PseG of Pseudaminic Acid Biosynthesis

A UDP-SUGAR HYDROLASE AS A MASKED GLYCOSYLTRANSFERASE

Received for publication, March 29, 2006, and in revised form, May 25, 2006 Published, JBC Papers in Press, May 25, 2006, DOI 10.1074/jbc.M602972200

Feng Liu and Martin E. Tanner

From the Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

The flagellin proteins in pathogenic bacteria such as Campylobacter jejuni and Helicobacter pylori are heavily glycosylated with the nine-carbon α-keto acid, pseudaminic acid. The presence of this posttranslational modification is absolutely required for assembly of functional flagella. Since motility is required for colonization, pseudaminic acid biosynthesis represents a virulence factor in these bacteria. Pseudaminic acid is generated from UDP-N-acetylgalactosamine in five biosynthetic steps. The final step has been shown to involve the condensation of 2,4-diacetamido-2,4,6-trideoxy-L-altrose (6-deoxy-AltdiNAc) with phosphoenolpyruvate as catalyzed by the enzyme pseudaminic acid synthase, NeuB3. The 6-deoxy-AltdiNAc used in this process is generated from its nucleotide-linked form, UDP-6-deoxy-AltdiNAc, by the action of a hydrolase that cleaves the glycosidic bond and releases UDP. This manuscript describes the first characterization of a UDP-6-deoxy-AltdiNAc hydrolase, namely PseG (Cj1312) from C. jejuni. The activity of this enzyme is independent of the presence of divalent metal ions, and the values of the catalytic constants were found to be $k_{cat} = 27 \text{s}^{-1}$ and $K_m = 174 \text{µM}$. The enzyme was shown to hydrolyze the substrate with an overall inversion of stereochemistry at C-1 and to utilize a C–O bond cleavage mechanism during catalysis. These results, coupled with homology comparisons, suggest that the closest ancestors to the hydrolase are members of the metal-independent GT-B family of glycosyltransferases that include the enzyme MurG.

In recent years, it has become increasingly clear that the posttranslational glycosylation of proteins not only occurs in eukaryotic organisms but also plays important roles in the biology of prokaryotes (1–4). One such example is the O-linked glycosylation of the flagellin proteins in the pathogenic Gram-negative bacteria, Campylobacter jejuni and Helicobacter pylori. The former organism is the major cause of gastrointestinal illness in North America and has been implicated as a causative agent of Guillain–Barré syndrome (5, 6). The latter causes gastritis that can lead to peptic ulceration and gastric cancer (7).

The main flagellar modification in these organisms involves the nine-carbon α-keto acid, pseudaminic acid (Pse)² (Fig. 1A) (8–10). This compound and its derivatives can account for up to 10% of the total mass of the flagellar glycoproteins. Recent reports have begun to uncover the pathway for pseudaminic acid biosynthesis and several of the enzymes have now been identified and characterized. The pathway begins by the action of a dehydratase, PseB, on UDP-N-acetylgalactosamine to generate a 6-deoxy-4-keto derivative, UDP-2-acetamido-2,6-dideoxy-β-L-arabinofuranose-4-hexulose (11). Unlike most known sugar nucleotide dehydrogenases, the reaction is accompanied by an inversion of configuration at C5 and the initially formed product is of the L-configuration (as is required for Pse biosynthesis). In the second step, an aminotransferase, PseC, installs an amino group at C4 to give UDP-4-amino-2,4,6-trideoxy-β-L-altrose (12). It is then proposed that an as yet unidentified acetyltransferase acetylates the 4-amino group and generates UDP-2,4-diacetamido-2,4,6-trideoxy-β-L-altrose (UDP-6-deoxy-AltdiNAc). A subsequent hydrolysis would cleave the UDP-linkage and generate the free sugar 2,4-diacetamido-2,4,6-trideoxy-L-altrose (6-deoxy-AltdiNAc). This compound has recently been shown to serve as a substrate for pseudaminic acid synthase, NeuB3, which condenses it with phosphoenolpyruvate to give Pse (13). Finally, the Pse is converted into its activated form, CMP-Pse, presumably by a CTP-dependent synthetase, and is thereby readied for use by the transferases involved in flagellar glycosylation.

In this paper, the C. jejuni UDP-6-deoxy-AltdiNAc hydrolase (PseG or Cj1312) responsible for the formation 6-deoxy-AltdiNAc is identified for the first time. It is found to be a member of the GT-B superfamily of metal-independent glycosyltransferases and catalyzes the reaction via a C–O bond cleavage mechanism with inversion of stereochemistry at C-1.

EXPERIMENTAL PROCEDURES

Materials and General Methods—UDP-N-acetylgalactosamine, lactate dehydrogenase (Type II from rabbit muscle), and pyruvate kinase (Type II from rabbit muscle) were purchased from Sigma. ¹⁸O-Enriched H₂O (95%) was purchased...
from Cambridge Isotope Laboratories. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard (14). ¹H and ³¹P NMR spectra were obtained on Bruker AV300/AV400 NMR spectrometers. Mass spectra were obtained on a Waters Micromass LCT mass spectrometer using electrospray ionization-mass spectrometry (ESI-MS).

