the nucleotide adopts a single conformation.

A close-up view of the region surrounding the dTDP is displayed in Fig. 3b. The deoxyribose adopts the C2'-endo pucker. The C-4 carbonyl oxygen of the thymine base is located within 2.7 Å of N91 of Arg33. There are no other direct contacts between the thymine ring and the protein, but rather N-3 and the C-2 carbonyl oxygen interact with ordered water molecules. Likewise, there are no side chains or ordered water molecules located within 3.2 Å of the 3-hydroxyl group of the deoxyribose. The α-phosphoryl oxygens of dTDP are surrounded by three waters and the side chains of Tyr119 and Arg65. There is a water molecule located within 3.1 Å of the bridging oxygen. The β-phosphoryl oxygens form electrostatic interactions with three water molecules and the guanidinium groups of Arg121 and Arg15. Note that Arg15 is contributed by subunit 1, and hence part of the FdtA active site is formed by the second subunit in the dimer as a result of domain swapping.

Although the crystals were grown in the presence of 20 mM d-fucose (which is 6-deoxy-d-galactose), there was no obvious electron density near the dTDP moiety that clearly indicated an ordered carbohydrate. However, there was a string of electron density near the nucleotide that was modeled as ordered water molecules, and this density could possibly correspond to a fucose molecule at very low occupancy. These solvent molecules fill in a substantial “hole” in the active site, and thus it can be speculated that this is the region where the sugar of the dTDP-4-keto-6-deoxyglucose is positioned. As displayed in Fig. 3c, there is an intriguing cluster of histidine residues located near this hole. Two of these histidine residues, 49 and 51, are absolutely conserved among the amino acid sequences reported thus far for putative sugar isomerases. To test their possible roles in catalysis, three site-directed mutant proteins, H49N, H51N, and H49N/H51N, were subsequently constructed, and their structural and biochemical properties analyzed.

Activities of the Site-directed Mutant Proteins—To test the activity of all of the recombinant proteins, the HPLC elution profile for the sequential reactions catalyzed by the dehydratase, RmlB, and wild-type FdtA (Scheme 2) was first compared with a control experiment to which no FdtA was added. As can be seen in Fig. 4a, in the absence of FdtA, the...
HPLC elution profile contained two large peaks. ESI mass spectrometry confirmed that the larger peak (Fig. 4a, peak 1) corresponded to both the substrate (dTDP-glucose, 563 g/mol) and the product (dTDP-4-keto-6-deoxyglucose, 547 g/mol) of the dehydratase reaction. In Fig. 4a, the peak denoted by an asterisk corresponded to dTMP, which was present in the laboratory-prepared dTDP-glucose samples used for the assays. Addition of FdtA resulted in two new peaks (Fig. 4a). The main compound in the new peak (Fig. 4a, peak 2) had a molecular weight identical to that of the 4-keto derivative, indicating that wild-type FdtA was active. In Fig. 4a, peak 3 corresponded to dTDP. The presence of dTDP is consistent with a previous study by Melancon et al. (11), where it was noted that the 3-keto derivative is inherently unstable and readily breaks down to yield the free sugar and dTDP.

To further confirm the above results, the previous experiments were repeated but this time in the presence of FdtB. As noted in Scheme 2, FdtB is a pyridoxal phosphate-dependent aminotransferase that functions on the 3-keto position of the sugar. The HPLC elution profile for the reaction lacking FdtA consisted of two distinct peaks, a sharper, larger peak and a second broader peak (Fig. 4b). On the basis of ESI mass spectrometry, Fig. 4b, peak 1, contained both dTDP-glucose and the 4-keto-6-deoxy derivative. The second peak (Fig. 4b, peak 3) contained a compound with a molecular weight of 546.1 g/mol, which is consistent with the replacement of the keto group with an amine and indicates that the aminotransferase reaction is not strictly specific for the 3-position of the sugar. Addition of FdtA resulted in a new peak (Fig. 4b, peak 2) and a decrease in the area of peaks 1 and 3. The molecular weight of the compound eluted in the new peak was consistent with that of dTDP-3-amino-3,6-dideoxy-α-D-galactose, the product of the FdtB reaction.

**FIGURE 3. The FdtA active site.** Shown in a is the electron density corresponding to the dTDP ligand in subunit 2. The map was calculated with coefficients of the form (\(F_o - F_c\)), where \(F_o\) was the native structure factor amplitude, and \(F_c\) was the calculated structure factor amplitude with the atoms corresponding to dTDP excluded from the calculation. The map was contoured at 3\(\sigma\). The \(\beta\)-strands are numbered as in Fig. 1. Note that \(\beta\)-strand 3 is labeled with an asterisk to emphasize that it is contributed by the second subunit of the dimer. A close-up view of the hydrogen-bonding pattern surrounding the nucleotide is presented in b. The positions of ordered water molecules are indicated by the red spheres. Ordered ethylene glycol molecules are labeled “EG.” Potential hydrogen bonds between the nucleotide and the protein or solvent (within 3.2 Å) are indicated by the dashed lines. An expanded view of the active site pocket, which shows the three histidine cluster, is presented in c.
structures were determined and refined to $\sim 2.5$ Å resolution. For each structure, the electron density maps were well ordered with only minor changes in side chain orientations. Importantly, the active site geometries for the mutant proteins and the wild-type enzyme were nearly identical (supplemental Fig. 2, a–c). The $\alpha$-carbons of the wild-type dimer and the H49N, H51N, and H49N/H51N proteins superimpose with root mean square deviations of 0.25, 0.26, and 0.27 Å, respectively. Hence the loss of catalytic activity with the H49N mutant protein is not a result of gross structural perturbations.

