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Identification and Characterization of NeuB3 from *Campylobacter jejuni* as a Pseudaminic Acid Synthase*

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Wayne K. Chou[‡], Scott Dick[§], Warren W. Wakarchuk[§], and Martin E. Tanner[‡1]

From the [‡]Department of Chemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z1 and the [§]Institute for Biological Science, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

Campylobacter jejuni* and *Campylobacter coli* are the main causes of bacterial diarrhea worldwide, and *Helicobacter pylori* is known to cause duodenal ulcers. In all of these pathogenic organisms, the flagellin proteins are heavily glycosylated with a 2-keto-3-deoxy acid, pseudaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid). The presence of pseudaminic acid is required for the proper development of the flagella and is thereby necessary for motility in, and invasion of, the host. In this study we report the first characterization of NeuB3 from *C. jejuni* as a pseudaminic acid synthase; the enzyme directly responsible for the biosynthesis of pseudaminic acid. Pseudaminic acid synthase catalyzes the condensation of phosphoenolpyruvate (PEP) with the hexose, 2,4-diacetamido-2,4,6-trideoxy-L-altrose (6-deoxy-AltdiNAc), to form pseudaminic acid and phosphate. The enzymatic activity was monitored using ^{1}H and ^{31}P NMR spectroscopy, and the product was isolated and characterized. Kinetic analysis reveals that pseudaminic acid synthase requires the presence of a divalent metal ion for catalysis and that optimal catalysis occurs at pH 7.0. A coupled enzymatic assay gave the values for k_{cat} of 0.65 ± 0.01 s^{-1}, K_mPEP of 6.5 ± 0.4 μM, and K_m6-deoxy-AltdiNAc of 9.5 ± 0.7 μM. A mechanistic study on pseudaminic acid synthase, using [2-^{18}O]PEP, shows that catalysis proceeds through a C-O bond cleavage mechanism similar to other PEP condensing synthases such as sialic acid synthase.

Campylobacter jejuni, *Campylobacter coli*, and *Helicobacter pylori* are flagellated, motile, Gram-negative bacteria that colonize the gastrointestinal tract of people worldwide. *C. jejuni* and *C. coli* are the main causative agents of bacterial diarrhea (1, 2), and *H. pylori* causes duodenal ulcers (3). Their flagellin proteins are heavily glycosylated with a 9-carbon 2-keto-3-deoxy acid, pseudaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid) (Fig. 1*A*) (4–7). The production of pseudaminic acid is important for the development of flagella in these organisms as evidenced by aflagellate *C. jejuni* (8) and *H. pylori* (6) mutants unable to produce pseudaminic acid. Motility is a key factor implicated in the colonization of the viscous gastrointestinal tract of the host and therefore flagella are necessary for their invasiveness (1, 9–11). By studying the enzymes involved in the biosynthesis of pseudaminic acid, methods can be developed for combating these pathogenic organisms.

Pseudaminic acid shares great structural similarity with another 9-carbon 2-keto-3-deoxy acid, *N*-acetylneuraminic acid (NeuNAc or sialic acid) (Fig. 1*B*). The biosynthesis of sialic acid has been well studied and the enzyme directly responsible for forming sialic acid is known as sialic acid synthase or *N*-acetylneuraminic acid synthase (SAS or NeuB). Sialic acid synthase catalyzes the condensation of phosphoenolpyruvate with *N*-acetyl-D-mannosamine (ManNAc) to form sialic acid and phosphate (12–14). Recent studies have reported on both the structure of NeuB and its mechanism of action (15, 16). Catalysis was demonstrated to proceed through an overall C-O bond cleavage process where the *si*-face of phosphoenolpyruvate (PEP)[2] initially attacks the aldehyde of the open chain form of ManNAc and water adds to the C-2 position forming a tetrahedral intermediate (Fig. 2*a*). The tetrahedral intermediate then collapses to form orthophosphate and the open chain form of sialic acid that cyclizes to the pyranose form in solution. Formation of the tetrahedral intermediate was proposed to proceed in a stepwise fashion through an oxocarbenium ion intermediate. Two other well studied PEP condensing enzymes that utilize very similar mechanisms are 2-keto-3-deoxy-D-*manno*-octulosonate-8-phosphate synthase, which catalyzes the condensation of PEP and D-*arabino*-5-phosphate (17, 18), and 2-keto-3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, which catalyzes the condensation of PEP and D-erythrose-4-phosphate (19, 20). A key mechanistic experiment performed with all three of these synthases was the use of [2-^{18}O]PEP as a substrate during enzymatic incubations (see labeled atoms in Fig. 2). In each case, catalysis resulted in the production of ^{18}O-labeled inorganic phosphate indicating that a C-O bond cleavage process was occurring (15, 19, 21).

To date none of the enzymes of pseudaminic acid biosynthesis have been characterized, however; it is reasonable to assume that a pseudaminic acid synthase exists that would catalyze the condensation of PEP with the hexose, 2,4-diacetamido-2,4,6-trideoxy-L-altrose (6-deoxy-AltdiNAc) (Fig. 1*A*). Three genes that show homology to *neuB* were identified in *C. jejuni*, where one encoded a sialic acid synthase (*neuB1*) and the other two genes were thought to encode proteins that modify the flagellin proteins (*neuB2* and *neuB3*) (8, 16). NeuB3 shows 35.0% identity to the NeuB from *Neisseria meningitidis* and was found to be essential for the formation of flagella because an insertional mutation in the gene resulted in aflagellate *C. jejuni* (8). In this study we report the first identification and characterization of the activity of NeuB3 as a pseudaminic acid synthase that catalyzes the condensation of PEP with 6-deoxy-AltdiNAc to form pseudaminic acid.