Cloning of pseG—The pseG gene (Cj1312) was amplified from C. jejuni (strain NCTC 11168) genomic DNA by PCR. Oligonucleotide primers, including overhangs for ligation-independent cloning, were: 5'-GGTGATGAGGTCGATGAAAGTGCCTTTTAAAAG-3' (sense) and 5'-AGAGGAGATTAGACCTCAATCTTATACCTCCA-3' (antisense). To a 200-μl tube were added: 5 μl of 10× PCR reaction buffer (Invitrogen), 1.5 μl of 50 mM MgCl₂, 1 μl of dNTP mix (PCR grade, Invitrogen), 125 ng of each primer, 100 ng of template DNA, 1.25 units of Taq polymerase (Invitrogen) and distilled H₂O to make a total volume of 50 μl. PCR was conducted on an iCycler Thermal Cycler (Bio-Rad) with the following cycles: 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 90 s at 72 °C, and one cycle of 10 min at 72 °C. The PCR product was cloned into a pET-30 Xa/LIC vector (Novagen) according to the manufacturer’s instructions. The resulting recombinant plasmid, which encodes a His₆-tag on the N-terminal of the target pseG protein, was amplified in NovaBlue Giga Singles competent Escherichia coli cells (Novagen).

Overexpression and Purification of His₆-tagged pseG, pseB, and pseC—The recombinant pseG plasmid was transformed into E. coli BL21 (DE3) competent cells which were incubated in 10 ml LB medium containing 50 mg/liter kanamycin at 37 °C/225 rpm for 10 h. The overnight culture was then poured into 500 ml of LB medium containing 50 mg/liter kanamycin and shaken at 37 °C/225 rpm until an A₆₀₀ of 0.6 had been reached. The culture was allowed to continue to grow for 5 h after 70 mg/liter of isopropyl β-D-galactopyranoside was added. Cells were harvested by centrifuging at 6000 × g for 30 min and then resuspended in 10 ml of phosphate buffer (20 mM, pH 8.0) containing 2 mM dithiothreitol, 1 mg/liter of aprotinin, and 1 mg/liter pepstatin A. The cells were lysed by passage through a French pressure cell at 20,000 p.s.i. The lysate was centrifuged at 6000 × g for 1 h and passed through a 0.22-μm filter.

A column containing 10 ml of chelating Sepharose Fast Flow resin (GE Healthcare) was charged with 20 ml of 100 mM NiSO₄, washed with 20 ml of distilled H₂O and 30 ml of sodium phosphate buffer (20 mM, pH 8.0, containing 0.5 M NaCl and 5 mM of imidazole). The clarified lysate was loaded onto the column and eluted with the same buffer containing increasing amounts of imidazole in a stepwise fashion (5 mM, 125 mM, and 500 mM). Eluate fractions that were eluted with 500 mM imidazole showed absorbance at 280 nm were collected. These fractions were concentrated using Amicon Ultra Centricons (Millipore) before flash freezing with liquid N₂ in the presence of 10% glycerol and 2 mM dithiothreitol.

The preparation of the plasmids used in the overexpression of His₆-tagged PseB (Cj1293) and PseC (Cj1294) has been described previously (11). The conditions used for overexpression of these proteins were identical to those described above for PseG with the exception that the growth media used to prepare PseC contained 50 mg/liter ampicillin in place of kanamycin. The clarified lysates obtained following lysis and filtering were used directly without further purification.

Chemoenzymatic Synthesis of UDP-6-deoxy-AltdiNAc—Samples of the crude lysates containing PseB and PseC (100 μl each) were added to 100 ml of triethanolamine HCl buffer (50 mM, pH 8.0) containing 450 mg of UDP-N-acetylglucosamine disodium salt, 0.1 M monosodium glutamate, 0.25 mM pyridoxal 5'-phosphate, and 0.25 mM NAD⁺. This solution was incubated for 4 h at 37 °C and the reaction progress was monitored by negative ESI-MS. It was determined that >90% of the UDP-GlcNAc (m/z 606, M – H⁺) was converted to UDP-6-deoxy-4-amino-2-diacetamido-1-altrose (m/z 589, M – H⁺) during this time. Enzyme was then removed by centrifugation and centrifugal ultrafiltration. The resultant filtrate was loaded onto a 220-ml column of DEAE-cellulose (DE52, Whatman Inc.) and eluted with a linear gradient of 0.1–0.5 M triethylammonium bicarbonate buffer. The eluant was monitored at A₂₅₄nm and UV-active fractions were analyzed by ESI-MS. Those containing UDP-6-deoxy-4-amino-2-diacetamido-1-altrose were lyophilized to dryness.

The lyophilized UDP-6-deoxy-4-amino-2-diacetamido-1-altrose (310 mg) was stirred with 0.5 g of silver acetate and 0.8 ml of acetic anhydride in 25 ml of methanol at room temperature for 12 h. Negative ESI-MS showed that the starting material was completely converted to UDP-6-deoxy-AltdiNAc (m/z 631, M – H⁺) during this time. After filtration and evaporation of the solvent under reduced pressure, the product was loaded onto a DE52 anion exchange column and subjected to linear gradient elution as described above. After lyophilization, the product was dissolved in 10 ml of H₂O and passed through a 20-ml column of Amberlite IR120 resin (sodium form, Aldrich). The eluant was re-lyophilized to give 173 mg of the UDP-6-deoxy-AltdiNAc disodium salt. ¹H NMR(D₂O) δ 1.29 (d, 3H, J₆,₂ 0.6 Hz, H-6), 2.06 (s, 3H, CH₃), 2.09 (s, 3H, CH₃), 3.91 (dd, 1H, J₅,₆ 4.1 Hz, H-5), 4.23 (m, 2H, J₅,₆ 2.9 Hz), 4.97 (d, 1H, J₄,₅ 2.7 Hz), 5.03 (m, 2H, J₄,₅ 2.7 Hz), 5.30 (m, 2H, J₃,₄ 4.1 Hz), 5.60 (d, 1H, J₃,₄ 2.2 Hz, H-3), 6.19 (d, 1H, J₂,₃ 2.2 Hz, H-2), 6.21 (d, 1H, J₁,₂ 2.2 Hz, H-1), 7.88 (m, 2H, H-1’, and H-5’), 8.00 (d, 1H, J₁,₂ 8.14 Hz, H-6). ³¹P NMR(D₂O) δ -10.31 (d, Jₒ₋ₓ,ₓ 20.0 Hz, Pₓ).

