Expression, Purification, and Biochemical Characterization of WbpP, a New UDP-GlcNAc C4 Epimerase from Pseudomonas aeruginosa Serotype O6*

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B-band lipopolysaccharide is an important virulence factor of the opportunistic pathogen Pseudomonas aeruginosa. WbpP is an enzyme essential for B-band lipopolysaccharide production in serotype O6. Sequence analysis suggests that it is involved in the formation of N-acetylgalactosamonic acid. To test this hypothesis, overexpression and biochemical characterization of WbpP were performed. By using spectrophotometric assays and capillary electrophoresis, we show that WbpP is a UDP-GlcNAc C4 epimerase. The $K_m$ for UDP-GlcNAc and UDP-GalNAc are 197 and 224 $\mu M$, respectively. At equilibrium, 70% of UDP-GalNAc is converted to UDP-GlcNAc, whereas the yield of the reverse reaction is only 30%. The enzyme can also catalyze the interconversion of non-acetylated substrates, although the efficiency of catalysis is significantly lower. 15 and 40% of UDP-Glc and UDP-Gal, respectively, are converted at equilibrium. WbpP contains tightly bound NAD(H) and does not require additional cofactors for activity. It exists as a dimer in its native state. This paper is the first report of expression and characterization of a C4 UDP-GlcNAc epimerase at the biochemical level. Moreover, the characterization of the enzymatic function of WbpP will help clarify ambiguous surface carbohydrate biosynthetic pathways in P. aeruginosa and other organisms where homologues of WbpP exist.

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that can cause life-threatening infections in patients with cystic fibrosis or burn wounds. (1). It produces a wide variety of virulence factors such as proteases, toxins, alginate, and lipopolysaccharides (LPS). Two forms of LPS have been identified as follows: the antigenically conserved A-band LPS, and the variable O-antigen or B-band. B-band LPS is particularly important in the initial steps of the infection and particularly for evasion of host defenses and colonization (2, 3). It contributes to causing initial tissue damage and inflammatory responses in the lungs of patients with cystic fibrosis (2). P. aeruginosa mutants deficient in B-band LPS biosynthesis are more sensitive to serum killing (1, 4, 5) and more susceptible to phagocytosis (6) than wild-type bacteria. They are found almost avirulent in mouse models (2). B-band LPS is the basis for classification of P. aeruginosa in 20 different serotypes. Among these, serotypes O6 and O11 are the most clinically relevant in epidemiological studies (7). To date, the prognosis for a cystic fibrosis patient infected with either serotype of P. aeruginosa is rather poor due to intrinsic multidrug resistance of P. aeruginosa. Such resistance is due partly to a highly impermeable outer membrane and partly to the presence of multidrug efflux pumps (8–10). Hence, B-band LPS biosynthesis has become an important target for drug discovery.

The genetics of B-band LPS biosynthesis are well documented in serotypes O5, O6, and O11 (11–13) and were thoroughly reviewed recently (14). For each of these serotypes, the entire cluster of genes responsible for B-band LPS synthesis has been sequenced, and putative pathways for the synthesis of the corresponding O-antigens have been proposed based on homology studies. In serotype O11, the functional role of these genes awaits further studies. However, in serotypes O5 and O6, extensive functional characterization has been performed by knockout construction and complementation analysis, using not only genes from P. aeruginosa but also homologues found in other organisms. Despite these efforts, ambiguities persist that can only be alleviated by direct biochemical characterization of the proteins involved. Such a characterization will also allow screening for inhibitors that might be useful for therapeutic purposes, especially if performed for enzymes found in the clinically relevant serotype O6.

The O-antigen of B-band LPS of serotype O6 consists of a tetrasaccharide repeat of $\alpha$-a-3-O-acetyl, 6-amino-2-deoxy-2-formamido-D-galacturonate acid-$\alpha$-L-Rha-$\alpha$-a-2-acetamido-2,6-dideoxy-$\beta$-D-glucose-$\alpha$-a-2-acetamido-2,6-dideoxy-2-formamido-$\beta$-D-galactopyranose. The product of the epimerization reaction, UDP-GalNAc, is a precursor of surface-associated carbohydrate synthesis (12, 18, 19). The product of the epimerization reaction, UDP-GlcNAc, is a product of the tetrasaccharide repeat of $\alpha$-a-3-O-acetyl, 6-amino-2-deoxy-2-formamido-D-galacturonate acid-$\alpha$-L-Rha-$\alpha$-a-2-acetamido-2,6-dideoxy-$\beta$-D-glucose-$\alpha$-a-2-acetamido-2,6-dideoxy-2-formamido-$\beta$-D-galactopyranose. The enzyme can also catalyze the interconversion of non-acetylated substrates, although the efficiency of catalysis is significantly lower. 15 and 40% of UDP-Glc and UDP-Gal, respectively, are converted at equilibrium. WbpP contains tightly bound NAD(H) and does not require additional cofactors for activity. It exists as a dimer in its native state. This paper is the first report of expression and characterization of a C4 UDP-GlcNAc epimerase at the biochemical level. Moreover, the characterization of the enzymatic function of WbpP will help clarify ambiguous surface carbohydrate biosynthetic pathways in P. aeruginosa and other organisms where homologues of WbpP exist.