There are two potential mechanisms proposed for pseudaminic acid synthase. The first and most likely mechanism is a C-O bond cleavage mechanism similar to that of sialic acid synthase (Fig. 2*A*). The second potential mechanism involves P-O bond cleavage whereby a direct attack of water onto the phosphate group of PEP generates phosphate and the enolate anion of pyruvate (Fig. 2*B*). The enolate then attacks the

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[1] To whom correspondence may be addressed. Tel.: 604-822-9453; Fax: 604-822-2847; E-mail: mtanner@chem.ubc.ca.

[2] The abbreviations used are: PEP, phosphoenolpyruvate; 6-deoxy-AltdiNAc, 2,4-diacetamido-2,4,6-trideoxy-L-altrose; NeuNAc, *N*-acetylneuraminic acid; ManNAc, *N*-acetyl-D-mannosamine; ESI-MS, electrospray ionization-mass spectrometry; MES, 4-morpholineethanesulfonic acid.

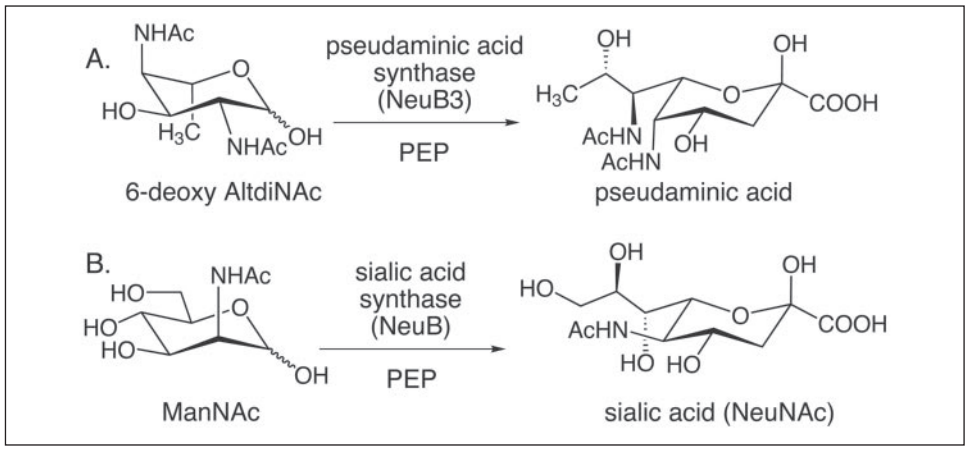

FIGURE 1. **Reactions catalyzed by pseudaminic acid synthase (*NeuB3*) and sialic acid synthase (*NeuB*).** *A*, NeuB3 catalyzes the condensation of PEP with 6-deoxy-AltdiNAc to form pseudaminic acid and orthophosphate. *B*, NeuB catalyzes the condensation of PEP with ManNAc to form sialic acid and orthophosphate.

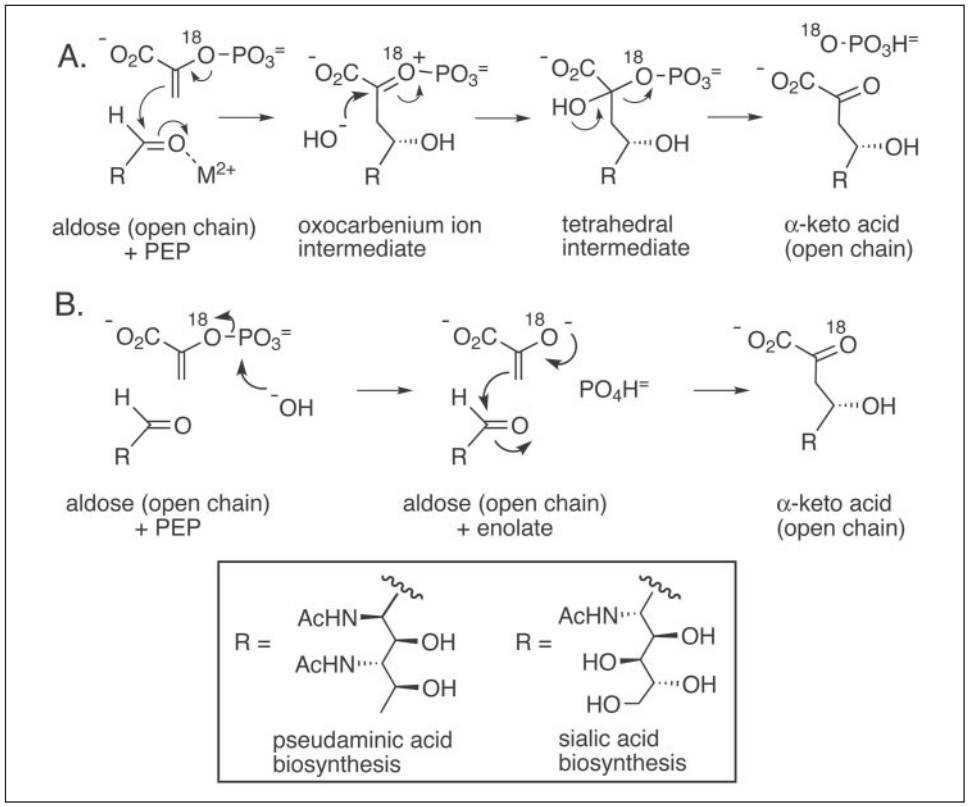

FIGURE 2. **Potential mechanisms for the reaction catalyzed by pseudaminic acid synthase (*NeuB3*) and sialic acid synthase (*NeuB*).** *A*, a C-O bond cleavage mechanism. *B*, a P-O bond cleavage mechanism. ^{18}O isotopic labels are included to highlight the fate of the bridging phosphate oxygen of PEP during catalysis.

