Bioorganic Chemistry 36 (2008) 312–320

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Mechanistic studies on PseB of pseudaminic acid biosynthesis: A UDP-*N*-acetylglucosamine 5-inverting 4,6-dehydratase

James P. Morrison [a], Ian C. Schoenhofen [b], Martin E. Tanner [a,*]

[a] Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Z1
[b] Institute for Biological Sciences, National Research Council, Ottawa, Ont., Canada, K1A OR6

ARTICLE INFO

Article history:
Received 18 July 2008
Available online 9 October 2008

Keywords:
UDP-*N*-acetylglucosamine 5-inverting 4,6-dehydratase
PseB
Inverting dehydratase
Pseudaminic acid
Campylobacter jejuni

ABSTRACT

UDP-*N*-acetylglucosamine 5-inverting 4,6-dehydratase (PseB) is a unique sugar nucleotide dehydratase that inverts the C-5″ stereocentre during conversion of UDP-*N*-acetylglucosamine to UDP-2-acetamido-2,6-dideoxy-β-L-*arabino*-hexos-4-ulose. PseB catalyzes the first step in the biosynthesis of pseudaminic acid, which is found as a post-translational modification on the flagellin of *Campylobacter jejuni and Helicobacter pylori*. PseB is proposed to use its tightly bound $NADP^+$ to oxidize UDP-GlcNAc at C-4″, enabling dehydration. The α,β unsaturated ketone intermediate is then reduced by delivery of the hydride to C-6″ and a proton to C-5″. Consistent with this, PseB from *C. jejuni* has been found to incorporate deuterium into the C-5″ position of product during catalysis in D_2O. Likewise, PseB catalyzes solvent isotope exchange into the H-5″ position of product, and eliminates HF from the alternate substrate, UDP-6-deoxy-6-fluoro-GlcNAc. Mutants of the putative catalytic residues aspartate 126, lysine 127 and tyrosine 135 have severely compromised dehydratase, solvent isotope exchange, and HF elimination activities.

1. Introduction

Pseudaminic acid is a nine carbon α-keto acid (nonulosonate) that is similar in structure to the sialic acids (Fig. 1A). It is found as an O-linked post-translational modification of the flagellin proteins in the pathogenic bacteria *Campylobacter jejuni and Helicobacter pylori* [1,2]. The presence of pseudaminic acid is crucial for the assembly of functional flagella, and is therefore required for bacterial motility and invasion of the host intestinal tract [2,3,4,5,6]. Recently, the biosynthesis of pseudaminic acid has been elucidated, and the first step is catalyzed by a UDP-*N*-acetylglucosamine (UDP-GlcNAc) 5-inverting 4,6-dehydratase (PseB or FlaA1) that converts UDP-GlcNAc to UDP-2-acetamido-2,6-dideoxy-β-L-*arabino*-hexos-4-ulose (UDP-4-keto-6-deoxy-L-IdoNAc) (Fig. 1A) [7,8,9].

Sugar nucleotide 4,6-dehydratases are $NAD(P)^+$-dependent oxidoreductases that are members of the short chain dehydrogenase/reductase (SDR) family and are responsible for the biosynthesis of 6-deoxyhexoses. They catalyze an overall redox neutral reaction in which the C-4 hydroxyl is oxidized to a ketone and the C-6 hydroxyl is reduced to a methyl group. Most studies to date have focused on the dTDP-glucose, CDP-glucose and GDP-mannose 4,6-dehydratases, all of which catalyze reactions in which the stereochemical configuration at C-5″ is unchanged [10,11,12]. More recently, two dehydratases have been discovered that act on UDP-GlcNAc. PglF produces UDP-2-acetamido-2,6-dideoxy-α-D-*xylo*-hexos-4-ulose (UDP-4-keto-6-deoxy-GlcNAc) that is used in the biosynthesis of the 2,4-diacetamido derivative, UDP-*N*,*N*′-diacetylbacillosamine, a precursor in the biosynthesis of N-linked Pgl glycoproteins (Fig. 1B)[8,13]. UDP-*N*,*N*′-diacetylbacillosamine is also known to serve as a precursor for the nonulosonic acid, *N*,*N*′-diacetyllegionaminic acid in certain bacteria [14]. Like most sugar nucleotide 4,6-dehydratases, PglF gives product with a retention of configuration at C-5″. The other UDP-GlcNAc-handling dehydratase is PseB of pseudaminic acid biosynthesis, and this enzyme is unique among the dehydratases in that it generates product with an inverted stereochemistry at C-5″ (L-configuration). In the biosynthesis of other 6-deoxy-L-hexoses such as L-fucose and L-rhamnose, the inversion of configuration at C-5″ occurs subsequent to the dehydratase step [15,16].

Since PseB is a potential target for the development of antibiotics and is the first example of a 5-inverting sugar nucleotide 4,6-dehydratase, we chose to investigate the mechanism of the *C. jejuni* enzyme in greater detail. A recent report on the structure of PseB (or FlaA1) from *H. pylori* in complex with bound substrate provided an excellent starting point for such studies [17]. The aim was to use site-directed mutagenesis in combination with solvent isotope incorporation studies and the reactions of alternate substrates to uncover the precise roles of active site residues in catalysis.

* Corresponding author. Fax: +1 604 822 2847.
E-mail address: mtanner@chem.ubc.ca (M.E. Tanner).