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The abbreviations used are: LPS, lipopolysaccharide; DMAB, p-dimethylaminobenzaldehyde; SDR, short chain dehydrogenase/reductase; CE, capillary electrophoresis; PAGE, polyacrylamide gel electrophoresis. IPTG, isopropyl-1-thio-$\beta$-D-galactopyranoside; CV, column volumes.
identity with WcdB, an enzyme thought to be involved in the formation of GalNAcA residues present in the Vi polysaccharide of Salmonella typhi (19). Disruption of the wbpP gene in a knockout mutant results in loss of B-band LPS production in P. aeruginosa, and this deficiency is fully alleviated after complementation by the wcdB homologue (12). Although no biochemical evidence is available for either WbpP or WcdB, sequence comparisons with other proteins and carbohydrate composition analysis suggest that they are C4 epimerases that transform UDP-Glc into UDP-GalNAc in vivo.

A functional assignment relying mainly on homology studies is particularly problematic in the case of putative epimerases. Epimerases belong to the short chain dehydrogenase/reductase (SDR) enzyme family. This family includes enzymes responsible for a wide variety of functions (20–22). Most of these enzymes possess common features that include the presence of alternating α-helices. The conserved catalytic triad is highlighted in bold. The GXXGXXG signature for nucleotide-binding proteins and the GXXGXXG signature for NAD(P) binding proteins is highlighted in bold. Secondary structure predictions were made using the Expasy molecular biology software, expasy.hcuge.ch.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unless stated otherwise all chemical reagents used were from Sigma. Restriction enzymes and T4 DNA ligase were from Life Technologies, Inc. Pwo DNA polymerase was from Roche Molecular Biochemicals. The dNTPs were from Perkin-Elmer. The His$_a$-histidine tag antibody was from Qiagen (Santa Clarita, CA). Agar was from Difco. All kits or enzymes were used following the manufacturer’s instructions.

**Cloning and Overexpression of WbpP in the pET System—**WbpP was cloned into the Ncol and EcoRI sites of a pET23 derivative (26) with an N-terminal histidine tag. The sequence of the primers used to amplify wbpP by polymerase chain reaction from genomic DNA (strain IATS O6) were 5’CAATGCGCATGGGATGATGCTTATGAAG3’ and 5’TAAAGAATTCTCATTCAAAAACATGAG3’ for the top and bottom primers, respectively. The polymerase chain reaction consisted of 100 ng of genomic DNA, 0.5 μM each primer, 0.2 mM each dNTP, 4 mM MgCl$_2$, and 1 μl buffer in a total of 50 μl. A 5-min denaturation at 94 °C was done before addition of DNA polymerase (1.5 units of Pwo). This was followed by 15 cycles of 1 min at 94 °C, 45 s at 55 °C, and 90 s at 72 °C. A final 7-min elongation was performed at 72 °C. The constructs obtained were checked by restriction analysis and sequencing.

The construct was subsequently transformed into the expression strain BL21(DE3)/pLysoS (Novagen, Madison, WI) with ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) selection. For protein expression, a strain of an overnight culture were inoculated into 10 ml of LB in the presence of ampicillin and chloramphenicol. The culture was grown at 30 °C. When the A$_{600}$ nm reached 0.6, IPTG (Promega, Madison, WI) was added to a final concentration of 0.15 mM and expression was allowed to proceed for 5–6 h at 30 °C. Cells were harvested by centrifugation at 5,000 × g for 15 min at 4 °C and the pellet was stored at −20 °C until needed. Expression was monitored by SDS-PAGE analysis, with Coomasie blue staining or Western immunoblot using the penta-His anti-histidine tag antibody as instructed by the manufacturer.

**Purification of WbpP by Chromatography—**Cells sedimented from 100 ml of induced culture were resuspended in 10 ml of buffer A (5 mM imidazole, 20 mM Tris, pH 8, 0.1 M NaCl). The cells were briefly sonicated (macrotip, sonicator XL2020 Heat Systems Inc., power set to 4, 2 min total, 5 s on, 5 s off) on ice. Cell debris was removed by centrifugation at 13,000 × g for 15 min at 4 °C, and the supernatant was