aldehyde of the open chain form of 6-deoxy-AltdiNAc producing the open chain form of pseudaminic acid. Precedence for this mechanism comes from pyruvate kinase (22) and PEP carboxykinase (23) where catalysis occurs through formation of the enolate anion of pyruvate via nucleophilic attack at the phosphate group of PEP. In this article we use [2-^{18}O]PEP to show that, like sialic acid synthase, pseudaminic acid synthase also employs a C-O bond cleavage mechanism.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Purine nucleoside phosphorylase and phosphoenolpyruvate were purchased from Sigma. ^{18}O-Enriched water (95%) was purchased from Icon Isotopes. 2-Amino-6-mercapto-7-methylpurine ribonucleoside was purchased from Berry and Associates. Protein concentrations were determined by the method of Bradford (24) using bovine serum albumin as the standard. Concentrations of stock substrate solutions were determined enzymatically using the reported assay conditions with high concentrations of NeuB3 (64 μg) and excess co-substrate. NMR spectroscopy was performed on a Bruker AV300 or AV400 spectrometer. ESI-MS was performed on a Bruker Esquire LC mass spectrometer.

Cloning, Overexpression, and Purification of NeuB3—The *neuB3* gene, Cj1317, was obtained from the genome strain *C. jejuni* 11168 by PCR amplification with Pwo polymerase according to manufacturer's conditions (Roche) (8). The primers used were: 5′-CCCCCCCATATGCAAATAGGAAATTTTAAC-3′ (forward sequence) and 5′-CCCCCCGTCGACTCATCATTGGAAATCTCCTTGTTTAAAG-3′ (reverse sequence). The gene was then cloned into the expression plasmid pCWori+ as an NdeI-SalI fragment, and the construct was maintained in *Escherichia coli* AD202, which was used for enzyme production (25). Cells were grown at 37 °C in 500 ml of Luria-Bertani (LB) medium supplemented with 100 μg ml^{-1} ampicillin. Overexpression of NeuB3 was induced through the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside at an A_{600} of 0.60, with growth at 37 °C for 6 h. Cells were harvested through centrifugation at 5,000 × *g* for 30 min, resuspended

in Tris-HCl buffer (20 mM, pH 7.0) containing pepstatin A (1 mg/liter) and aprotonin (1 mg/liter), and lysed with two passes through a French press. The cell lysate was centrifuged at 7,000 × *g* for 1.5 h, passed through a 0.45-μm filter, and loaded directly onto a 5-ml Hi-trap™ Q-Sepharose column (Amersham Biosciences) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.0). NeuB3 was eluted with a linear gradient of 0 to 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.0). Fractions containing active enzyme were desalted 2 times by concentration and reconstitution with 20 mM Tris-HCl buffer (pH 7.0) using Amicon Ultra Centricons (Millipore), and then flash frozen with 10% glycerol. Protein samples were determined to be >90% pure by SDS-PAGE. The molecular mass was determined to be 38,670 using ESI-MS (38,647 predicted).

NMR Incubation Studies and Tests for Activity—6-Deoxy-AltdiNAc was synthesized according to literature procedures (26, 27). A solution of Tris-DCl buffer prepared in D_2O (700 μl, 10 mM, pD 7.4) containing 6-deoxy-AltdiNAc (10 mM) and PEP (20 mM) was placed in an NMR tube. Initial ^1H and proton-decoupled ^{31}P NMR spectra were taken. The solution was removed from the tube and mixed with 50 mg of NeuB3 (buffer exchanged with the deuterated Tris-HCl buffer (pD 7.4)) and 1 mM $MgCl_2$ to a total volume of 1 ml. After incubation of the reaction mixture for 5 min at 25 °C, Chelex-100 resin (~20 mg) was added and ^1H and ^{31}P NMR spectra were retaken.

C-O Versus P-O Bond Cleavage—[2-^{18}O]PEP disodium salt was prepared according to literature reported procedures (28, 29). The extent of ^{18}O incorporation at the C-2 position was determined to be 54% by mass spectrometry: ESI-MS (MeOH) *m/z* 169 ($M - H^+$, ^{18}O, 100), 167 ($M - H^+$, ^{16}O, 85). ^{31}P NMR spectroscopy was used to determine that the position of the label was at the C-2 position: ^{31}P NMR δ −2.999 (s, P-^{16}O, PEP), −3.018 (s, P-^{18}O, PEP).

A solution of Tris-DCl buffer prepared in D_2O (700 μl, 10 mM, pD 7.4) containing 6-deoxy-AltdiNAc (10 mM) and 20 mM [2-^{18}O]PEP was placed in an NMR tube, and Chelex-100 resin (~20 mg previously washed with D_2O) was added. An initial proton-decoupled ^{31}P NMR spectrum was obtained with the following parameters: spectral frequency of 121.5 MHz, sweep width of 2437 Hz, acquisition time of 13.4 s, pulse delay of 2 s, and pulse width of 10 μs. The solution was decanted from the Chelex resin and mixed with 50 mg of NeuB3 (buffer exchanged with the deuterated Tris-HCl buffer (pD 7.4)) and 1 mM $MgCl_2$. The reaction was incubated for 5 min at 25 °C and Chelex-100 resin (~20 mg previously washed with D_2O) was added. After a 1-h time period to allow complete complexation of the metals, another proton-decoupled ^{31}P NMR spectrum was acquired with the same parameters: ^{31}P NMR δ 0.0 (s, Pi-^{16}O), −0.022 (s, Pi-^{18}O), −3.010 (s, P-^{16}O, PEP), −3.029 (s, P-^{18}O, PEP).

