WbpO, a UDP-N-acetyl-d-galactosamine Dehydrogenase from Pseudomonas aeruginosa Serotype O6*

WbpO is associated with B-band lipopolysaccharide biosynthesis in Pseudomonas aeruginosa serotype O6. This protein is thought to catalyze the enzymatic conversion of UDP-N-acetyl-d-galactosamine (UDP-GalNAc) to UDP-N-acetyl-d-galactosaminuronic acid (UDP-GalNAcA). WbpO was overexpressed with a C-terminal hexahistidine tag. The soluble form of expressed WbpO (WbpOSol) exhibited a secondary structure with 29.2% $\alpha$-helix and 20.1% $\beta$-strand. However, no enzymatic activity could be detected using either high performance anion exchange chromatography or capillary electrophoresis-mass spectrometry analysis. An insoluble form of expressed WbpO was purified in the presence of guanidine hydrochloride by immobilized metal ion affinity chromatography. After refolding, this preparation of WbpO (designated as WbpORf) exhibited a secondary structure at pH 7.5 to 8.2, and it was enzymatically active. Capillary electrophoresis-mass spectrometry analysis showed that WbpORf catalyzed the conversion of UDP-GalNAc to UDP-GalNAcA. 26 and 22% of the substrate could be converted to UDP-GalNAcA in the presence of NAD+ and NADP+ as the cofactors, respectively. The $K_m$ values of WbpORf for UDP-GalNAc, NAD+, and NADP+ were 7.79, 0.65, and 0.44 mM, respectively. WbpORf can also catalyze the conversion of UDP-GlcNAc to UDP-GlcNAcA. In conclusion, this is the first report of the overexpression, purification, and biochemical characterization of an NAD+/NADP+-dependent UDP-GalNAc dehydrogenase. Our results also complete the biosynthetic pathway for GalNAcA that is part of the O-antigen of P. aeruginosa serotype O6 lipopolysaccharide.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that poses a threat to compromised individuals including those with cystic fibrosis, trauma, and burn wounds (1). Lipopolysaccharide (LPS)1 is one of many virulence factors in P. aeruginosa, and it is also implicated as the surface antigen interacting with cystic fibrosis transmembrane regulator protein on epithelial cells of healthy individuals (2). P. aeruginosa produces simultaneously two forms of LPS, designated A-band and B-band (3). A-band LPS is a common antigen and is composed of trisaccharide repeating units of $\alpha$-d-rhamnose. B-band LPS O-antigen is serotype-specific. It is composed of a heteroplymer of di- to pentasaccharide repeating units. Due to its importance in virulence and its potential as a vaccine component, LPS in P. aeruginosa has been extensively studied at the molecular level (4).

The International Antigenic Typing Scheme serotype O6 is the most clinically prevalent serotype among all P. aeruginosa strains (5–7). Its B-band O-antigen is a repeating linear tetrasaccharide containing $\alpha$-L-rhamnose, $\alpha$-N-acetyl-$\alpha$-D-2,6-dideoxy-glucosamine (N-acetyl-$\alpha$-D-quinovosamine), and two $\alpha$-d-galactosaminuronic acid residues, one of which is formylated and the other acetylated (GalNAcA) (8, 9). Structural analysis of polysaccharides with mass spectrometry, high performance liquid chromatography (10), and NMR (11–16) has indicated that a-0-GalNAcA is one of the most common acidic sugars identified in the O-antigens or capsular polysaccharides of virulent Gram-negative bacteria. Reddy et al. (11) suggested that bacterial capsules and LPS containing acidic sugar repeats play an important part in bacteria and host cell interactions. Examples of a-0-GalNAcA containing polysaccharides among bacteria include the following: O-antigen polysaccharide of P. aeruginosa serotype O6 (4), Pseudomonas solanacearum (12), Pseudomonas fluorescens biovar B (17), Shigella-like Escherichia coli O121 (16), Acinetobacter spp. (13), and Vibrio anguillarum (12); as well as capsular polysaccharide of Salmonella typhi (18), Staphylococcus aureus (19), and Vibrio vulnificus (11, 15). Although the genes that are involved in the biosynthesis of a-0-GalNAcA have been described in a few studies (1, 18, 19), the enzyme activities of these genes have not been elucidated. At present UDP-GalNAcA is not commercially available; therefore, a better understanding of the enzyme involved in the synthesis of this uronic acid sugar would provide the methodology for its production.