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**Fig. 1.** Comparison of the primary and secondary structural features of three members of the short chain dehydrogenase/reductase family. WbpP from P. aeruginosa serotype O6, the C4 UDP-Glc epimerase GaLE from E. coli, and the dTDP-glucose-4,6-dehydratase RFFG from E. coli. +, identical amino acids; *, homologous amino acids; green letters, α-helices; pink letters, β-sheets; red letters, γ-helices. The GXXGXXG signature for NAD(P) binding proteins is highlighted in bold. Secondary structure predictions were made using the Expasy molecular biology software, expasy.hcuge.ch.
applied to a 3-ml fast flow chelating Sepharose column (Amersham Pharmacia Biotech) previously loaded with nickel sulfate (30 ml of 0.1 M) and equilibrated with 5 column volumes (CV) of buffer A. Loading of the sample as well as all washing and elution steps were done by gravity. After loading of the sample, the column was washed with 10 CV of buffer A. Buffer B was then added to the column (20 ml of 0.1 M NaCl). Elution was carried out with 3 CV of buffer C (1 mM imidazole, 20 mM Tris, pH 8, 0.1 M NaCl). Fractions were collected every 1 CV. Most of the protein was eluted in fraction 2 as seen by SDS-PAGE analysis. This fraction was subjected to further purification by anion exchange chromatography after dialution 1/30 in 50 mM Tris, pH 8. Half of it was loaded onto a 1-ml column of Sephacryl S-200 (Amersham Pharmacia Biotech). The column was washed with 30 CV of Tris buffer, and the protein was eluted with 3 CV of 50 mM Tris, pH 8, 0.5 M NaCl. Fractions were collected every 1 CV, and most of the protein was recovered in fraction 2. This fraction was desalted by overnight dialysis (cut-off 3500 Da) in 50 mM Tris, pH 8, at 4 °C. The dialyzed samples were concentrated by overlay with PEG 8000 (Sigma) for 2–3 h at 4 °C. Protein quantitation was done using the BCA reagent (Pierce). The purified enzyme was either used fresh or stored at −20 °C in 25% glycerol or 20% adonitol in 50 mM Tris, pH 8, without any significant loss of activity.

**Determination of the Oligomerization Status by Gel Filtration Analysis**—A 45 × 1.6-cm column containing 90 ml of Sephacryl G-100 (Sigma) was used for gel filtration. Standard curves were prepared to determine the oligomerization status of WbpP. The column was equilibrated in 50 mM Tris, pH 8, containing 100 mM NaCl and run at 1.4 ml/min. Molecular weight standards (Sigma, 12–150 kDa) were applied onto the column one by one (50–200 μg each in 200 μl). WbpP was applied onto the column either as a concentrated or a diluted solution (200 μg or 50 μg/200 μl deposited). Protein elution was monitored at 280 nm.

**Extraction of NADH from Purified WbpP**—A freshly purified and extensively dialyzed sample of WbpP at 1.75 mg/ml in 50 mM Tris, pH 8, was used for the extraction and quantification of bound NAD(H).

WbpP (175 μg) was incubated in the presence of 10 μM of proteinase K for 45 min at 37 °C. Total digestion of WbpP was checked by SDS-PAGE analysis and Coomassie staining. After complete digestion, WbpP was subjected to chemical reduction by successive additions of 1 μl of 10 mg/ml sodium borohydride (Fisher) every 30 min for 2 h. The proteolysis step was included prior to reduction to ensure quantitative reduction and recovery of NAD(H). The absorption spectrum was recorded before and after chemical reduction between 230 and 450 nm using a DU582 spectrophotometer (Beckman Instruments, Fullerton, CA) equipped with a 50-μl microcell. Serial dilutions of NAD* (Sigma) ranging from 40 to 50 μM were prepared in 50 mM Tris, pH 8, and were incubated at 37 °C for the same amount of time as WbpP with or without chemical reduction. The precise concentration in NAD was calculated using ε_{240 nm} = 17400 M⁻¹ cm⁻¹ and the efficiency of reduction was calculated using ε_{260 nm} = 6270 M⁻¹ cm⁻¹.

**Determination of the Enzymatic Conversion of UDP-GlcNAc and UDP-GalNAc Using δ-Dimethylaminobenzaldehyde (DMAB)**—The enzymatic reactions were quenched after 45 min at 37 °C by neutralization of the final sugar nucleotide concentrations ranging from 0.075 to 1.75 mM, and the reactions were quenched after 3 min of incubation. Capillary electrophoresis (CE) analysis was performed using a PACE 5000 system (Beckman Instruments, Fullerton, CA) with UV detection. The running buffer was 25 mM sodium tetraborate, pH 9.4. Three total reactions were run, 20 min each, at a detector position of 57 cm, with a detector flow of 50 cm. The capillary was conditioned before each run by washing with 0.2 mM NaOH for 2 min, water for 2 min, and running buffer for 2 min. Samples were introduced by pressure injection for 4 s, and the separation was performed at 22 kV. Peak integration was done using the Beckman PACE Station software. The calculation of kinetic parameters was done using the PRISM software.

**Study of the Requirement for NAD** or Divalent Cations for Enzymatic Activity—To assess the requirements for NAD* or divalent cations for the enzymatic activity of WbpP, reactions were carried out with or without NAD* (1 mM final concentration) and with or without divalent cations (4 mM final concentration of MnCl₂, MgCl₂, or CaCl₂) monitored by capillary electrophoresis as described above.

**Spectrophotometric Study of the Epimerization of UDP-Glc and UDP-Gal by WbpP**—The enzymatic reactions were performed in 20 mM Tris, pH 8, with 39 μg of freshly purified enzyme and 0.8 mM sugar nucleotide in a total reaction volume of 44 μl. Time course studies were performed over 2 h at 37 °C. After incubation for the required amount of time, the reactions were quenched by acid hydrolysis of the UDP moiety as described above. The reaction mixtures were concentrated by overlay with PEG 8000 (Sigma) previously loaded with nickel sulfate (30 ml of 0.1 M), and the protein was eluted with 3 CV of 50 mM Tris, pH 8, with 39 μg of freshly purified enzyme and 0.8 mM sugar nucleotide in a total reaction volume of 44.8 μl. The total sugar nucleotide concentrations in the enzymatic reactions ranged from 0.045 to 2.009 mM. The reactions were quenched after 15 min of incubation at 37 °C. The samples were analyzed by CE in the same conditions as described above, and the K_m and V_max values were determined using the PRISM software.