Isolation and Characterization of Pseudaminic Acid—Enzyme was removed from the reactions by centrifugal ultrafiltration and the resulting filtrate was loaded onto a 10-ml column of Dowex AG1-X8 resin (formate form) pre-equilibrated with water. A stepwise gradient of 0–1.0 M formic acid in water with 0.2 M increments (50 ml per increment) was used to elute the pseudaminic acid from the column. Pseudaminic acid eluted from the column in the 0.2 and 0.4 M fractions that were concentrated *in vacuo* and then lyophilized. Pseudaminic acid was characterized using ^1H, ^{13}C, two-dimensional COSY, HMQC, and one-dimensional TOCSY NMR spectroscopy (in 10 mM deuterated phosphate buffer pD 7.4), and negative ESI-MS mass spectrometry. ^1H (D_2O) δ 1.0 (d, 3 H, $J_{8,9}$ 6.5 Hz, H-9), 1.95 (dd, 1 H, $J_{3ax,4}$ 12.2 Hz, $J_{3ax,3eq}$ 13.3 Hz, H-3ax), 1.99 (dd, 1 H, J_{3eq3ax} 13.3, $J_{3eq,4}$ 4.6 Hz, H-3eq), 1.86 (s, 3 H, CH3), 1.89 (s, 3 H, CH3), 3.91 (dd, 1 H, $J_{5,6}$ 1.7 Hz, $J_{6,7}$ 10.3 Hz, H-6), 4.01 (dq, 1 H, $J_{7,8}$ 3.7 Hz, $J_{8,9}$ 6.5 Hz, H-8), 4.03 (dd, 1 H, $J_{6,7}$ 10.3, $J_{7,8}$ 3.7, H-7), 4.05 (ddd, 1 H, $J_{3eq,4}$ 4.6, $J_{3ax,4}$ 12.2, $J_{4,5}$ 5.5, H-4), 4.13 (dd, 1 H, $J_{4,5}$ 5.5, $J_{5,6}$ 1.7, H-5). ^{13}C NMR (D_2O) δ 15.2 (C-9), 21.8 (CH_3-NAc), 22.0 (CH_3-NAc), 34.8 (C-3), 48.8 (C-5), 52.9 (C-7), 65.2 (C-4), 66.8 (C-8), 69.9 (C-6), 96.4 (C-2), 173.7, 174.6, 176.3 (C = O). -ve ESI-MS (H_2O) *m/z* 333 ($M - H^+$), *m/z* 315 ($M - H_2O$).

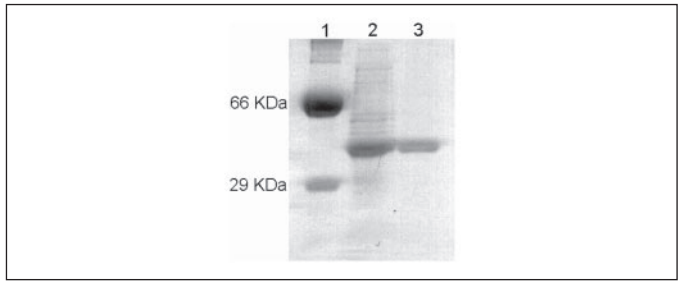

FIGURE 3. **SDS-PAGE gel showing the expression and purification of recombinant pseudaminic acid synthase (*NeuB3*) using ion-exchange chromatography.** *Lane 1* contains molecular mass standards of 66 and 29 kDa, *lane 2* shows crude cell extract, *lane 3* shows the purified pseudaminic acid synthase.

Enzyme Kinetics as Determined by a Continuous Coupled Assay—Enzyme kinetics were measured by a continuous coupled phosphate assay (30). A cuvette containing 100 mM Tris-HCl buffer (pH 7.0), 6-deoxy-AltdiNAc (variable), PEP (variable), 2-amino-6-mercapto-7-methylpurine ribonucleoside (200 μM), purine nucleoside phosphorylase (5 units buffer exchanged twice into 100 mM Tris-HCl buffer pH 7.0), and $MnCl_2$ (10 mM) was thermally equilibrated for 5 min at 37 °C. The enzymatic reaction was initiated by the addition of NeuB3 (3.2 μg) for a total assay volume of 500 μl and the enzymatic rate was calculated from the observed increase of absorption at A_{360} (using ϵ = 11,000 M^{-1} cm^{-1}). The K_m value for 6-deoxy-AltdiNAc was measured in the presence of 1 mM PEP (saturating), and that for PEP was measured in the presence of 1 mM 6-deoxy-AltdiNAc (saturating). Kinetic parameters were determined from initial velocities fit to Michaelis-Menten kinetics using the program *Grafit* (31).

pH Versus Rate Profile Experiment—The pH *versus* rate profile was constructed using a mixture of 50 mM Tris-HCl and 50 mM MES buffer at pH 6–9. Purine nucleoside phosphorylase was buffer exchanged twice in the same buffer at the different pH values. Saturating 6-deoxy-AltdiNAc (1 mM) and saturating PEP (1 mM) were used with the kinetic assay above. Initial rates were plotted against pH.

Metal Dependence Experiment—The metal dependence experiment was carried out with saturating 6-deoxy-AltdiNAc (1 mM) and saturating PEP (1 mM) at pH 7.0 using the kinetic assay above. Different divalent metal cations were independently added at a concentration of 10 mM and the initial velocities were determined. The rates were normalized to the fastest rate (Co^{2+}). Controls included adding 10 mM EDTA or no additives (enzyme as isolated) to the kinetic assay.

RESULTS AND DISCUSSION

Overexpression and Purification of NeuB3-C. jejuni—The *neuB3* gene, Cj1317, was obtained from the genome strain *C. jejuni* 11168 by PCR amplification and overexpressed in *E. coli*. NeuB3 was isolated using a single anion exchange chromatographic step and the resulting protein was found to be greater than 90% pure as determined by SDS-PAGE (Fig. 3). The molecular mass of NeuB3 was determined to be 38,670 ± 30 using electrospray ionization-mass spectrometry (38,647 predicted).