The sequencing and characterization of the complete cluster of wbp genes involved in the biosynthesis of P. aeruginosa serotype O6 LPS has recently been accomplished by our laboratory (20). Based on homology analysis, one of the genes of the O6 LPS biosynthetic cluster, wbpO, was thought to be an enzyme involved in the synthesis of $\alpha$-0-GalNAcA. Importantly, WbpO is implicated as the surface antigen interacting with cystic fibrosis transmembrane regulator protein on epithelial cells of healthy individuals (2).

* This work was supported in part by Grant MT14796 (to J. S. L.) from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipients of the Canadian Cystic Fibrosis Foundation.

† Current address: Dept. of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611-0880.

‡§ To whom correspondence should be addressed: Dept. of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Tel.: 519-824-4120, ext. 3823; Fax: 519-837-1820; E-mail: jiam@uoguelph.ca.

¶¶ To whom correspondence should be addressed: Dept. of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Tel.: 519-824-4120, ext. 3823; Fax: 519-837-1820; E-mail: jiam@uoguelph.ca.

1 The abbreviations used are: LPS, lipopolysaccharide; HPAEC, high performance anion exchange chromatography; CE-MS, capillary electrophoresis-mass spectrometry; MS-MS, tandem mass spectrometry; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; GdnHCl, guanidine HCl; UDP-GalNAcA, UDP-N-acetyl-$\alpha$-galactosaminuronic acid; IMAC, immobilized metal ion affinity chromatography.
a knockout mutant of wbpO is deficient in B-band LPS production (20). Furthermore, WbpO has significant homology to VipA (64% identity), an enzyme presumed to be involved in the formation of O-GalNAcA molecule of Vi-antigen in S. typhi (18). A wbpO mutant was successfully complemented by vipA, and the production of B-band LPS of P. aeruginosa O6 was fully restored (20). This indicated that WbpO and VipA are functional homologues of each other. These observations suggested that WbpO could be a UDP-N-acetyl-D-galactosamine (UDP-GalNAcA) dehydrogenase that catalyzes the conversion of UDP-GalNAcA to UDP-GalNAcA, which may be an intermediate in the synthesis of the α-1-fucosylation moiety of the B-band O-unit in serotype O6. This study is the first report of the expression, purification, and function identification of an enzyme, WbpO, involved in the synthesis of UDP-GalNAcA.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis**—Amino acid homology analysis of WbpO was accomplished by using Basic Local Alignment Search Tool (BLAST) through data base Non-redundant GenBank CDS (21). Protein motif analysis was performed by using the San Diego Supercomputer Center—Multiple EM for Motif Elicitation data base via the.

**Construction of WbpO with a C-terminal His Tag and Sequencing**—wbpO was amplified by polymerase chain reaction with the following primers. The upstream primer was 5′-TTT GGT ACA TAT GAA GGA TCT GAA GGT TGC A-3′, and the downstream primer was 5′-TAT TAC TCG AGA GAC AGG CGT AGA TCA GAC-3′, which contained Ndel and XhoI restriction sites, respectively. The downstream primer also contained the indicated mutation sites to change the original stop codon TAA to TCT coding serine. This polymerase chain reaction product was cloned into the Ndel and XhoI sites of pET30a expression vector (Novagen, Madison, WI) and the sequence encoding His tag on pET30a was in frame with wbpO. This construct was transformed into E. coli JM109 by CaCl2 transformation (22). Transformants were selected on Luria agar (Fisher) containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol with agitation and then inoculated at 2% (v/v) into 300 ml of fresh TB medium and grown at 37 °C to an absorbance at 600 nm of 0.6. Overexpression of the recombinant WbpO was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3.5 h. The cells were harvested by centrifugation at 6,000 × g for 10 min, and the cell pellet was stored at −20 °C.

**Protein Concentration**—Protein concentration was determined by the BCA method (24) following the procedure described by the manufacturer (Fierce), and bovine serum albumin was used as the standard. WbpO Purification and Refolding—Protein cell pellet (0.4 g, wet weight) was suspended in 20 ml of suspension buffer (20 mM Tris-HCl and 0.5 mM NaCl, pH 8) and sonicated on ice for 2 min with Sonicator Ultrasonic Processor XL 2020 (MANDEL Scientific Company Ltd., Guelph, Ontario, Canada). The cell extract was centrifuged at 10,000 × g for 20 min.