Chemical Hydrolysis and Characterization of the Product—To a 1-ml solution containing 6 mg of UDP-6-deoxy-AltdiNAc was added 50 μl of concentrated HCl. The mixture was incubated at 37 °C for 10 min and then loaded on a 10-ml column of DE52 anion exchange resin and eluted with distilled H₂O. The flow-through was collected, lyophilized, and re-dissolved in D₂O. ¹H NMR spectroscopy showed that the mixture of 6-deoxy-2,4-diacetamido-1-altrose anomers produced was identical to that obtained from chemical synthesis (13, 15). ¹H NMR(D₂O), δ 1.21 (d, 3H, J₆,₂ 0.6 Hz, H-6), 1.24 (d, 3H, J₅,₆ 6.5 Hz, H-5), 2.03 (s, 3H, β-CH₃), 2.04 (s, 3H, α-CH₃), 2.05 (s, 3H, α-CH₃), 2.09 (s, 3H, β-CH₃), 3.82 (dd, 1H, J₅,₆ 2.9 Hz, J₅,₆ 10.4 Hz, H-4), 3.91 (dd, 1H, J₃,₄ 3.2 Hz, J₃,₄ 2.9 Hz, H-3), 3.94 (dq, 1H, J₁,₂ 10.1 Hz, J₁,₂ 0.1 Hz, H-5), 4.05 (dd, 1H, J₁,₂ 2.8 Hz, J₁,₂ 4.3 Hz, H-2), 4.19 (dd, 1H, J₁,₂ 1.8 Hz, J₁,₂ 3.2 Hz, H-3), 5.05 (d, 3H, J₅,₆ 2.8 Hz, J₅,₆ 2.8 Hz, H-1’), 5.28 (d, 3H, J₁,₂ 1.8 Hz, H-1’).
**PseG of Pseudaminic Acid Biosynthesis**

**Enzyme Kinetics Using a Continuous Coupled Assay**—Enzyme kinetics were measured using a continuous coupled assay for UDP formation, under conditions slightly modified from those described by Chou et al. (19). Each cuvette contained 50 mM NaH₂PO₄ buffer (pH 7.5), 10 mM MgCl₂, 2 mM phenylpyruvate, 0.2 mM NADH, 20 units of lactate dehydrogenase, 18 units of pyruvate kinase, and the UDP-6-deoxy-Alt-diNAc concentration was varied from 25–2000 μM. The concentrations of stock UDP-sugar solutions were determined by measuring A₂₆₂ (ε = 9 890 M⁻¹ cm⁻¹). Enzymatic reactions were initiated by addition of 20 μl of enzyme solution (final concentration 7.9 nm). Rates were measured by monitoring the decrease A₄₄₀ at 37 °C. Kinetics parameters were determined by fitting initial velocities to the Michaelis-Menten equation using GraFit 4.0. No detectable background release of UDP was observed in the absence of added PseG.

**Metal Dependence Experiment**—Two aliquots of a solution containing UDP-6-deoxy-Alt-diNAc disodium salt (6 mg per aliquot) in 50 mM Tris-HCl buffer (pH 7.4) were prepared. To one aliquot was added MgCl₂ and to the other was added EDTA tetrasodium salt (each at a 10 mM final concentration in a total volume of 990 μl). PseG (10 μl of a 33 μM stock solution) was added to each sample and the mixtures were incubated for 2 h at room temperature. The progress of the reactions was monitored using ³¹P NMR spectroscopy with integration of the diphosphate signals.

**Reaction Stereochemistry and Deuterium Incorporation**—A glycerol stock solution of PseG (200 μl, 33 μM) was subjected to buffer exchange with a 20 mM Tris-DCl/D₂O buffer (pD 7.4, 200 μl final volume) using centrifugal ultrafiltration. This was added to a solution of UDP-6-deoxy-Alt-diNAc disodium salt (5 mg) dissolved in 800 μl of D₂O (1 ml final volume), and ¹H NMR spectra were acquired at timed intervals.

To assign the signals of the anomic products, a sample of UDP-6-deoxy-Alt-diNAc disodium salt was dissolved in D₂O and analyzed by nuclear Overhauser effect spectroscopy with irradiation at 5.28 ppm (H1 of the major anomer at equilibrium). An nuclear Overhauser effect enhancement of the H₅ signal was confirmed that the α-anomer was the predominant species at equilibrium. Assignment of the H₅ signal was achieved using COSY spectroscopy via an observed correlation with the H₆ methyl signal (see supplemental Figs. 3 and 4 for spectral details on this section).