Test for Activity of NeuB3—One of the main hurdles in studying pseudaminic acid synthase is that the putative substrate 6-deoxy-AltdiNAc is not commercially available. To obtain a sample it was necessary to chemically synthesize the compound in 12 steps from L-fucose using a protocol described in the literature (26, 27). To test whether NeuB3

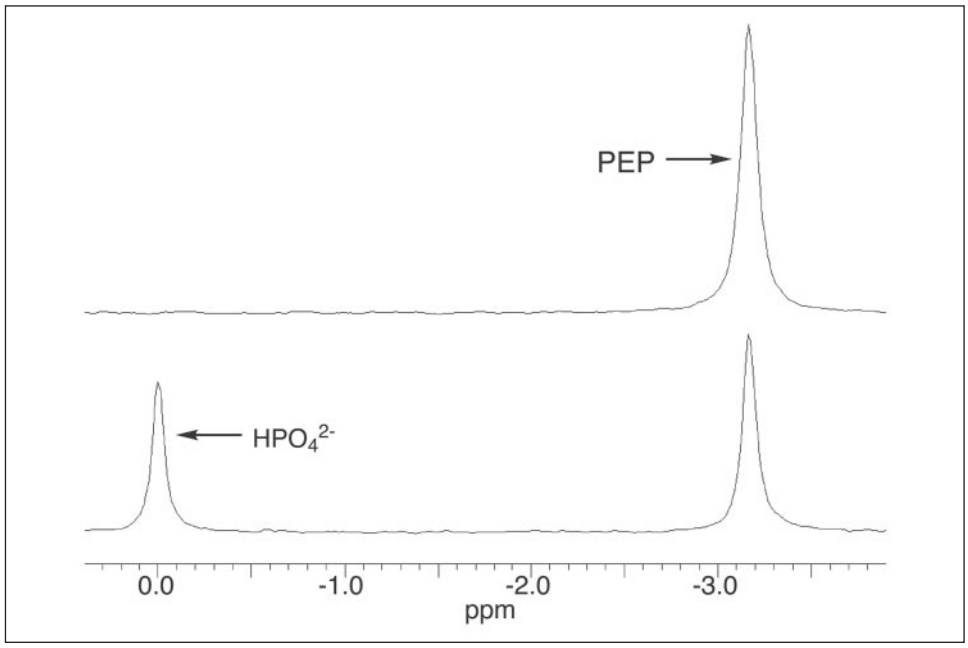

FIGURE 4. **^{31}P NMR spectra monitoring the conversion of PEP to phosphate by NeuB3.** The *upper panel* shows the spectrum before the addition of NeuB3. The *lower panel* shows the spectrum taken after 5 min of incubation.

showed pseudaminic acid synthase activity, 1 eq of 6-deoxy-AltdiNAc and 2 eq of PEP were incubated in deuterated buffer containing Mn^{2+} and NeuB3, and the reaction was monitored by ^{1}H and ^{31}P NMR spectroscopy. Before the addition of NeuB3, the ^{31}P NMR spectrum shows a single phosphorus peak corresponding to the phosphate group of PEP at −3.01 ppm (Fig. 4, *upper panel*). After the addition, a new peak appears at 0 ppm corresponding to inorganic phosphate released during catalysis (Fig. 4, *lower panel*). The identities of the phosphorus peaks were verified by spiking with PEP and phosphate standards (data not shown). Control incubations lacking either enzyme or 6-deoxy-AltdiNAc did not show the formation of orthophosphate under identical conditions, indicating that the reaction was not because of either background hydrolysis or a phosphatase impurity. In addition, ManNAc could not replace 6-deoxy-AltdiNAc as a substrate indicating that NeuB3 does not possess detectable sialic acid synthase activity.

The time course as observed by ^{1}H NMR spectroscopy shows the conversion of 6-deoxy-AltdiNAc to pseudaminic acid. The initial ^{1}H NMR spectra shows that 6-deoxy-AltdiNAc exists as a 3:1 ratio of the β to α anomer in solution (Fig. 5, *upper panel*). The anomeric ratio is apparent from integration of the two acetamido signals of 6-deoxy-AltdiNAc (β-anomer at 1.88 and 1.93 ppm, and α-anomer at 1.89 and 1.90 ppm) and also the anomeric protons (data not shown). After the addition of NeuB3, the complete conversion of the substrate into largely a single anomer of a new product is observed (acetamido peaks at 1.81 and 1.85 ppm). This would be expected for the pyranose form of a nonulosonic acid that would strongly favor the α-anomer in solution. Further support is seen in the appearance of the key characteristic peaks of pseudaminic acid because of the signals of the C-3 methylene protons (Fig. 5, *lower panel*). The H-3ax (axial) proton signal is a doublet of doublets that appears at 1.62 ppm and the H-3eq (equatorial) proton signal is a doublet of doublets that appears at 1.77 ppm (slightly obscured by the methyl *N*-acetyl proton signals) under these conditions. The fact that the H-3ax proton signal appears as a triplet is because of strong geminal coupling to the H-3eq proton ($J_{3ax,3eq}$ 13.3 Hz) and strong coupling to the H-4 proton ($J_{3ax,4}$ 12.2 Hz). The large $J_{3ax,4}$ coupling can only arise if H-3ax and H-4 have a *trans*-diaxial relationship. This indicates that PEP attacks the *si*-face of the aldehyde of 6-deoxy-AltdiNAc to generate an (*S*)-configuration at C-4.