The pellet containing the insoluble WbpO was dissolved in 5 ml of suspension buffer with the addition of 6 M guanidine HCl (GdnHCl) and 5 M imidazole. It was purified by immobilized metal ion affinity chromatography (IMAC) on a chelating Sepharose Fast Flow resin (Amersham Pharmacia Biotech) using nickel as a chelating agent, which has high selective binding for proteins with His6 tags, under denaturing conditions. The resin was first charged with 50 mM NiSO4 and then equilibrated with loading buffer (6 M GdnHCl, 5 mM imidazole in 10 mM Tris-HCl, pH 8). Crude WbpO in 6 M GdnHCl was loaded 3 times onto a 1.6-cm diameter column containing 3 ml of resin. The column was first washed with 20 bed volumes of loading buffer and then 4 bed volumes of 20 mM imidazole in loading buffer. WbpO was eventually eluted with 4 bed volumes of 200 mM imidazole in loading buffer and 10 bed volumes of 200 mM imidazole and 10 bed volumes of 500 mM imidazole. The refolding of WbpO began with reducing GdnHCl concentration from 6 to 2 M by dilution with 20 mM Tris-HCl, pH 8.0, at a rate of 0.5 mM/min−1. At the completion of this step, 5 mM reduced and 1 mM oxidized glutathione were added. A protease inhibitor mixture that contains 4-(2-aminoethyl)benzenesulfonyl fluoride, bestatin, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane, and N-[4-(2-aminophenyl)oxo-hexyloxyphosphoryl]-Leu-Trp(phosphoramidon) was added. The protein solution was then slowly stirred at 4 °C for 30 h to allow refolding, followed by slow dilution at an extremely low rate of 0.2 mM/min−1 and concentration with ultrafiltration. Ultrafiltration was performed under 50 pounds/square inch N2 in a stir cell with YM3 membrane (Amicon, Sunnyvale, CA) which has a molecular mass cut-off of 30 Da. GdnHCl concentration was below 200 mM, and WbpO was eluted onto 200 ml of Tris-HCl buffer, pH 8.0, with 3 buffer changes. All the purification and refolding procedures were performed between pH 7.5 and 8.0, which is well above the pI 5.8 of WbpO to minimize protein precipitation. Any precipitate observed during the refolding and dilution steps was removed by filtration through a 0.2-μm filter.

The supernatant of the cell extract containing the soluble WbpO (WbpOref) was subjected to purification under native conditions, which was carried out under the same conditions as the purification under denatured condition except that no GdnHCl was present. WbpOref was eluted with 40–120 mM imidazole and then dialyzed against 20 mM Tris-HCl, pH 8.0.

**Protein Analysis**—The protein fraction in 6 M GdnHCl eluted from IMAC column was dialyzed to 1 M GdnHCl and precipitated with trichloroacetic acid for analysis with 10% SDS-polyacrylamide gel electrophoresis (25). Western immunoblotting following SDS-polyacrylamide gel electrophoresis was performed according to Burnette (26) using Penta-His Antibody (Qiagen, Mississauga, Ontario, Canada) diluted to 1:1,000 in 3% bovine serum albumin in Tris-buffered saline as the first antibody.

**CD Spectroscopy**—CD of WbpO protein was measured with a JASCO J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) at 20 °C. Secondary structure fractions were tabulated using the JASCO Protein Secondary Structure Estimation Program (Japan Spectroscopic Co., Tokyo, Japan) which is based on the algorithm of Chang et al. (27), and our results were analyzed using the data base of Hennessy and Johnson (28). Spectra were recorded between 190 and 250 nm, and three spectra of each sample were obtained and their values averaged. All protein samples were adjusted to 0.3 mg/ml in 10 mM potassium phosphate buffer, pH 8.0, and were degassed before scanning. The base line was also corrected with 10 mM potassium phosphate buffer. Measurements were performed with a cell path length of 1 mm. Molar ellipticity was expressed in units of degree × cm2/molecule of amino acid residues. The secondary structure of WbpO based on its amino acid sequence was predicted using data base PredictProtein (29).