**¹⁸O Incorporation Experiment**—A solution of 50 mM Tris-HCl buffer (pH 7.4, 400 μl) was prepared using 50% H₂¹⁸O/50%H₂O and was divided into two equal aliquots. To one aliquot was added UDP-6-deoxy-Alt-diNAc (5.0 mg) and to the other was added 6-deoxy-Alt-diNAc (2.5 mg) and UDP (4.0 mg). PseG (10 μl of a 33 μM stock solution) was added to each sample, and the mixtures were incubated at room temperature. Isotope incorporation was monitored by both positive (sugar detection) and negative (UDP detection) ESI-MS as a function of time. The extent of incorporation into 6-deoxy-Alt-diNAc was deduced from the ratio of peaks at m/z 269 (¹⁶O, M + Na⁺) and m/z 271 (¹⁸O, M + Na⁺ + 2).

**RESULTS**

**Preparation of the Hydrolyase Substrate UDP-6-deoxy-Alt-diNAc**—One of the main difficulties in identifying the enzymes involved in Pse biosynthesis is the difficulty in obtaining the substrates for the advanced steps of the pathway. In previous work (13, 15), a chemical synthesis approach was used to obtain the 6-deoxy-Alt-diNAc that serves as the substrate for pseudaminic acid synthase. The length of the synthesis and the number of low yielding steps, however, make this approach impractical for a synthesis of UDP-6-deoxy-Alt-diNAc. For this compound an enzymatic approach would be superior, however, it is complicated somewhat by the fact that the putative acetyltransferase has not yet been identified. In this work, the dehydratase and aminotransferase enzymes, PseB and PseC (Fig. 1A), from C. jejuni were used to prepare UDP-4-amino-2,4,6-trideoxy-β-L-AltANAc in a multimilligram scale (11). This compound was purified by ion exchange chromatography, and then the C-4 amino group was chemically acetylated by acetic anhydride/silver acetate to give the hydrolase substrate, UDP-6-deoxy-Alt-diNAc (15). After purification, this material gave a mass spectrum and ³¹P NMR/¹H NMR spectra (see supplemental Fig. 1) that were consistent with the expected structure. To further confirm the structure of this compound, a small sample...
was subjected to a mild acidic hydrolysis that cleaved the glycoside from the UDP moiety. The $^1$H NMR spectrum of the resulting sugar proved to be identical to that of chemically synthesized 6-deoxy-AltdiNAc (see supplemental Fig. 2) (13, 15). This confirms that the stereochemical assignments recently made on the product of the PseB/C pair were correct (11).

**Identification of the UDP-6-deoxy-AltdiNAc Hydrolase from C. jejuni**—The search for enzymes involved in pseudaminic acid biosynthesis has been strongly influenced by an understanding of the biosynthetic pathway of the related α-keto acid, sialic acid (or N-acetylneuraminic acid, NeuAc) (16). In bacteria, sialic acid biosynthesis also begins with UDP-N-acetylgalcosamine, and the first step is catalyzed by the hydrolyzing UDP-GlcNAc 2-epimerase, NeuC (Fig. 1B) (17, 18). This enzyme catalyzes both an inversion of stereochemistry at C-2 and the hydrolysis of the glycosyl-UDP bond to give the free sugar, ManNAc. Mechanistic studies have shown that the reaction proceeds via an anti-elimination of UDP to give the enzyme-bound intermediate 2-acetamidoglucal, followed by the syn-addition of water to give ManNAc (17, 19). In a subsequent step the ManNAc is condensed with phosphoenolpyruvate by sialic acid synthase, NeuB, which is homologous to pseudaminic acid synthase and employs a similar reaction mechanism (20, 21). Finally, the sialic acid is activated as its CMP form by CMP-sialic acid synthetase, NeuA (22, 23).

Since the overall strategy employed in pseudaminic acid biosynthesis (the use of UDP-GlcNAc in ultimately forming a CMP-linked α-keto acid) is known to be the same as in sialic acid biosynthesis (24), and since the key syntheses are closely related (13, 20, 21), it seemed possible that the hydrolases may also be homologous. Such an enzyme could catalyze the elimination of UDP from UDP-6-deoxy-AltdiNAc to give a glycal intermediate and then hydrate the double bond of the glycal with overall retention of stereochemistry at C-2 to give the hydrolysis product, 6-deoxy-AltdiNAc. A C. jejuni gene, Cj1328 or NeuC2, was found to share 32.5% sequence identity with the Neisseria meningitidis NeuC and had been implicated in the biosynthesis of an acetamidino-containing derivative of pseudaminic acid, PseAm (25). NeuC2 was therefore overexpressed in E. coli both in a His-tagged form and as a fusion with the maltose-binding protein. In the former construct, the vast majority of the expressed protein was insoluble, and the small amounts of soluble protein that were retained on a metal affinity column showed no hydrolase activity with the substrate. The latter construct gave large quantities of soluble protein that could be purified on an amylose column; however, no hydrolase activity could be observed with the resulting protein either before or after cleavage of the maltose binding protein from NeuC2 using thrombin. It appears that NeuC2 is involved in the biosynthesis of PseAm, in a pathway that is distinct from Pse biosynthesis.

A second target for the C. jejuni hydrolase gene of Pse biosynthesis was identified from a report on proteins that were required for flagellar glycosylation in H. pylori (26). Two overlapping genes, neuA (HP0326a) and flmD (HP0326b), encoded for these proteins and mutations in these genes severely impaired the flagellar glycosylation process. The *neuA* gene product is homologous to the NeuA of sialic acid biosynthesis and likely encodes for a CMP-pseudaminic acid synthetase, although this has not yet been demonstrated. The flmD gene product was assigned as a putative glycosyltransferase and was thought to be involved in transfer of a carbohydrate on the flagellin protein. It was also noted that the homologs of these two genes colocalized in several other strains of flagellated bacteria, including C. jejuni (Cj1311/Cj1312), although in this case they did not overlap.