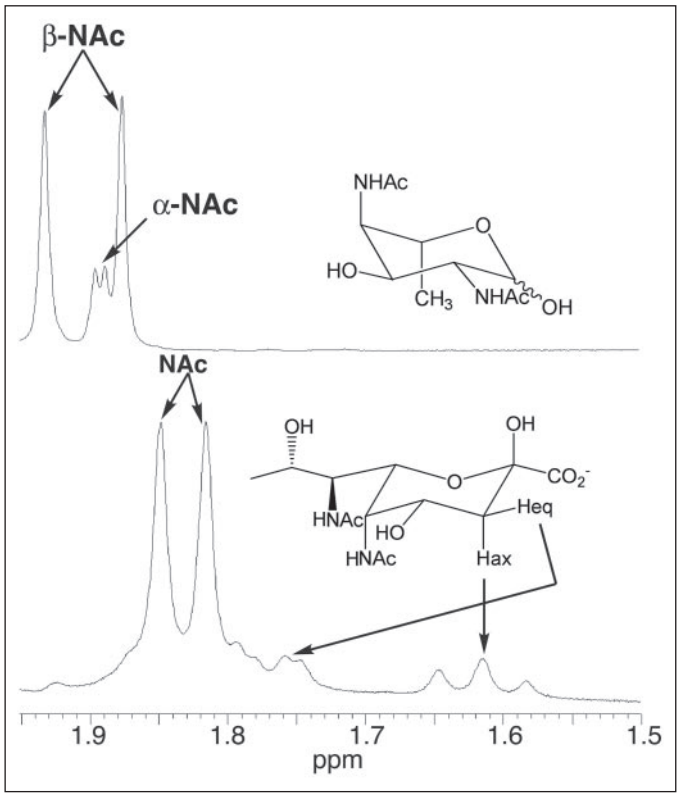

FIGURE 5. **^{1}H NMR spectra monitoring the conversion of 6-deoxy-AltdiNAc into pseudaminic acid by NeuB3.** The *upper panel* shows the spectrum taken before the addition of NeuB3. The *lower panel* shows the spectrum taken after 5 min of incubation.

Whereas previous studies have demonstrated that the absolute stereochemistry of pseudaminic acid from *Pseudomonas aeruginosa* is of the L-*glycero*-L-*manno* configuration, this has not been unequivocally shown in the case of *C. jejuni*. One can postulate reasonable biosynthetic routes to either enantiomer using very similar enzymatic pathways and therefore it was conceivable that either the D- or L-enantiomer of 6-deoxy-AltdiNAc could be the correct substrate for NeuB3. The fact that the L-isomer of 6-deoxy-AltdiNAc is the substrate of NeuB3 indi-

TABLE ONE

^{1}H NMR spectral assignment of pseudaminic acid

Proton signal (integration)	δ	Muliplicity	Coupling constants
	ppm		*Hz*
H-3eq (1 H)	1.81	dd	$J_{3eq,4} = 4.6$
H-3ax (1 H)	1.67	dd	$J_{3ax,3eq} = 13.3$
H-4(1 H)	4.05	ddd	$J_{3ax,4} = 12.2$
H-5 (1 H)	4.13	dd	$J_{4,5} = 5.5$
H-6 (1 H)	3.91	dd	$J_{5,6} = 1.7$
H-7 (1 H)	4.03	dd	$J_{6,7} = 10.3$
H-8 (1 H)	4.01	dq	$J_{7,8} = 3.7$
H-9 (3 H)	.99	d	$J_{8,9} = 6.5$
N-Ac (3 H)	1.86	s	
N-Ac (3 H)	1.89	s	

TABLE TWO

^{13}C NMR spectral assignment of pseudaminic acid

Carbon signal	δ
	ppm
C-2	96.4
C-3	34.8
C-4	65.2
C-5	48.8
C-6	70.0
C-7	52.9
C-8	66.8
C-9	15.3
CH_3-NAc	21.8
	22.0
C = O	173.7
	174.6
	176.3

cates that the stereochemical assignment of pseudaminic acid is correct as the previously assigned L-*glycero*-L-*manno* isomer (4, 32–35).

Isolation and Characterization of Pseudaminic Acid—The pseudaminic acid produced from the NeuB3 reaction was isolated using anion exchange chromatography and fully characterized. Mass spectral analysis (-ve ESI-MS) showed signals for the expected parental ion at *m*/*z* 333 ($M - H^+$) and the oxonium ion at *m*/*z* 315 ($M - H_3O^+$). The NMR spectra were similar to those previously reported in the literature for pseudaminic acid derivatives characterized from the lipopolysaccharide of *P. aeruginosa*, *Shigella boydii*, *Vibrio cholerae*, *Proteus vulgaris*, and the flagellin proteins of *C. jejuni* (4, 32–35). ^{1}H NMR spectroscopy was used to assign the relative configuration of the pyranose ring protons. The large coupling constants of $J_{3a,4}$ (12.2 Hz) and small coupling constants of $J_{4,5}$ (5.5 Hz) and $J_{5,6}$ (1.7 Hz) indicates that H-4 is axial, H-5 is equatorial, and H-6 is axial (TABLE ONE). The large $J_{6,7}$ of 10.3 Hz indicates that C-6 and C-7 have the *erythro* configuration as opposed to sialic acid, which has a small $J_{6,7}$ of 1.2 Hz and the *threo* configuration (33). In the ^{13}C NMR spectrum (TABLE TWO), the C-9 chemical shift is 15.3 ppm, which is similar to the chemical shift of the methyl group for *N*-acetyl L-allothreonine (17.5 ppm) indicating that C-7 and C-8 has the *erythro* conformation (*versus* 19.5 ppm with *N*-acetyl L-threonine) (33). The difference in the ^{1}H chemical shifts of H-3ax and H-3eq is small (0.14 ppm), indicating that the α-anomer is the predominant anomer because the β-anomer has a larger difference in chemical shift between the two proton signals (0.77 ppm) as seen in C-2 glycosylated pseudaminic acid that is β-linked (4, 32–35).