**Enzyme Reaction and HPAEC**—The enzyme reaction mixture contained 1.0 mM UDP-GalNAc, 2.5 mM β-nicotinamide adenine dinucleotide (NAD+), or β-nicotinamide adenine dinucleotide phosphate (NADP+), 6 mM MgCl2, and 10 mM Tris-HCl buffer, pH 8.2, in a total volume of 1 ml. The reaction was initiated by addition of 5 purified WbpO solution, then incubated at 37 °C for 1 h, quenched by boiling for 10 min, and stored at −20 °C for product analysis. Enzyme reaction was monitored by recording the absorbance of the solution at 340 nm which indicates NAD/P+H production. Sugar compositional analysis was performed by high performance anion exchange liquid chromatography (HPAEC) equipped with a pulsed amperometric detector on a Waters 600E (20) and a CarboPac PA1 anion exchange column (4 × 250 mm, Dionex, Sunnyvale, CA) equipped with a PA1 guard column, at a flow rate of 1.0 ml/min−1. Neutral and amino sugars were separated with 14 mM NaOH in the initial 10 min. Acidic sugars were then eluted by an application of a linear salt gradient up to 100 mM NaOH and 150 mM NaOAc within 10 min. The column was maintained in these conditions for 5 min, and then washed with 15 mM NaOH for 5 min before being re-equilibrated with 14 mM NaOH for 20 min.

The conversion ratio of UDP-GalNAc to UDP-GalNAcA was estimated based on the comparison of peak areas under substrate and product, respectively, from HPAEC.

**Cappillary Electrophoresis-Electrospray Mass Spectrometry (CE-MS) and Tandem Mass Spectrometry (MS/MS)**—Sugar analysis by CE-MS or CE-MS/MS was performed using a crystal model 310 CE instrument (AYI Unicam, Boston) coupled to an API 3000 mass spectrometer (PerkinElmer Life Sciences) via a micro-ion spray interface. The fused silica capillaries used were 192 (outer diameter) × 50 μm (inner diameter) (Polymicro Technologies, Phoenix, AZ). The separations were obtained on a 60-cm long capillary with 50 μm inner diameter, 50 μM KCl, and a voltage of 30 kV was applied. Collision-induced dissociation of selected precursor ions was achieved using nitrogen as collision gas at collision energies of typically 60–70 eV.

**Kinetic Characterization of WbpORf**—The enzyme reaction was performed at room temperature in 10 mM ammonium acetate, pH 8.5, with a total volume of 100 μl containing sugar (UDP-GalNAc or UDP-GlcNAc), NADP+, WbpOref (0.1–12 μM), and 2 mM dithiothreitol. The Km was
and $V_{\text{max}}$ values of WbpO$_{\text{RF}}$ were determined based on Michaelis-Menten equations, and the sugar concentrations added ranged from 2 to 40 mM, and the cofactor concentrations ranged from 0.2 to 5 mM. The enzyme reaction is monitored by following the reduction of NAD$^+$ at 340 nm using spectrophotometer (DU$^\text{TM}$ Series 520 spectrophotometer, Beckman Instruments, Mississauga, Ontario, Canada), and the initial velocities were measured during the first 30 s after the initiation of the reaction with NADP$^+$. NADP$^+$/H concentrations were determined using the absorbance at 340 nm with $e_{340} = 6,220 \text{ M}^{-1}\text{cm}^{-1}$.

**Determination of Cofactor Specificity**—For the determination of the $K_m$ value for the cofactors, an excess amount of sugar (40 mM) was used, and the cofactor concentrations ranged from 0.1 to 5 mM.

**Determination of Substrate Specificity of WbpO$_{\text{RF}}$**—UDP-GalNAc, UDP-GlcNAc, and UDP-Gal were used as the substrate, respectively, for the determination of the specificity of WbpO$_{\text{RF}}$, and the reactions were carried out in the presence of 5 mM NAD$^+$. The enzyme reactions using UDP-GalNAc or UDP-GlcNAc were analyzed by CE-MS/MS to identify the product.

**Requirement of Cations for the Enzyme Activity**—Enzyme reactions containing 10 mM final concentration of MgCl$_2$, MnCl$_2$, or KCl in addition to 6 mM UDP-GalNAc, 10 mM NAD$^+$, 2 mM dithiothreitol, and 2.4 $\mu$g of enzyme were monitored at 340 nm for 30 min at room temperature.