In our search for a sugar nucleotide hydrolase involved in Pse biosynthesis, a putative glycosyltransferase could represent a likely candidate since transfer of the glycoside to water as an acceptor would constitute a hydrolysis reaction. Further PSI-BLAST homology searching on the C. jejuni homolog, PseG or Cj1312, indicated that it most closely resembled N-acetylglucosaminyltransferases in the metal-independent GT-B superfamilly that includes the peptidoglycan biosynthetic enzyme MurG (27–29). PseG was therefore overexpressed in *E. coli* with an N-terminal His-tag. The soluble protein was purified by metal affinity chromatography and was found to be >95% pure as analyzed by SDS-PAGE (see supplemental Fig. 5). Subsequent analysis revealed that this enzyme was indeed a UDP-6-deoxy-AltdiNAc hydrolase.

**Analysis of the UDP-6-deoxy-AltdiNAc Hydrolase Reaction**—Incubation of the hydrolase with UDP-6-deoxy-AltdiNAc and monitoring the reaction by both $^3$P NMR spectroscopy and ESI mass spectrometry showed that free UDP was being generated along with a sugar whose mass was consistent with that of 6-deoxy-AltdiNAc. The sugar was isolated from all charged species by passage through an ion exchange column and its structure was confirmed by $^1$H NMR spectroscopy with comparison to a synthetic standard of 6-deoxy-AltdiNAc (13, 15). A brief survey of potential alternate substrates showed no detectable activity with UDP-GlcNAc and an extremely low level of activity (<1%) with the biosynthetic precursor, UDP-4-amino-2,4,6-trideoxy-β-L-altrose, indicating that the enzyme exhibits reasonable substrate specificity.

The kinetics of the hydrolysis reaction were monitored by a continuous coupled assay for UDP release that employs pyruvate kinase and lactate dehydrogenase. The reaction was found to obey Michaelis-Menten kinetics with kinetic constants of $k_{cat} = 26.6 \pm 0.6 \text{ s}^{-1}$, $K_m = 174 \pm 11 \text{ mM}$, and $k_{cat}/K_m = 1.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ (Fig. 2). The reasonably large value of the specificity constant suggests that UDP-6-deoxy-AltdiNAc hydrolysis is the biologically relevant reaction catalyzed by this enzyme.

The metal requirements of this enzyme were determined using a qualitative $^1$H NMR spectroscopic assay since the coupled assay requires the presence of metals for the function of pyruvate kinase (30). Two identical samples of UDP-6-deoxy-AltdiNAc were incubated with the hydrolase in the presence of either 10 mM Mg$^{2+}$ or 10 mM EDTA, and the extent of reaction was determined by integration of the substrate and product signals. The sample containing Mg$^{2+}$ was 65% complete during the incubation period and the sample containing EDTA was 55% complete in the same time period. The similarity of the values indicates that exogenous metal ions are not required for catalysis, however, the involvement of a tightly bound non-exchangeable metal ion cannot be ruled out. The slightly slower rate in the latter case may reflect a weak inhibition by EDTA.
Stereochemical and Mechanistic Analysis of the Hydrolase Reaction—To determine whether the hydrolase is an inverting or retaining enzyme, the anomeric stereochemistry of the first formed product was determined. The reaction was run in a deuterated buffer and analyzed by $^1$H NMR spectroscopy (Fig. 3). At the earliest time points, a major product was observed whose H1 signal appeared as a doublet at 5.04 ppm with a coupling constant ($J_{1,2}$) of 2.8 Hz. As the reaction proceeded, non-enzymatic mutarotation generated the other anomer whose $^1$H signal appeared as a doublet at 5.26 ppm with a coupling constant ($J_{1,2}$) of 1.8 Hz (Fig. 3, c and d). Although the conformational analysis of altrose derivatives is somewhat complicated by the population of both the $^4_{C_1}$ and the $^4_{C_4}$ chair forms (Fig. 4), only the $^4_{C_1}$ conformation of the $\alpha$-anomer bears H1 and H2 protons in a trans-diaxial relationship (note that for L-sugars, the $^4_{C_1}$ conformation of the $\alpha$-anomer places the anomeric hydroxyl in an equatorial position). Thus one would expect that the $\alpha$-anomer would have the larger coupling constant and correspond to the signal at 5.04 ppm. The identity of the major anomer at equilibrium was confirmed on a sample of the purified 6-deoxy-AltdiNAc using nuclear Overhauser effect spectroscopy. Irradiation of the H1 signal at 5.26 ppm resulted in an enhancement of the H5 signal (a doublet of quartets) at 3.94 ppm (see supplemental Fig. 3). This indicates that a 1,3 diaxial relationship exists between the two protons as would only be found in the $^4_{C_4}$ conformation of the $\beta$-anomer (Fig. 4). The observation that the $\alpha$-anomer is formed first in this reaction indicates that the enzyme is an inverting hydrolase. In addition to the stereochemical analysis, this experiment shows that solvent-derived deuterium is not incorporated into the C-2 position during catalysis. This is clear from the observed doublet in the H1 signals of the product (Fig. 3 d). Mass spectral analysis of the 6-deoxy-AltdiNAc generated in this experiment confirms that no (<5%) non-exchangeable deuterium is incorporated during catalysis.