**RESULTS**

**Protein Expression and Purification**—WbpP is a 37.7-kDa protein with a slightly acidic isoelectric point (pI = 5.99). It was expressed in the pET system as an N-terminally histidine-tagged protein. Provided that expression was carried out at low temperature (30 °C) and with a low concentration of inducer IPTG (0.15 mM), most of the protein was expressed in a soluble form (Fig. 2). It was expressed at a very high level since it represented 30–35% of total cellular proteins. It was readily purified to 90–95% by nickel chelation, and most of the contaminants were further eliminated by anion exchange chromatography to produce 95–98% pure protein. Therefore, the protein was purified only 3-fold to reach homogeneity. The yield obtained was 5–7 mg/100 ml of culture (Table I). The presence of the histidine tag was confirmed by Western immunoblot using an anti-histidine tag antibody (data not shown).

Results from gel filtration analysis suggest that WbpP exists as a dimer in its native form (data not shown). No apparent monomer or higher order oligomers were detected even in the presence of 100 mM salt or at low enzyme concentration.

**Determination of the Kinetic Parameters for UDP-Glc and UDP-Gal**—The spectrophotometric assays used for the Quantitation of GlcNAc and GalNAc—The spectrophotometric assay used to quantitate GlcNAc and GalNAc in enzymatic reactions relies on the use of DMAB which is specific for N-acetylhexosamines. Different colorimetric yields are obtained with different N-acetylhexosamines (27). For the two substrates relevant to this study, a much higher reaction yield (6 times) is obtained with GlcNAc than with GalNAc (Fig. 3A). The assay is very sensitive and allows discrimination between...
both substrates at low substrate concentration (0.15 mM). Moreover, the yields of reaction are additive. Hence, the composition of a mixture of GlcNAc and GalNAc obtained after enzymatic conversion can be calculated from standard curves established with each substrate separately (Fig. 3B).

### Functional Characterization of WbpP Using the DMAB Assay—

The results obtained for WbpP using the DMAB assay are consistent with a UDP-GlcNAc C4 epimerase activity. When the enzymatic reaction was performed with UDP-GlcNAc, the total yield of the reaction with DMAB decreased (Fig. 3C). This is consistent with the formation of GalNAc that reacts poorly with DMAB. Alternatively, when the enzymatic reaction was performed with UDP-GalNAc, the yield of the reaction with DMAB increased. This is consistent with the formation GlcNAc that reacts strongly with DMAB. The activity was dependent on the quantity of enzyme added (Fig. 3C). Maximum substrate conversions obtained were approximately 30% for UDP-GlcNAc and 70% of UDP-GalNAc. Less enzyme was required to obtain maximum substrate conversion for UDP-GalNAc than for UDP-GlcNAc. The specific activity of purified WbpP was 5.6 and 2.3 units/mg with regard to UDP-GalNAc and UDP-GlcNAc, respectively (Table 1). This represents only a 2-fold increase of the specific activity. This apparent low level of purification in terms of specific activity is due to the fact that the protein was expressed at a very high level.

### Characterization of the C4 UDP-GlcNAc Epimerase Activity by Capillary Electrophoresis Analysis—

Capillary electrophoresis was used to confirm the identity of the reaction products after enzymatic conversion of UDP-GlcNAc or UDP-GalNAc by WbpP by comparison with standard compounds. Under analytical conditions, UDP-GlcNAc and UDP-GalNAc are well resolved, with peaks at 11.6 and 12.3 min, respectively. Fig. 4A shows time-course studies of the epimerization of UDP-GlcNAc and UDP-GalNAc by WbpP. The reaction was performed with a 0.2 mM substrate substrate. The reaction was stopped by addition of stop buffer and the samples were subjected to capillary electrophoresis analysis. The results obtained for WbpP using the DMAB assay are consistent with a UDP-GlcNAc C4 epimerase activity. When the enzymatic reaction was performed with UDP-GlcNAc, the total yield of the reaction with DMAB decreased (Fig. 3C). This is consistent with the formation of GalNAc that reacts poorly with DMAB. Alternatively, when the enzymatic reaction was performed with UDP-GalNAc, the yield of the reaction with DMAB increased. This is consistent with the formation GlcNAc that reacts strongly with DMAB. The activity was dependent on the quantity of enzyme added (Fig. 3C). Maximum substrate conversions obtained were approximately 30% for UDP-GlcNAc and 70% of UDP-GalNAc. Less enzyme was required to obtain maximum substrate conversion for UDP-GalNAc than for UDP-GlcNAc. The specific activity of purified WbpP was 5.6 and 2.3 units/mg with regard to UDP-GalNAc and UDP-GlcNAc, respectively (Table 1). This represents only a 2-fold increase of the specific activity. This apparent low level of purification in terms of specific activity is due to the fact that the protein was expressed at a very high level.