Enzyme Kinetics of NeuB3 as Determined by a Continuous Coupled Assay—The kinetic pararmeters of the NeuB3 reaction were determined using a continuous coupled assay for phosphate release (30). Initial reaction velocities (v_o) were determined for each substrate by varying its concentration in the presence of saturating amounts of the other substrate. The kinetic data obeyed Michaelis-Menten kinetics (Fig. 6) and gave kinetic constants of $k_{cat} = 0.65 \pm 0.01\ s^{-1}$, K_mPEP = 6.5 ± 0.4 μM and K_m6-deoxy-AltdiNAc of 9.5 ± 0.7 μM (100 mM Tris-HCl, pH 7.0, 10 mM Mn^{2+} and 37 °C). Whereas the turnover number is similar to that observed with sialic acid synthase, the K_m values were considerably lower, potentially reflecting the lower concentration of 6-deoxy-AltdiNAc that is available in the cell. A qualitative screen of maximal rate *versus* pH gave a bell-shaped profile and indicated that optimal activity was observed at pH 7. Finally, an assessment of the metal ion requirements for pseudaminic acid synthase was performed (TABLE THREE). When the enzyme was assayed directly as isolated, only low levels of activity were observed. Activity was gained upon the addition of various divalent metal ions with the highest values observed

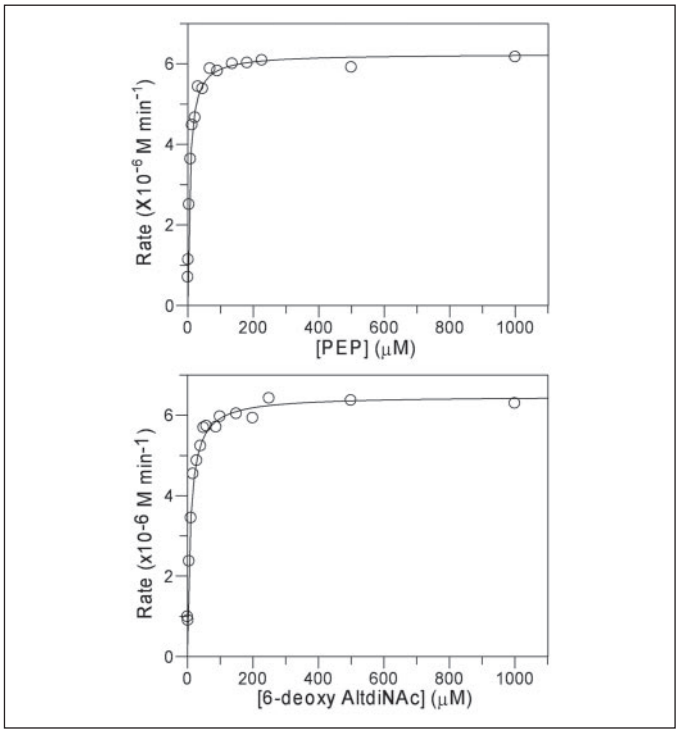

FIGURE 6. **Enzyme kinetic plots of initial velocity *versus* substrate concentration.** The *upper plot* shows the kinetic plot of initial velocity *versus* PEP concentration at a saturating concentration of 6-deoxy-AltdiNAc (1 mM). The *lower plot* shows the kinetic plot of initial velocity *versus* 6-deoxy-AltdiNAc concentration and saturating concentrations of PEP (1 mM). The kinetic parameters as determined by fitting the data to Michaelis-Menten kinetics are as follows: $k_{cat} = 0.65 \pm 0.01\ s^{-1}$, K_mPEP = 6.5 ± 0.04 μM, K_m6-deoxy-AltdiNAc = 9.5 ± 0.07 μM.

in the presence of Mn^{2+} and Co^{2+}. The addition of EDTA completely abolished the activity of the synthase. These observations indicate that pseudaminic acid synthase requires a divalent cation for activity and that the bound metal is largely lost during isolation of the enzyme. Similar observations have been made with sialic acid synthase, NeuB, which apparently uses the divalent cation to increase the electrophilicity of the substrate aldehyde (15). A recent x-ray crystal structure of NeuB complexed with Mn^{2+}, PEP, and *N*-acetylmannosaminitol (a reduced form of ManNAc bearing a hydroxyl group at C-1) showed that the divalent metal ion was in close proximity (2.5 Å) to the C-1 hydroxyl group of the substrate analog (Protein Data Bank file 1XUZ). This indi-

cates that in the Michaelis complex the Mn^{2+} would be in a favorable position for activation of the aldehyde (15).

Mechanistic Studies on Pseudaminic Acid Synthase—To probe the mechanism of pseudaminic acid synthase, [2-^{18}O]PEP was synthesized with a 54% incorporation of the label (28, 29). The location of the ^{18}O label was determined using ^{31}P NMR spectroscopy because the substitution of ^{16}O for ^{18}O in a singly bonded position to phosphorus results in a small upfield shift for the labeled phosphorus atom (36, 37). By monitoring the enzymatic reaction of [2-^{18}O]PEP and 6-deoxy-AltdiNAc with ^{31}P NMR spectroscopy, the fate of the ^{18}O label can be determined. The initial ^{31}P NMR spectrum of [2-^{18}O]PEP with 6-deoxy-AltdiNAc shows two phosphorus signals, one for unlabeled PEP at −3.01 ppm and the other for [2-^{18}O]PEP shifted slightly upfield at −3.03 ppm (Fig. 7, *upper panel*). After addition of NeuB3 and incubation for 5 min, two new peaks appear corresponding to inorganic phosphate (0 ppm) and ^{18}O-labeled inorganic phosphate (−0.02 ppm) (Fig. 7, *lower panel*). The ratio of ^{16}O to ^{18}O in the inorganic phosphate produced was the same as that of the starting material indicating that the label was fully retained and that C-O bond cleavage was occurring during catalysis.