An experiment was also devised to determine whether the hydrolase utilized a P–O or a C–O bond cleavage mechanism. The reaction was run in a buffer prepared from 50% $H_2^{18}O$ and 50% $H_2^{16}O$ and the products were immediately analyzed by mass spectrometry. The observed mass of the UDP that was generated was identical to that observed in a control reaction run in 100% $H_2^{16}O$. The signals for 6-deoxy-AltdiNAc generated in this experiment confirms that no (<5%) non-exchangeable deuterium is incorporated during catalysis.

FIGURE 2. Enzyme kinetic plots of initial velocity versus substrate concentration. The kinetic parameters as determined by fitting the data to Michaelis-Menton kinetics are as follows: $k_{cat} = 26.6 \pm 0.6 \text{ s}^{-1}$, $K_m = 174 \pm 11 \text{ mM}$.

FIGURE 3. $^1$H NMR spectra monitoring the time course of the conversion of UDP-6-deoxy-AltdiNAc into $\alpha$-6-deoxy-AltdiNAc and $\beta$-6-deoxy-AltdiNAc. The regions of the spectra showing the anomeric proton (H1) of the 6-deoxy-AltdiNAc compounds are displayed.
into the sugar during catalysis. To confirm that the label had not been incorporated into the product subsequent to the hydrolysis event, a sample of 6-deoxy-AltdiNAc was incubated with the hydrolase under identical conditions. While a slow incorporation of label could be observed over an extended incubation period, the rate of incorporation was several orders of magnitude too slow to account for the incorporation observed during the hydrolase reaction. This result shows that a solvent-derived oxygen atom is incorporated into the sugar during hydrolysis and is consistent with a C–O bond cleavage mechanism in which water directly attacks the anomeric carbon to displace UDP.

**DISCUSSION**

A unique aspect of the biosynthesis of sialic acid and many of its derivatives is that UDP-N-acetylgalactosamine serves as the precursor to the nine-carbon α-keto acid (16). This necessitates that hydrolysis of the UDP-linkage and generation of the free hexose occurs immediately prior to coupling with the three-carbon pyruvate unit. Ultimately, the sialic acid derivative is activated as a CMP-linked nucleotide that serves as a substrate for the appropriate transerase enzymes. In the biosynthesis of sialic acid itself, it is also necessary to invert the configuration at C-2 of the hexose to give ManNAc as the substrate for sialic acid synthase (Fig. 1B). Both the inversion and hydrolysis are catalyzed by the hydrolyzing UDP-GlcNAc 2-epimerase, NeuC (17–19). In the case of pseudaminic acid biosynthesis (Fig. 1A), there is no need for an inversion of stereochemistry at C-2 of the hexose since its absolute configuration is retained in the final nine-carbon α-keto acid (16). In this work we identify PseG (Cj1312) as the C. jejuni UDP-6-deoxy-AltdiNAc hydrolase of pseudaminic acid biosynthesis for the first time. This finding strongly suggests that FlmD (HP0326b) also plays this role in H. pylori (26).

The amino acid sequence of PseG does not show significant homology (>25% identity) with any proteins of proven function; however, it has been annotated as a putative glycosyltransferase. Iterative PSI-BLAST searches show that PseG is related to UDP-N-acetylglucosaminyl transferases, most notably the MurG enzymes involved in peptidoglycan biosynthesis (see supplemental Fig. 6). MurG is a member of the GT-B superfamily of enzymes (family 28) that are metal-independent inverting glycosyltransferases (27, 29). Structural studies on the complex between the E. coli MurG and UDP-GlcNAc have revealed key conserved residues in the C-terminal half of the enzyme that are important for sugar nucleotide binding (28). Although overall homologies between PseG and MurG are low (<15% identity), all of these residues are present in the PseG sequence and they consistently align with those in the MurG sequences. The GGS loop of the GT-B family members (residues 190–192 in E. coli MurG) provides the serine that interacts directly with the β-phosphate of the sugar nucleotide and is conserved as GGT in PseG (residues 165–167). The conserved glutamate and glutamine residues that contact the ribose hydroxyls and the hexose hydroxyls (Glu209 and Gln208 in E. coli) are also conserved as Glu239 and Gln238 in PseG.

The finding that PseG is related to a family of glycosyltransferases raises the notion that the true role of the enzyme may actually be to act as a transferase and that the hydrolase activity is simply an alternate reaction that occurs in the absence of an appropriate acceptor molecule. Several lines of reasoning argue against this possibility. First, the measured catalytic constants for the hydrolysis reaction are comparable/superior to those obtained for glycosyl transfer with related glycosyltransferases (31, 32). This indicates that hydrolysis is the normal reaction catalyzed by the enzyme and that UDP-6-deoxy-AltdiNAc is the true substrate. In cases where glycosyltransferases have been observed to show a background hydrolysis reaction, the rates are orders of magnitude slower than that of the normal transferase reactions (33–35). This ensures that the enzymes do not needlessly hydrolyze their substrates in an unproductive fashion. Second, PseG is clearly involved in the flagellar glycosylation process. The PseG gene is located in the gene cluster that contains all of the known and putative enzymes responsible for the biosynthesis of CMP-pseudaminic acid (notably Cj1317 encoding for Pse synthase and Cj1311 encoding for a putative CMP-Pse synthetase) (1, 13). Furthermore, its homolog in H. pylori, FlmD or HP0326b, has been shown to be required for flagellin glycosylation (26). Most importantly, during the writing of this manuscript a report appeared showing that mutations of the PseG gene resulted in the loss of motility in the C. jejuni strain 81–176 (36). The only glycosyltransferases that would be required in flagellin glycosylation would be CMP-pseudaminyltransferases that would likely resemble CMP-sialyltransferases. Since the structurally characterized CMP-sialyltransferase from C. jejuni was found to display a GT-A-like fold, it is highly unlikely that PseG (a putative GT-B enzyme) catalyzes an analogous reaction (37, 38). Finally, the hexose, 6-deoxy-AltdiNAc, is otherwise unknown as a carbohydrate residue utilized by C. jejuni, and it is unlikely that a glycosyltransferase would exist that accepts its UDP-linked form as a substrate. Together this reasoning strongly suggests that the true role of PseG is to act as a UDP-6-deoxy-AltdiNAc hydrolase in pseudaminic acid biosynthesis.