### TABLE I

| Fraction                  | Vol. (ml) | Conc. (g/l) | Protein (mg) | Yield (%) | Substrate | Total units | Specific activity | Purification (x) |
|---------------------------|-----------|-------------|--------------|-----------|-----------|--------------|------------------|------------------|
| Total cell extract        | 10        | 5.2         | 52           | 100       | UDP-GlcNAc| 11           | 2.6              | 1                |
| Soluble fraction          | 10        | 3.3         | 33           | 64        | UDP-GlcNAc| 133          | 2.6              | 1                |
|                           |           |             |              |           | UDP-GlcNAc| 35           | 1.1              | 1                |
|                           |           |             |              |           | UDP-GalNAc| 113          | 3.4              | 1.3              |
| Anion exchange            | 3.5       | 2.8         | 9.7          | 19        | UDP-GlcNAc| 19           | 2.0              | 1.8              |
|                           | 5         | 1.2         | 5.8          | 11        | UDP-GlcNAc| 45           | 4.6              | 1.8              |
|                           |           |             |              |           | UDP-GalNAc| 45           | 4.6              | 1.8              |

*Conc. refers to the total protein concentration of the fraction tested for activity.

* One unit is defined as the amount of enzyme that allows conversion of 1 μmol of substrate in 1 min under our experimental conditions. The reactions were performed using 8.8 μl of enzyme fraction or cell extract and 0.75 mM substrate in a total volume of 44 μl. The activity was determined using the DMAB assay.

* Total cells extracts produce a high background of UDP-GlcNAc-modifying activity (9.5 units), mostly associated with the membrane fraction. In addition, the preferred direction of the reaction with WbpP is toward UDP-GlcNAc production (see kinetic data). Hence, very little difference is observed on total cell extracts expressing WbpP (20.5 units) or not (9.5 units) when reactions are performed with UDP-GlcNAc as a substrate. Therefore, the controls for analysis of total cell extracts or soluble fraction containing WbpP were total cell extract or soluble fraction of the same *E. coli* strain used for expression of WbpP but harboring the empty pET23 vector only. Also, for UDP-GlcNAc, the reference used for the purification is the specific activity obtained with the soluble extract only, where unspecified UDP-GlcNAc modification was not observed.

**Fig. 3. Study of the epimerization of UDP-GlcNAc and UDP-GalNAc by WbpP using the DMAB assay.** A, standard curves obtained with each compound separately. Open circles, UDP-GlcNAc; open squares, UDP-GalNAc. B, comparison of the experimental data (closed triangles) obtained for mixtures of UDP-GalNAc and UDP-GlcNAc of different proportions (constant total sugar nucleotide concentration of 0.75 mM) and the theoretical data (open triangles) calculated from the standard curves from A. C, activity of WbpP as a function of the amount of enzyme added. The reactions were performed with 0.75 mM substrate in a total volume of 35 μl for 8 min at 37 °C. Closed circles, UDP-GlcNAc; closed squares, UDP-GalNAc.
shows that UDP-GlcNAc and UDP-GalNAc are inter-converted into one another by WbpP, thus confirming its C4 epimerase activity on these substrates. At equilibrium, the yields of enzymatic conversion are the same as calculated from the DMAB assay data.

Determination of the Kinetic Parameters for UDP-GlcNAc and UDP-GalNAc by Capillary Electrophoresis—Time course experiments performed with different enzyme dilutions indicate that the rate of conversion of UDP-GlcNAc is much slower than that of UDP-GalNAc at equal enzyme dilution (Fig. 5). Initial rate conditions were selected by choosing the enzyme dilutions that allow transformation of less than 10% of the substrate in 3 min, for substrates concentrations ranging from 0.02 to 1.75 mM. The $K_m$ and $V_{max}$ parameters of WbpP for each substrate were determined under these initial rates conditions (Table II). The $K_m$ values derived from Eadie-Hofstee plots are 224 and 197 μM for UDP-GlcNAc and UDP-GalNAc, respectively. The enzyme shows an equal affinity for these substrates.

Determination of the Physicochemical Parameters: Optimal pH, Temperature, and Storage Conditions—WbpP has a broad pH range of activity, with significant activity observed for pH 6.5 and an optimum between pH 7 and 8 (data not shown). The enzyme is also active over a wide range of temperatures (data not shown) with an optimum between 37 and 42 °C. The enzyme can be kept active without any significant loss of activity when stored at −20 °C in 25% glycerol or 20% adonitol in 20 mM Tris, pH 8 (data not shown).