**DISCUSSION**

Sugars represent the most abundant biomolecules in the Earth’s biomass and are involved in such important physiological processes as the immune response, cell–cell interactions, and fertilization. In recent years, there has been considerable research interest in the more unusual di- and tri-deoxy sugars that possess antigenic or antibiotic activities (25). In general, the 2,6-dideoxy- and 2,3(4),6-trideoxy sugars are involved in antibiotic biosynthesis (9, 26), whereas the 3,6-dideoxy sugars are found predominantly on the outer surface of the cell membranes of both Gram-positive and Gram-negative bacteria (27). In Gram-negative bacteria, these modified sugars are found in the O-antigen, the outermost portion of the lipopolysaccharide (28). In Gram-positive bacteria, which do not synthesize lipopolysaccharide, these sugars are found attached to S-layer proteins (29).

The pathways involved in the production of these unusual sugars represent a rich source of intriguing enzymes. FdtA, the focus of this study, represents a novel type of isomerase first characterized by Pfoestl et al. in 2003 (10). Strikingly, as indicated in Scheme 2, whereas glucose 1-phosphate serves as the starting material for the production of dTDP-Fucp3NAc, the stereochemistry about C-4 of the sugar changes to that of galactose in the reaction catalyzed by FdtA. As noted previously, the biochemical properties for a similar 3,4-ketoisomerase from the $\delta$-mycaminose biosynthetic pathway of *S. fradiae* have recently been reported (11). In Ty1a, however, the stereochemistry about C-4 is retained.

From the x-ray crystallographic and biochemical data presented here, it is now known that both His$^{49}$ and His$^{51}$ in FdtA play critical roles in catalysis. On the basis of these data, we have modeled the substrate of FdtA into the active site cleft as presented in Fig. 5a. This modeling was accomplished by first anchoring the nucleoside portion of the substrate into a similar position as that observed for dTDP. By a series of rotations about the phosphoryl groups of the substrate, it was possible to position C-3 of the sugar near the imidazole nitrogen of His$^{49}$. As can be seen, this model also places His$^{51}$ near the sugar C-3 oxygen and His$^{98}$ near the sugar C-4 oxygen.

A possible mechanism for FdtA can be envisioned whereby His$^{49}$ removes the hydrogen from the sugar C-3 and shuttles it to the sugar C-4 on the same side of the glycosyl group. This would result in inversion of configuration about C-4. His$^{51}$ might function in catalysis by shuttling protons between the C-3 and C-4 oxygens. The postulated role for His$^{98}$ is consistent with the lack of measured enzymatic activity when it is substituted with an asparagine. The fact that the enzymatic activity of FdtA is considerably reduced when His$^{51}$ is changed to an asparagine is also consistent with the reduction of catalytic activity.
with its proposed role as a proton shuttle. The residual activity in the H51N mutant protein might result from another residue, such as His95, fulfilling the proton transfer role, albeit at a less-than-optimal rate. Additional site-directed mutagenesis and x-ray crystallographic analyses are presently underway to further clarify the catalytic mechanism of FdtA.

Both FdtA and Tyl1a function on the same substrate and catalyze similar isomerization reactions, although as noted in Tyl1a, the configuration about C-4 is retained. It can thus be speculated that in Tyl1a there must be two active site bases situated on opposite faces of the sugar. One base would be required for abstracting the hydrogen from the sugar C-3 and donating it to the C-4 oxygen, whereas the other would be required to abstract the hydroxyl group hydrogen on C-3 and transfer it to C-4. On the basis of an amino acid sequence alignment, it is known that Tyl1a contains two histidines that are homologous to His49 and His51 in FdtA, but the similarities between these two proteins extend far beyond these two residues (supplemental Fig. 1). An expanded view of the FdtA active site is given in Fig. 5b. The only differences are at Ile11, Phe48, His46, and Met97 in FdtA, which are His25, HAH(K/R)X(L/I)XQX,G,S, where X can be any amino acid. In FdtA, this sequence, which contains the two conserved histidine residues, forms part of the active site. This sequence will serve as a hallmark for this type of sugar isomerases, and it will be of value in assigning function to additional uncharacterized ORFs as they become available. Furthermore, it can be speculated that those enzymes that retain configuration about C-4 of the sugar, such as Tyl1a, contain an arginine at the homologous position of His95 in FdtA, whereas those that are inverting contain a histidine residue.

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