**RESULTS**

**Sequence Analysis**—Comparison of the sequence of WbpO (GenBank$^\text{TM}$ accession number AF 035937) with other proteins in the GenBank$^\text{TM}$ data base indicated that WbpO has high homology to VipA (64% identity) and CapL (57% identity). VipA is involved in the biosynthesis of S. typhi Vi-antigen, a homopolymer polysaccharide consisting of GalNacA (18, 30), and CapL is thought to be involved in the biosynthesis of the same sugar of the capsular polysaccharide in S. aureus (18). WbpO showed moderate homology with EpsD, a putative NDP-GalNAc dehydrogenase in Burkholderia solanacearum, with an identity of 29% (31). It also demonstrated homology with UDP-ManNAcA dehydrogenases in Methanobacterium thermoautotrophicum (B7, 32% identity) (32), Pyrococcus horikoshii (B8, 31% identity) (33), Methanococcus jannaschii (Y428, 30% identity) (34), and E. coli (35), respectively. Four conserved motifs located at the N terminus of these sequences (Fig. 1) were found using San Diego Supercomputer Center/Multiple EM for motif elicitation alignment tool. These motifs also coincide with the conserved secondary structures. Motif-1 contains a GXGXXG consensus (where $X$ represents any amino acid and $G$ represents glycine) in a $\beta\beta$ secondary structure, indicating the possible involvement of nucleotide (i.e., NAD$^+$ or NADP$^+$) in the WbpO function. Part of Motif-2 is in $\beta$-strand structure conformation, whereas the whole Motif-3 containing 27 amino acids is in $\alpha$-helical structure. However, Motif-2, -3, and -4 do not correspond to any known functional signature. These alignment results clearly suggest that WbpO could be an enzyme involved in the formation of uronic acid in polysaccharide biosynthesis. Furthermore, complementation data previously obtained by our laboratory demonstrated that the knockout mutant of WbpO could be cross-complemented by VipA from S. typhi to restore the production of B-band LPS in O6 (20). All the above evidence led to the assignment of the function of WbpO as a putative UDP-GalNAc dehydrogenase that produces UDP-GalNacA.

**Overexpression of WbpO**—WbpO was overexpressed by induction with 1 mM IPTG in TB medium (Fig. 2A, lane 4). Results from Western immunoblotting with Penta-His$^\text{TM}$ Antibody (B), Lane 1, molecular mass marker proteins. Lane 2, vector pET30a without wbpO insert. Lanes 3 and 4, overexpression of WbpO before (lane 3) and after (lane 4) induction with 1 mM IPTG. Lane 5, inclusion body proteins from E. coli BL21 (DE3)pLysS cells expressing WbpO. Lane 6, IMAC purification of protein in lane 5. Lane 7, refolded WbpO. Lane 8, soluble fraction of expressed proteins of E. coli cell expression WbpO. Lane 9, IMAC purification of protein of lane 8.

**Purification and Refolding of WbpO**—The purification of soluble form of WbpO by IMAC under native conditions yielded >95% pure WbpO (Fig. 2A, lane 6), and the same purity of WbpO was also achieved by the purification of the insoluble form with IMAC under denaturing conditions (Fig. 2A, lane 9). Effective refolding of WbpO required optimization of the ratios.
of reduced and oxidized glutathione. The final concentrations of 5 and 1 mM of reduced and oxidized glutathione, respectively, were deemed optimal for achieving the highest yield of 2 mg cell pellet of WbpO with approximately 99% purity (Fig. 2A, lane 7).

CD Analysis of WbpO—The CD spectrum of WbpO sol showed a minimum at 208 nm and a shoulder at 220 nm (Fig. 3), which are the characteristics of proteins that have an \( \alpha \)-helical conformation (36–38). The CD spectrum of refolded WbpO (WbpORf) showed different patterns and weaker ellipticities than that of WbpO sol, and these results are consistent with the calculations, 29.2% for WbpO sol and 22.0% for WbpORf, respectively, as shown in Table I. WbpORf also showed altered CD spectrum with a minimum circular dichroism signal at 198 nm, which indicated an increased content of \( \beta \)-turn in the secondary structure (39, 40).

CD spectra were recorded with WbpORf, which had been dialyzed to pH 8.2, 7.5, 6.5, and 5.5, respectively. As the pH decreased, the CD spectra were shifted toward the UV region suggesting an increased percentage of turns in the secondary structure of the protein. In particular, the CD spectrum of WbpORf at pH 5.5 demonstrated high proportions of turns and coils with much less \( \alpha \)-helices (10.9%) and \( \beta \)-strands (12.1%) (Table I). Since WbpO has a predicted pI of 5.8, we suspect that the loss of secondary structure at pH 5.5 corresponds to isoelectric precipitation of the protein. These observations also showed that the secondary structure of WbpORf was relatively stable in the range of pH 7.5–8.2. Consequently, a pH between 8 and 8.2 was chosen to carry out enzymatic reactions.