Substrate Specificity—A glucose-specific spectrophotometric assay (28) was used to study the substrate specificity for WbpP. By using this assay, it was shown that WbpP can use UDP-Glc as a substrate (Fig. 6), but the identity of the reaction product is unknown. Also, UDP-Glc was produced when the reaction was performed with UDP-Gal as a substrate. These results are consistent with a C4 epimerase activity on the non-acetylated substrates UDP-Glc and UDP-Gal. From these results, the product of UDP-Glc modification by WbpP is expected to be UDP-Gal, but its identity needs to be confirmed by analytical methods. Also, the rate of conversion was significantly higher for UDP-Gal than UDP-Glc at equal enzyme dilutions (Fig. 6). At equilibrium, approximately 40% of UDP-Gal was transformed to UDP-Glc, whereas only 15% of UDP-Glc was modified by the enzyme. Capillary electrophoresis analysis confirmed without ambiguity that WbpP has C4 epimerase activity on UDP-Glc and UDP-Gal (Fig. 7) and confirmed that the maximum conversions were 40 and 17% for UDP-Gal and UDP-Glc, respectively.

Determination of the Kinetic Parameters for UDP-Glc and UDP-Gal by Capillary Electrophoresis—The kinetic parameters determined under initial rates conditions are summarized in Table II. The $K_m$ values for UDP-Glc and UDP-Gal are 237 and 251 μM, respectively. The $V_{max}$ values are 54 and 82 pmol/min.

Analysis of NAD$^+$ or Divalent Cation Requirements by Capillary Electrophoresis—The addition of NAD$^+$, Mg$^{2+}$, Ca$^{2+}$, or Mn$^{2+}$ to the reaction mixture was not necessary for the C4 epimerase activity of WbpP, would it be on the acetylated or non-acetylated forms of the substrates as determined by capillary electrophoresis (data not shown).

Extraction of NAD$^+$ /NADH from Purified WbpP—Tightly bound NAD$^+$ /NADH could be extracted from highly purified and extensively dialyzed WbpP after complete digestion with proteinase K. The released nucleotide was reduced to NADH by sodium borohydride treatment. A yield of 0.7–0.8 mol of NAD(II)/mol of WbpP was calculated from the absorbance at 340 nm (data not shown). This indicates that WbpP binds to the nucleotide tightly during its synthesis.

DISCUSSION

UDP-GlcNAc is an essential precursor of surface carbohydrate biosynthesis (29), both in bacteria, where it is the precursor of peptidoglycan, capsule, or lipopolysaccharide biosynthesis, and in humans, where it is the main precursor involved in cell surface sialylation (30). Although the requirements of UDP-GlcNAc-modifying enzymes such as C2 and C4 epimerases or C6 dehydratases (12, 13, 30–32) have been inferred from in vivo experiments and structural analysis of various surface carbohydrates, very little information is available at the biochemical level on the enzymes responsible for such activities.

WbpP is a small protein essential for the biosynthesis of B-band LPS in P. aeruginosa serotype O6 (12). Prior to this study, the exact function of this enzyme was unknown. Sequence analysis showed that it belongs to the short chain dehydrogenase/reductase (SDR) family. The variety of enzymatic functions represented in the SDR family doesn’t allow for a specific functional assignment for WbpP. Most enzymes be-

FIG. 4. Capillary electrophoresis analysis of the epimerization of UDP-GlcNAc and UDP-GalNAc by WbpP at equilibrium. The reactions were performed in a total volume of 35 μl with 1.5 mM substrate and 17 μg of enzyme. They were incubated at 37 °C for 2 h. 1, UDP-GalNAc alone; 2, UDP-GlcNAc alone; 3, UDP-GalNAc + WbpP; 4, UDP-GlcNAc + WbpP.
UDP-Gal by WbpP using the glucose oxidase-coupled assay. Measurements were made per time point on the same enzymatic reaction. The reactions were performed in a total volume of 35 μl with 1.5 mM substrate and 17 μg of enzyme. They were incubated at 37 °C for 2 h. 1, UDP-Gal alone; 2, UDP-Glc alone; 3, UDP-Gal + WbpP; 4, UDP-Glc + WbpP.

longing to this family share the same initial steps of catalysis resulting in the formation of a 4-hexosulose intermediate that can subsequently lead to the formation of a variety of new carbohydrates such as epimers, deoxy sugars, or branched carbohydrates. Therefore, belonging to this family is not a sufficient criteria for specific functional assignment. Comparisons of the LPS composition of organisms that exhibit WbpP or a homologue suggested that WbpP might be a C4 epimerase specific for UDP-GlcNAc. The validity of such an assignment is supported by successful complementation of a wbpP null mutant of P. aeruginosa by an S. typhimurium homologue, wcdB. This homologue of wbpP has been shown to be involved in the biosynthesis of a homopolymer of α-1,4 2-deoxy-2-N-acetylgalactosamine uronic acid (19). However, another homologue, WbpK, showing 51% homology to WbpP, is localized in the gene cluster for B-band LPS biosynthesis in P. aeruginosa serotype O5 (PAO1) where its function is at present unknown. The O5 LPS contains FucNAc, which was previously proposed to arise from epimerization of UDP-GlcNAc to UDP-GalNAc followed by dehydration and reduction to UDP-FucNAc. Hence, a UDP-GlcNAc C4 epimerase activity was also expected to exist in serotype O5. WbpKO5 was the best candidate for such an epimerase as judged by its high level of homology to WbpPO6. Complementation analysis using a WbpKO5 knockout showed that WbpPO6 is not able to rescue LPS biosynthesis in PAO1 (this study, data not shown). This suggests that WbpPO6 and WbpKO5 have a different function and/or substrate specificity despite their high level of sequence conservation. Hence, in addition to providing the first description of a UDP-GlcNAc C4 epimerase at the biochemical level, the characterization of WbpP will also be useful to clarify ambiguous biosynthetic pathways for LPS biosynthesis in organisms that possess homologues of WbpP.