There are three reasonable mechanisms that the hydrolase could employ during catalysis (Fig. 5). One possibility involves a P–O bond cleavage process in which water attacks the β-phosphorus of the sugar nucleotide and displaces the hexose moiety (Path A). Another possibility involving C–O bond cleavage is that the water attacks at the anomeric carbon and displaces UDP (Path B). This would be the expected mechanism if the
enzyme bears close resemblance to a glycosyltransferase (39). The third possibility also involves C–O bond cleavage and resembles the mechanism employed by the hydrolyzing UDP-GlcNAc 2-epimerase, NeuC, which also shares a GT-B fold (17, 19, 40). In this case an anti-elimination of UDP would generate a glycal intermediate, and hydration of the glycal with retention of configuration at C-2 would give the final product (Path C). The observation that solvent derived 18O is incorporated into the hexose and not into UDP demonstrates that a C–O bond cleavage mechanism is at play and rules out Path A. In addition, the observation that the hydrolase is an inverting enzyme is inconsistent with this mechanism. While it is difficult to conclusively distinguish between Paths B and C, two lines of evidence favor the former. First, no solvent-derived deuterium was incorporated into the C-2 position of 6-deoxy-AltdiNAc during catalysis. This is consistent with the mechanism shown in Path B. In the case of Path C, the proton that was removed from C-2 of the substrate might exchange with bulk solvent during the lifetime of the glycal intermediate, and solvent isotope incorporation would take place during the hydration step. This type of exchange is observed with the hydrolyzing NeuC of sialic acid biosynthesis. Second, the observation that the reaction proceeds with inversion of stereocchemistry at C-1 indicates the enzyme more closely resembles an inverting glycosyltransferase such as MurG (28). In contrast, the NeuC enzyme catalyzes a hydrolysis involving the retention of stereocchemistry at C-1. When taken together with the observed homology to family 28 glycosyltransferases, it is reasonable to assume that UDP-6-deoxy-AltdiNAc hydrolase employs a mechanism involving the direct attack of water at C-1 (Path B).

The closest precedence to the reaction catalyzed by UDP-6-deoxy-AltdiNAc hydrolase is that seen with the enzyme GDP-mannose hydrolase. This is also an inverting hydrolase that utilizes a C–O bond cleavage mechanism during catalysis (41). However, GDP-mannose hydrolase is a member of the Nudix family of enzymes and employs a divalent cation that binds to the α- and β-phosphates of the substrate in a bidentate fashion (42). Thus, the enzymes UDP-6-deoxy-AltdiNAc hydrolase (PseG) and UDP-GlcNAc 2-epimerase (NeuC) that are involved in the biosynthesis of pseudaminic and sialic acid, respectively, appear to be unique examples of sugar nucleotide hydrolases that have evolved from the metal-independent GT-B family of glycosyltransferases.

Acknowledgments—We thank Warren W. Wakarchuk, Ian C. Schoenhofen, and Susan M. Logan for helpful discussions regarding this manuscript and for supplying the NeuC2-MBP, PseB, and PseC expression plasmids.