Enzyme Reaction and HPAEC Analysis—To assess the enzymatic activity of WbpO, HPAEC was used to analyze the product of the typical enzyme reaction. Neither NAD(P)\(^+\) nor UDP-GalNAc would bind on the column, and these compounds were eluted during the first 10-min wash with 14 mM NaOH with only a slight separation. When the reaction with WbpORf was analyzed by HPAEC, a new peak was observed after 8.49 min elution with the salt gradient (Fig. 4). Uronic acids are known to bind to the CarboPac PA1 column in our operating conditions and elute with NaOAc gradient (41). Hence the new peak that we observed is consistent with uronic acid formation, and it was collected for further CE-MS/MS analysis to determine its identity. HPAEC analysis also indicated the requirement of NAD(P)\(^+\) for the catalysis to occur. In contrast to WbpO, WbpORf did not show any detectable enzyme activity. Moreover, precipitation of a small amount of WbpORf was observed during the 1-h incubation of the enzyme assay, whereas refolded WbpORf was stable in the reaction mixture.

CE-MS/MS—To identify the product of the enzyme reaction of WbpO, CE-MS analysis was performed on the collected peak from HPAEC as well as the whole enzymatic reaction of WbpO. An ion peak at \( m/z \) 620.0, which is consistent with the mass of the expected product UDP-GalNAcA (\( m/z \) 621), was observed in both samples. The substrate (UDP-GalNAc) peak at \( m/z \) 605.0 was also observed in the CE/MS on the whole enzyme reaction. Both of the product (\( m/z \) 620.0) and substrate (\( m/z \) 605.0) were further analyzed by MS/MS.

MS/MS analysis of the product peak \( m/z \) 620.0 is shown in Fig. 6, and the fragments corresponding to each peak are depicted in Table II. In addition to the parent peak at \( m/z \) 620.0, other peaks at \( m/z \) 174.5, 254.5, 506.0, 522.0, and 540.0 were observed and matched to the fragments of GalA-O, GalNAcA-PO\(_3\)-PO\(_2\)-Rib, GalNAcA-PO\(_3\)-PO\(_2\)-Rib-NH, and GalNAcA-PO\(_3\)-PO\(_2\)-Rib-NH\(_2\)=CH\(_2\), respectively (Table II). These
six fragments exhibited diagnostic signature for the existence of the carboxyl group attached to the Gal ring. It also indicated the attachment of GalNAcA to the phosphate moiety of UDP.

Two other fragments corresponding to m/z 408.0 for Gal-PO3-PO3-Rib and m/z 426.0 for GalN-PO3-PO3-Rib that did not contain carboxyl group provided further evidence to indicate the attachment of the carboxyl group to the Gal ring rather than to other moieties.

The ion peaks at m/z 78.5 (PO3) and m/z 158.5 (PO3–PO3) provided evidence for the existence of a diphosphate moiety. The appearance of another three peaks at m/z 133.5 for PO2-Rib, m/z 272.5 for PO2-PO3-Rib, and m/z 290.5 for PO3-PO3-Rib further suggested the attachment of the diphosphate moiety to the ribose ring as a part of UDP. By comparing to the MS/MS results of the substrate UDP-GalNAc (m/z 605.0, data not shown), the ion peak at m/z 620.0 is identified to correspond to UDP-GalNAcA.

In addition to the m/z 620.0 peak, two additional new peaks at m/z 565.0 and 575.0 (data not shown) were observed in the CE/MS analysis of both collected peak from HPAEC and the whole WbpO enzymatic reaction. Further MS/MS analysis had identified them to be the breakdown fragments of the product UDP-GalNAcA by losing acetylamino and acetyl groups, respectively.

**Physicokinetic Characterization of WbpO**—The production of the product UDP-GalNAcA or NAD(P)H was linear over time within the initial 10 min of the enzyme reaction and reached a maximum after 30 min. The enzyme activity is relatively constant over a wide temperature range from 37 to 56 °C, and the optimum pH is 8.5.

**Kinetic Characterization of WbpO**—The measurement of the initial velocities for the calculation of kinetic parameters

### TABLE II

| Composition of fragmenta | Mass       |
|--------------------------|-----------|
| UDP-GalNAcA (product)    | 620.0     |
| GalA + O                 | 174.5     |
| GalNA + PO3              | 254.5     |
| GalNAc + PO3 + PO3 + Rib | 506.0     |
| GalNAc + PO3 + PO3 + Rib + NH | 522.0   |
| GalNAc + PO3 + PO3 + Rib + NHCH2 | 540.0 |
| Gal + PO3 + PO3 + Rib    | 408.0     |
| GalN + PO3 + PO3 + Rib   | 426.0     |
| PO3 + PO3                | 78.5      |
| PO3 + PO3                | 158.5     |
| PO3 + Rib                | 133.5     |
| PO3 + PO3 + Rib          | 272.5     |
| PO3 + PO3 + Rib          | 290.5     |