As mentioned previously, the existence of UDP-N-acetylgalactosamine 4-epimerase activity has been inferred from the analysis of the surface carbohydrates of a variety of organisms or even mammalian tissues. However, the experimental demonstration of the existence of the activity has only been reported on two occasions. The first one was the description of both UDP-GlcNAc and UDP-GalC4 epimerase activity associated with a protein fraction isolated from porcine submaxillary gland (33). In this study, the purified enzyme performs with equal or higher efficiency on the non-acetylated substrates than on the acetylated ones. Hence, it is doubtful that the activity arises from a genuine UDP-GlcNAc C4 epimerase but rather is a side reaction of a standard GalE homologue. The sequence of the enzyme was not provided to resolve the question. In the second case, a UDP-N-acetylgalactosamine 4-epimerase activity was linked with the gneA locus in Bacillus subtilis (34). Assays were performed using whole cell extracts, and the enzyme was not purified. Considering that the substrate and product involved in this reaction are shared by a variety of sugar nucleotide-modifying enzymes, results obtained using whole cell extracts are not unequivocal. The biochemical characterization described in this study and performed in vitro using overexpressed and purified enzyme is the first unambiguous demonstration of the existence of a specific UDP-GlcNAc C4 epimerase and provides the first kinetic analysis of such an enzyme.

### Table II

| Substrate     | Km (μM) | Vmax (pmol/min) | Enzyme | kcat (min⁻¹) | kcat/km (min⁻¹) |
|---------------|---------|-----------------|--------|--------------|-----------------|
| UDP-GalNAcα   | 197 ± 15| 840 ± 25        | 3.1    | 271 ± 7      | 1375 ± 142      |
| UDP-GlcNAcα   | 224 ± 17| 741 ± 22        | 6.2    | 120 ± 3      | 536 ± 57        |
| UDP-Galβ      | 251 ± 16| 82 ± 3          | 436    | 0.188 ± 0.007| 0.749 ± 0.06    |
| UDP-Glcβ      | 237 ± 53| 54 ± 6          | 436    | 0.124 ± 0.014| 0.523 ± 0.18    |

α Three independent experiments were performed where the range of substrate concentrations was shifted toward lower concentrations, and the enzyme was used at higher dilutions to refine the value of the parameters obtained. The results presented in this table are the results of the last experiment.

β Two independent experiments were performed and analyzed using the spectrophotometric assay to get an estimation of the Km and Vmax parameters. A third experiment was performed with a wider substrate concentration range including 5 points below the estimated Km to refine the values of the parameters. Very similar kinetic parameters were obtained in the three experiments, but the error was considerably lower using CE data. Therefore, the results presented in this table are the results of the last experiment that were obtained by CE analysis.
Although numerous spectrophotometric assays are available to study the UDP-Glc C4 epimerase activity, none is available for the UDP-GlcNAc C4 epimerase activity. Most assays rely on the coupling of the epimerization reaction to a secondary enzymatic reaction that is usually very specific for the substrate or product in its non-acetylated form (28, 35). A spectrophotometric assay using DMAB was designed to measure C4 epimerase activity on the N-acetylated substrates, UDP-GlcNAc and UDP-GalNAc. The results obtained with the DMAB assay as described in this study are consistent with a C4 epimerase activity involving UDP-GlcNAc and UDP-GalNAc. But other activities resulting in the production of different N-acetylated osamines derivatives with different reactivities toward DMAB cannot be excluded. Hence, capillary electrophoresis was used to provide the proof for the identity of the reaction products. The results from CE analysis clearly confirmed that WbpP is a UDP-GlcNAc C4 epimerase.

The kinetic analysis was carried out under initial rate conditions using the standard Michaelis-Menten model. One of the assumptions of this model is that no product can be used as a substrate. The initial rate conditions used in our study ensured that no more than 10% of the substrate was used up by the enzyme, hence maintaining product re-conversion to a minimum. The kinetic analysis revealed that WbpP has the same affinity for UDP-GalNAc and UDP-GlcNAc, but the reaction proceeds at a faster rate for the former than the latter. Moreover, the \( \frac{k_{cat}}{K_m} \) shows that for an equal amount of enzyme present in the reaction, the conversion of UDP-GalNAc to UDP-GlcNAc is more efficient than the reverse reaction. This is also apparent at equilibrium where 70% of UDP-GalNAc are converted to UDP-GlcNAc, whereas only 30% of UDP-GlcNAc are converted to UDP-GalNAc. Hence, in vitro, the equilibrium is shifted toward the production of UDP-GlcNAc. Such a shift of the equilibrium toward the production of the glucose isomer has been previously reported for GalE from E. coli (35). However, this is opposite to what is expected in vivo and in the pathway proposed for O-antigen biosynthesis in serotype O6. The use of the product by the next enzyme involved in the B-band LPS biosynthetic pathway pulls the equilibrium toward the production of UDP-GalNAc in vivo. This could be part of a regulatory mechanism. When the biosynthesis of LPS is down-regulated as a function of varying environmental conditions (36), the UDP-GlcNAc stock is not depleted by the activity of WbpP and as a function of varying environmental conditions (36), the mechanism. When the biosynthesis of LPS is down-regulated at equilibrium where 70% of UDP-GalNAc are converted to UDP-GlcNAc, whereas only 30% of UDP-GlcNAc are converted to UDP-GalNAc. Hence, in vitro, the equilibrium is shifted toward the production of UDP-GlcNAc. This is opposite to what is expected in vivo and in the pathway proposed for O-antigen biosynthesis in serotype O6. The use of the product by the next enzyme involved in the B-band LPS biosynthetic pathway pulls the equilibrium toward the production of UDP-GalNAc in vivo. This could be part of a regulatory mechanism. When the biosynthesis of LPS is down-regulated as a function of varying environmental conditions (36), the UDP-GlcNAc stock is not depleted by the activity of WbpP.