REFERENCES

1. Szymanski, C. M., and Wren, B. W. (2005) Nat. Rev. Microbiol. 3, 225–237
2. Messner, P. (2004) J. Bacteriol. 186, 2517–2519
3. Schmidt, M. A., and Riley, L. W. (2003) Trends Microbiol. 11, 554–560
4. Upreti, R. K., Kumar, M., and Shankar, V. (2003) Proteomics 3, 363–379
5. Yuki, N. (2005) Curr. Opin. Immunol. 17, 577–582
6. Butzler, J.-P. (2004) Clin. Microbiol. Infect. 10, 868–876
7. van Amsterdam, K., van Vliet, A. H. M., Kusters, J. G., and van der Ende, A. (2006) FEMS Microbiol. Rev. 30, 131–156
8. Goon, S., Kelly, J. F., Logan, S. M., Ewing, C. P., and Guerry, P. (2003) Mol. Microbiol. 50, 659–671
9. Schirrm, M., S., E. C., Aubry, A. J., Austin, J., Thibault, P., and Logan, S. M. (2003) Mol. Microbiol. 48, 1579–1592
10. Thibault, P., Logan, S. M., Kelly, J. F., Brisson, J.-R., Ewing, C. P., Trust, T. J., and Guerry, P. (2001) J. Biol. Chem. 276, 34862–34870
11. Schoenhofen, I. C., McNally, D. J., Vinogradov, E., Whitfield, D., Young, N. M., Dick, S., Wakarchuk, W. W., Brisson, J.-R., and Logan, S. M. (2006) J. Biol. Chem. 281, 723–732
12. Schoenhofen, I. C., Lunin, V. V., Julien, J.-P., Li, Y., Ajami, E., Matte, A., Cyclger, M., Brisson, J.-R., Aubry, A., Logan, S. M., Bhatia, S., Wakarchuk, W. W., and Young, N. M. (2006) J. Biol. Chem. 281, 8907–8916
13. Chou, W. K., Dick, S., Wakarchuk, W. W., and Tanner, M. E. (2005) J. Biol. Chem. 280, 3592–35928
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
15. Liaw, A., and Sharon, N. (1973) Carbohydr. Res. 30, 109–126
16. Tanner, M. E. (2005) Bioorg. Chem. 33, 216–228
17. Murkin, A. S., Chou, W. K., Wakarchuk, W. W., and Tanner, M. E. (2004) Biochemistry 43, 14290–14298
18. Vann, W. F., Daines, D. A., Murkin, A. S., Tanner, M. E., Chaffin, D. O., Rubens, C. E., Vionnet, J., and Silver, R. P. (2004) J. Bacteriol. 186, 706–712
19. Chou, W. K., Hinderlich, S., Reutter, W., and Tanner, M. E. (2003) J. Am. Chem. Soc. 125, 2455–2461
20. Gunawan, J., Simard, D., Gilbert, M., Lovering, A. L., Wakarchuk, W. W., Tanner, M. E., and Strynadka, N. C. (2005) J. Biol. Chem. 280, 3555–3563
21. Sundaram, A. K., Pitts, L., Muhammad, K., Wu, J., Beutenbaugh, M., Woolard, R. W., and Vann, W. F. (2004) Biochem. J. 383, 83–89
22. Munster-Kuhnel, A. K., Tiralongo, J., Krapp, S., Weinhold, B., Ritz-Sedlacek, V., Jacob, U., and Gerardy-Schahn, R. (2004) Glycobiology 14, 43–51
23. Mosimann, S. C., Gilbert, M., Dombrowski, D., To, R., Wakarchuk, W. W., and Strynadka, N. C. (2001) J. Biol. Chem. 276, 8190–8196
24. S., E., Aubry, A. J., Logan, S. M., Guerry, P., Kelly, J. F., Young, N. M., Thibault, P., and Guerry, P. (2004) Anal. Chem. 76, 619–625
25. Logan, S. M., Kelly, J. F., Thibault, P., Ewing, C. P., and Guerry, P. (2002) Mol. Microbiol. 46, 587–597
26. Josenhans, C., Vosbelein, L., Friedrich, S., and Suerbaum, S. (2002) FEMS Microbiol. Lett. 210, 165–172
27. Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003) J. Mol. Biol. 328, 307–317
28. Hu, Y., Chen, L., Ha, S., Gross, B., Falcone, B., Walker, D., Mokhtarzadeh, M., and Walker, S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 845–849
29. Wrabl, J. O., and Grishin, N. V. (2001) J. Mol. Biol. 314, 365–374
30. Kachmar, J. F., and Boyer, P. D. (1953) J. Biol. Chem. 200, 669–682

PseG of Pseudaminic Acid Biosynthesis

FIGURE 5. Potential mechanisms for the reaction catalyzed by UDP-6-deoxy-AltdiNAc hydrolase (PseG). Path A, a P–O bond cleavage mechanism involving direct attack of water at phosphorus. Path B, a C=O bond cleavage mechanism involving direct attack of water at carbon. Path C, a C–O bond cleavage mechanism involving glycal formation. Isotopic labels indicate the consequence of carrying out the reaction in H218O.


20908 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 30 • JULY 28, 2006
31. Chen, L., Men, H., Ha, S., Ye, X.-Y., Brunner, L., Hu, Y., and Walker, S. (2002) Biochemistry 41, 6824–6833
32. Losey, H. C., Peczuh, M. W., Chen, Z., Eggert, U. S., Dong, S. D., Pelczer, I., Kahne, D., and Walsh, C. T. (2001) Biochemistry 40, 4745–4755
33. Lairson, L. L., Chiu, C. P. C., Ly, H. D., He, S., Wakarchuk, W. W., Strynadka, N. C., and Withers, S. G. (2004) J. Biol. Chem. 279, 28339–28344
34. Zhang, Y., Wang, P. G., and Brew, K. (2001) J. Biol. Chem. 276, 11567–11574
35. Ciesla, W. P., Jr., and Bobak, D. A. (1998) J. Biol. Chem. 273, 16021–16026
36. Guerry, P., Ewing, C. P., Schirm, M., Lorenzo, M., Kelly, J., Patarini, D., Majam, G., Thibault, P., and Logan, S. (2006) Mol. Microbiol. 60, 299–311
37. Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., and Imberty, A. (2006) Glycobiology 16, 29R–37R
38. Chiu, C. P. C., Watts, A. G., Lairson, L. L., Gilbert, M., Lim, D., Wakarchuk, W. W., Withers, S. G., and Strynadka, N. C. (2004) Nat. Struct. Biol. 11, 163–170
39. Lairson, L. L., and Withers, S. G. (2004) Chem. Commun. 20, 2243–2248
40. Campbell, R. E., Mosimann, S. C., Tanner, M. E., and Strynadka, N. C. J. (2000) Biochemistry 39, 14993–15001
41. Legler, P. M., Massiah, M. A., Bessman, M. J., and Mildvan, A. S. (2000) Biochemistry 39, 8603–8608
42. Mildvan, A. S., Xia, Z., Azurmendi, H. F., Saraswat, V., Legler, P. M., Massiah, M. A., Gabelli, S. B., Bianchet, M. A., Kang, L.-W., and Amzel, L. M. (2005) Arch. Biochem. Biophys. 433, 129–143