a The abbreviations for the mass peaks identified are as follows: A, COOH; Gal, C5H6O3 (galactose ring); Rib, C5H8O3 (ribose ring).
was performed by using different dilutions of the enzyme to ensure less than 10% conversion of the substrate within the initial 30 s. The kinetics of the enzyme reaction of WbpORf followed the Michaelis-Menten model. The $K_m$ values of WbpORf derived from the double-reciprocal plots are 7.89 for UDP-GalNAc and 0.65 and 0.44 mM for NAD$^+$ and NADP$^+$, respectively (Table III).

**Substrate Specificity of WbpORf—**WbpORf can also use UDP-GlcNAc as the substrate, and the product of this enzyme reaction was identified as UDP-GlcNAcA by CE-MS/MS (data not shown). The $K_m$ value for UDP-GlcNAc using NAD$^+$ was 22.2 mM.

WbpORf showed very low activity using UDP-Gal as the substrate. In this reaction, despite using 5 times more enzyme, the conversion of the cofactor NAD$^+$ within 30 min is less than 10% compared to that using UDP-GalNAc as the substrate (data not shown).

**Requirement of Cations for the Enzyme Activity and Enzyme Stability**—The enzymatic reactions of WbpORf in the presence of MgCl$_2$, MnCl$_2$, or KCl were also recorded within the initial 30 min. In the presence of 10 mM MgCl$_2$ or MnCl$_2$, there was approximately 9% increase in NADH generation, whereas KCl did not have any effect on the enzyme reaction (data not shown).

In terms of shelf life, the enzyme retained ≥80% activity after being preserved in 25% glycerol or 20% adonitol for 2 weeks at −20 °C.

### DISCUSSION

All proteins shown in Fig. 1 possess a NAD(P)$^+$ binding domain (Motif-1) that is composed of about 30 amino acids with a predicted secondary structure of $\beta \beta \beta$ (18, 42). This domain is highly conserved among NAD(P)$^+$-dependent dehydrogenases (18, 43). Therefore, the sequence conservation observed in Motif-2 and Motif-3 also coincides with structure conservation since Motif-2 and Motif-3 are in $\beta$-strand and $\alpha$-helix conformation, respectively, in WbpO. The identification of the NAD(P)$^+$ binding domain and amino acid sequence homology and gene complementation data strongly suggest that WbpO is a NAD(P)$^+$-dependent dehydrogenase. Since all the conserved motifs in WbpO were close to the N terminus of the sequence, a His$_6$ tag was added at the C terminus of WbpO to facilitate its purification and identification.

The small amount of WbpO expressed before the induction with IPTG was likely due to a leaky expression of the wbpO-pET30a vector by the host (lane 3, Fig. 2, A and B). Since all experiments were performed at pH 8 well above the pI (5.8) of WbpO, the observed instability of purified WbpO$_{sol}$ might be due to the incorrect folding of the protein during expression rather than isoelectric precipitation. Since we observed >90% of the expressed WbpO in an insoluble form and the WbpO$_{sol}$ was unstable in solutions, the refolding experiment was a worthwhile task for characterizing the enzyme activity of WbpO.

By optimizing the refolding conditions such as adjusting the reduced to oxidized glutathione ratios, most of the contaminating proteins could be precipitated and were subsequently removed by filtration. Moreover, to obtain a properly refolded protein, the use of low protein concentration, i.e. <0.1 mg/ml, is necessary to avoid any intermolecular disulfide bond formation during the refolding procedure. Unlike the fast process in vivo, in vitro disulfide bond formation is an extremely slow reaction and could take hours to days (45). In our case, refolding of WbpO was optimal at approximately 30 h. It is also important to perform dilution and concentration steps slowly to minimize the precipitation of the target protein. Other refolding procedures, which involved sequential dialysis with decreased concentrations of urea (46, 47) followed by renaturation of the protein immobilized to affinity resin (48) and renaturation of protein in 20% glycerol (49), generally result in very low yields because of protein precipitation problems. The procedure described in this paper provided an optimal condition to achieve high purity and high yield of WbpO with tight secondary structure.