For both series of substrates, the enzyme is active without requiring addition of exogenous NAD\(^+\) or divalent cations such as Mg\(^{2+}\), Mn\(^{2+}\), or Ca\(^{2+}\). However, the mechanism of C4 epimerization implies the participation of a NAD\(^+\) molecule as an essential coenzyme (37). This molecule is predicted to be bound in the Rossman fold delineated by the alternating \( \alpha \)-helix and \( \beta \)-sheet structures and the GXXGXXG motif at the N terminus of the protein. The binding site has been mapped by NMR (43) and crystallography studies (24, 44, 45) in GalE from E. coli. In GalE, the NAD\(^+\) molecule is a redox cofactor responsible for reversibly and non-stereospecifically dehydrogenating carbon 4 in the pyranosyl rings of UDP-Glc and UDP-Gal. This NAD\(^+\) molecule does not dissociate from the enzyme either in the course of catalysis or between catalytic cycles. However, an NAD\(^+\)-independent epimerase that carries its function via carbon-carbon bond cleavage rather than by a simple deprotonation-reprotonation mechanism was recently described (46). In the case of WbpP, the enzyme can still perform the epimerization of both UDP-Gal and UDP-Glc with \( K_m \) values of the same order as those for the acetylated substrates. However, the \( k_{cat} \) and \( V_{max} \) values clearly indicate that the catalysis is ~1000-fold less efficient with these substrates than with the acetylated ones. Moreover, the \( k_{cat}/K_m \) ratio indicates that the binding is quite poor, especially for UDP-Glc. This is reflected by the fact that the epimerization of the non-acetylated substrates requires the presence of significantly higher amounts of enzyme than the epimerization of the acetylated substrates.

As observed for the acetylated substrates, the equilibrium is also shifted toward the production of UDP-Glc, but the maximum percentages of substrate conversion are much lower than in the previous case. Only 40% of UDP-Gal are converted to UDP-Glc at equilibrium, and around 12% of UDP-Glc are converted to UDP-Gal. Although WbpP can epimerize the non-acetylated substrates in vitro, the poor efficiency of catalysis and high amounts of enzyme necessary to carry such reactions indicate that these reactions are unlikely to happen in vivo and that the acetylated forms of the substrates are the preferred ones in vivo. Determination of the three-dimensional structure and site-directed mutagenesis studies of WbpP will help decipher the molecular basis for substrate specificity in this enzyme. In P. aeruginosa, a genuine UDP-Glc C4 epimerase activity is required for the synthesis of the galactose residue found in the LPS core. Since our data show that UDP-Glc is not the preferred substrate for WbpP, this activity might be carried by a yet uncharacterized homologue of WbpP. This is consistent with the fact that inactivation of WbpP by gentamicin cassette insertion and allelic replacement does not result in the production of a truncated core in serotype O6 (12). This is also consistent with the observation that Southern blotting experiments using the \( wbpP \) gene as a probe reveal the existence of homologues in all 20 serotypes of \( P. \) aeruginosa that share common core structural motifs.

Overall, the \( K_m \) values determined for WbpP and its different substrates are within the range of values reported in the literature for GalE epimerases from different sources (28, 33, 35, 41, 42). The wide variety of methods employed to assay activity and the varying degrees of purity of enzyme preparations used in these studies do not allow for more detailed comparisons of the kinetic parameters.
Most SDR enzymes exist as dimers or tetramers in their native state (20). Our gel filtration data suggest that WbpP also forms a dimer. However, in contrast to what has been previously described for a UDP-GlcNAc C2 epimerase (47), no allosteric behavior was observed for WbpP.

In conclusion, this paper describes the first overexpression, purification, and biochemical characterization of a C4 epimerase that shows strong specificity for the UDP-GlcNAc and UDP-GalNAc. Moreover, the unambiguous functional assignment of WbpP provides valuable information to help clarify surface carbohydrate biosynthetic pathways in a variety of organisms where no biochemical data were available.

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Expression, Purification, and Biochemical Characterization of WbpP, a New UDP-GlcNAc C4 Epimerase from *Pseudomonas aeruginosa* Serotype O6

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