PEP-condensing enzymes that catalyze C-O bond cleavage processes must inherently proceed through a tetrahedral intermediate. Therefore the proposed catalytic mechanism of pseudaminic acid synthase is the attack of the C-3 of PEP to the aldehyde of the open chain form of 6-deoxy-AltdiNAc and an attack of water to the C-2 of PEP to form a tetrahedral intermediate (Fig. 2*A*). The tetrahedral intermediate then collapses to release inorganic phosphate and form the open chain form of pseudaminic acid that cyclizes to the pyranose form in solution. The formation of the tetrahedral intermediate could occur in a concerted fashion if water attacks the C-2 of PEP at the same time as the C-3 of PEP attacks the aldehyde. The tetrahedral intermediate could also form in a stepwise manner through an oxocarbenium ion intermediate (shown in Fig. 2*A*) that is subsequently attacked by water to form the tetrahedral intermediate. The most convincing evidence for the oxocarbenium ion intermediate comes from the work done on 2-keto-3-deoxy-D-*manno*-octulosonate-8-phosphate synthase where a cationic mimic of the oxocarbenium ion intermediate was found to be a potent inhibitor (37–39).

TABLE THREE

Metal dependence study of pseudaminic acid synthase

All percent activity values are normalized to the rate of catalysis of NeuB3 with Co^{2+}.

Additive (10 mM)	Activity
	%
Co^{2+}	100
Mn^{2+}	91
Mg^{2+}	48
Ca^{2+}	30
Ni^{2+}	26
Enzyme as isolated	4
EDTA	0

Conclusion—This is the first identification and kinetic characterization of NeuB3 from *C. jejuni* as a metal-dependent pseudaminic acid synthase that catalyzes the condensation of PEP with 6-deoxy-AltdiNAc. The characterization of the enzymatically produced pseudaminic acid verifies that the stereochemical assignment of pseudaminic acid is correct as the L-*glycero*-L-*manno* configuration. A mechanistic ^{18}O labeling study shows that catalysis proceeds through a C-O bond cleavage mechanism similar to other PEP condensing enzymes.

Pseudaminic acid production is important for the invasiveness of these pathogenic organisms. The discovery of the activity of NeuB3 as a pseudaminic acid synthase furthers our understanding of the biosynthetic pathway of pseudaminic acid and acts as a novel target for drug development. Other derivatives of pseudaminic acid have also been identified such as *N*-formamido derivatives, *N*-acetamidino derivatives and *N*-dihydroxypropionyl derivatives (4–6, 32–35). It is likely that they are biosynthesized from derivatives of 6-deoxy-AltdiNAc that may be alternate substrates of pseudaminic acid synthase. In this light, it is interesting to note that sialic acid synthase has been shown to catalyze the condensation of PEP with longer *N*-acyl substituents (16). Other isomers of pseudaminic acid have also been isolated from the lipopolysaccharide of *P. aeruginosa* and *Legionella pneumophila* (40–43). These include legionaminic acid (5,7-diacetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid), a C-4 epimeric derivative (5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*talo*-non-2-ulosonic acid), and a C-8 epimeric derivative of legionaminic acid (5,7-diacetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid). It is also likely that there are closely related PEP-condensing synthases that are responsible for the biosynthesis of these isomers.

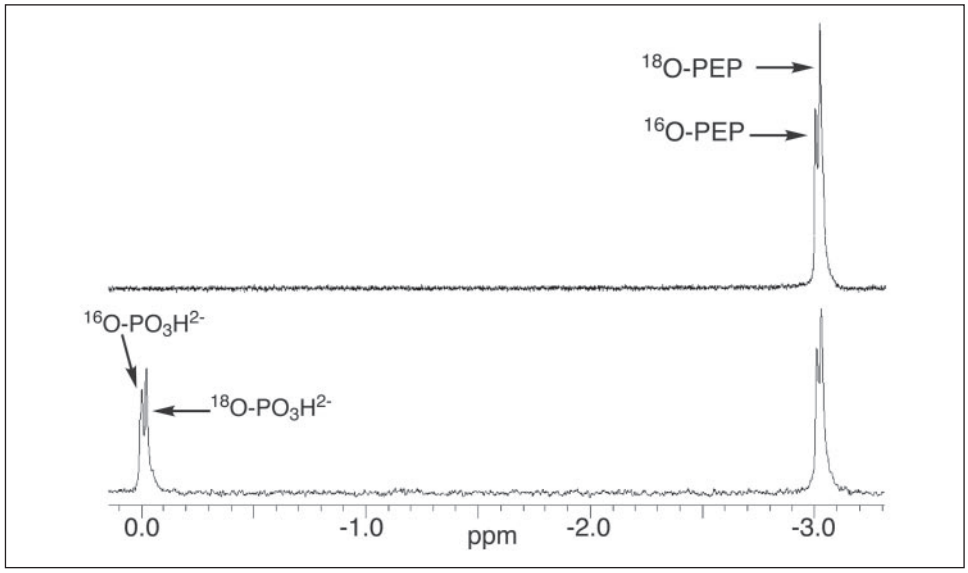

FIGURE 7. **^{31}P NMR spectra monitoring the conversion of partially labeled [2-^{18}O]PEP into ^{18}O-labeled phosphate by NeuB3.** The *upper panel* shows the spectrum before the addition of NeuB3. The *lower panel* shows the spectrum taken after 5 min of incubation.

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Identification and Characterization of NeuB3 from *Campylobacter jejuni* as a Pseudaminic Acid Synthase

Wayne K. Chou, Scott Dick, Warren W. Wakarchuk and Martin E. Tanner

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