The proportion of $\alpha$-helices observed in both WbpO$_{sol}$ (29.2%) and WbpO$_{fr}$ (22.0% at pH 8.2) are lower than the predicted value (38.3%) based on the amino acid sequence of WbpO, whereas the $\beta$-strand contents of these two forms of WbpO (20.1 and 19.6%, respectively), are higher than the predicted value of 16.8%. However, the total percentage (49.2%) of $\alpha$- and $\beta$-structure of WbpO$_{sol}$ is consistent with that of some other dehydrogenases (48–51%) (50, 51). The differences between CD spectra of WbpO$_{sol}$ and WbpO$_{fr}$ reflect the differences in the structures of these two forms of WbpO. In WbpO$_{fr}$ the total percentages of $\alpha$-helix and $\beta$-strand are not as high as those in WbpO$_{sol}$ and suggested that the secondary structure of WbpO$_{sol}$ was not as tight as that of WbpO$_{fr}$. Interestingly, the fact that WbpO$_{sol}$ has no detectable enzyme activity implied that it might have an incorrect secondary structure. This interpretation was substantiated by the observation that WbpO$_{sol}$ was prone to precipitation when being used in an enzyme assay. Moreover, the gradual removal of GdnHCl during refolding of WbpO may cause more disulfide bonds forming on the surface of the protein so that high negative ellipticities at 198 nm were observed on CD spectrum of WbpO$_{fr}$ (39, 40).

Furthermore, the optimum pH of 8.2 for the enzymatic reaction of WbpO is in good agreement with the highest secondary structure content at pH 8.2 from CD analysis.

In our HPAEC analysis, we observed a new peak eluted from NaOAc gradient which is consistent with the results shown by previous studies (41) that acidic sugars are eluted from Carbopac column in NaOAc gradient. Since UDP-GalNAcA was not commercially available to be used as a standard in the HPAEC, we had to perform CE-MS/MS to identify the product (UDP-GalNAcA). Running the samples through CE prior to MS analysis allowed further separation of the product and facilitated the interpretation of the MS spectra.

From the MS/MS analysis, all the fragments related to the existence of m/z 174.5, 254.5, 506.5, 522.0, and 620.0 and loss of m/z 408.0 and 426.0 of the carboxyl group implied that the galactose ring remained intact. These observations also indi-

### Table III

| Sugar substrate | Parameters for | $K_m$ | $V_{max}$ | Enzyme | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------------|----------------|------|---------|-------|-----------|--------------|
| UDP-GalNAc      | UDP-GalNAc$^a$ | 7.78 $\pm$ 0.19 | 955 $\pm$ 48 | 19.5 | 47.8 | 6.13 |
| UDP-GalNAc      | NAD$^+$        | 0.65 $\pm$ 0.03 | 523 $\pm$ 88 | 19.5 | 26.8 | 41.23 |
| UDP-GlcNAc      | NADP$^+$       | 0.44 $\pm$ 0.02 | 330 $\pm$ 50 | 19.5 | 16.9 | 38.41 |
| UDP-GalNAc      | UDP-GlcNAc$^a$ | 22.2 $\pm$ 1.20 | 214 $\pm$ 15 | 39.0 | 5.5 | 0.25 |

$a$ Reactions were performed with NAD$^+$ as the cofactor.
cate that the only possible position of the carboxylation would be at C-6 of the galactose moiety by replacing the –CH₂OH in UDP-GalNAc with –COOH. From the CE-MS/MS analysis on the enzyme reaction of UDP-ManNAc dehydrogenase (60). The higher Km value for WbpOₜₜ could be due to the fact that our kinetic parameters were obtained with a refolded protein. As shown in the structural analysis by CD, WbpOₜₜ showed less secondary structures than of WbpO expressed in a soluble form.

As mentioned above, Creuzenet et al. (57) recently reported the UDP-GlcNAc C₄ epimerase function of WbpP, which catalyzes the metabolic step that provides the substrate, UDP-GlcNAc, for WbpO. Thus, by achieving the enzymatic characterization of WbpO, our laboratory has verified the proposed two-step biosynthetic pathway of UDP-GalNAcA (see Refs. 3, 20, 66, and 67, Fig. 7) which is the precursor of one residue of the trisaccharide repeating unit of the O-antigen in LPS of P. aeruginosa serotype O6.

To date, WbpO is the first UDP-GalNAc dehydrogenase that has been characterized at the molecular and biochemical level. The results in this study have provided significant biological and structural evidence to establish the function of WbpO as a NAD(P)⁺-dependent UDP-GalNAc dehydrogenase (Fig. 5, see Ref. 65) that catalyzes Reaction 1.