0045-2068/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2008.08.004

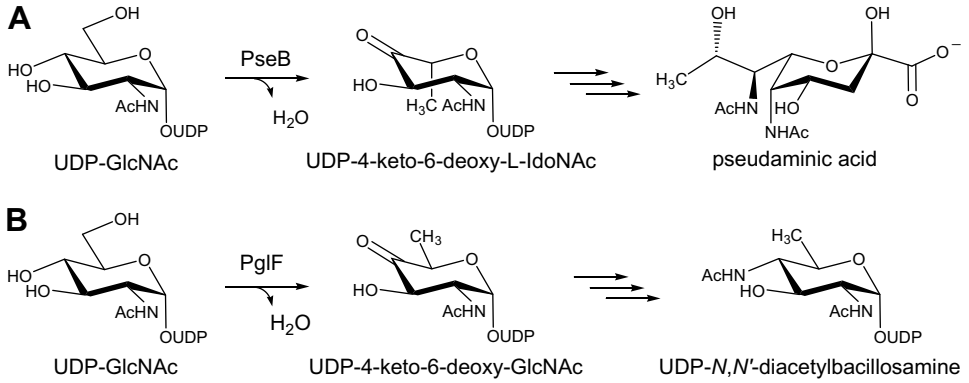

Fig. 1. (A) The 5-inverting dehydratase PseB is the first enzyme in the biosynthesis of pseudaminic acid from UDP-GlcNAc. (B) The non-inverting dehydratase PglF is the first enzyme in the biosynthesis of UDP-*N*,*N*′-diacetylbacillosamine from UDP-GlcNAc.

2. Materials and methods

2.1. Instrumentation and general methods

All chemicals were purchased from Sigma–Aldrich and used without further refinement unless otherwise noted. Dry solvents were distilled fresh, using CaH_2 (CH_2Cl_2, pyridine) or Na/benzophenone (THF) as drying agent. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard [18]. ^{1}H NMR spectra were obtained on a Bruker AV300 or AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. ^{31}P NMR spectra were recorded on these spectrometers at 121.5 or 162 MHz, respectively. ^{19}F NMR spectra were obtained on the Bruker AV300 spectrometer at 282.3 MHz. Mass spectrometry was performed by electrospray ionization (ESIMS) using a Waters Micromass LCT mass spectrometer at the Mass Spectrometry/Microanalysis Laboratory at UBC. An Orion fluoride electrode (model 96-09BN), interfaced with a Fischer Scientific Accumet 925 pH/ion meter, was used to monitor fluoride release.

2.2. Site-directed mutagenesis to prepare wild-type and mutant pseB

The plasmid pNRC20.3 contains the pseB gene (also referred to as cj1293) from *C. jejuni* (NCTC 11168) in a pET30 vector, which encodes for a C-terminal hexahistidine tag to the expressed protein [8]. pNRC20.1, an earlier plasmid preparation to pNRC20.3, encodes two mutations to the nucleic acid sequence of pseB: g328t and t384c. The g328t mutation codes for a D110Y mutation in the protein sequence, and the t384c mutation is silent. In the first round of mutagenesis the g328t mutation was corrected according to the protocol in the QuikChange Site-Directed Mutagenesis Kit from Stratagene, using Platinum Pfx DNA polymerase, Dpn I restriction enzyme, and dNTPs from Invitrogen. The corrected plasmid encoding His-tagged wild-type pseB was then used to prepare the mutant plasmids. Oligonucleotide primers used are listed below, with the mutated nucleotides underlined. The g328t mutation was corrected by mutagenesis using the following primers: 5′-CG GTGCGCAAAATGTCATCGACGCTTGTTTTGAAAATGG-3′ (forward), and: 5′-CCATTTTCAAAACAAGCGTCGATGACATTTTGCGCACCG-3′ (reverse). This created the plasmid encoding the gene pseB (t384c) which was used to express His-tagged wild-type PseB. Using the plasmid encoding the gene pseB (t384c), the following primers were used to generate the plasmid encoding the gene pseB D126N (t384c): 5′-TGTATCGCTCTTAGTACGAATAAGGCCTGTAAT CCTG-3′ (forward), and 5′-CAGGATTACAGGCCTTATTCGTACTAAGA GCGATACA-3′ (reverse). The plasmid encoding the gene pseB K127A was prepared using the following primers and the plasmid encoding the gene pseB (t384c) as template. These primers encode for the K127A mutation and correct the silent mutation: 5′-GCTCTT AGTACGGATGCGGCTTGTAATCCTG-3′ (forward), and 5′-CAGGATTA CAAGCCGCATCCGTACTAAGAGC-3′ (reverse). The plasmid encoding the gene pseB Y135F (t384c) was prepared using the following primers and the plasmid encoding the gene pseB (t384c) as template: 5′-GTAATCCTGTAAATTTATTCGGTGCAACCAAACTTGC-3′ (forward), and 5′-GCAAGTTTGGTTGCACCGAATAAATTTACAGGATT AC-3′ (reverse). Gene sequences were confirmed by sequencing the entire gene.

2.3. Overexpression and purification of wild-type and mutant PseB and PglF

The plasmid pNRC40.1 bears a truncated version of the pglF (Cj1120c) gene from *C. jejuni* (NCTC 11168) that has been described in detail previously [8].

The recombinants were transformed into chemically competent *Escherichia* coli BL21(DE3) cells, which were incubated overnight at 37 °C with shaking at 225 rpm in 10 mL Luria–Bertani (LB) medium containing 30 μg mL^{-1} kanamycin. The overnight cultures were poured into 500 mL LB medium containing 30 μg mL^{-1} kanamycin and grown at 37 °C with shaking at 225 rpm until an OD_{600} of 0.6–0.9 was reached. Cells were induced for overexpression by addition of 120 mg L^{-1} (0.5 mM) IPTG, and the cultures were allowed to continue growth until an OD_{600} of 1.6–1.8 was reached (∼6 h). Cells were harvested at 4000 rpm for 30 min, and resuspended in 10 mL TEM lysis buffer (10 mM Tris–HCl, 2.5 mM EDTA, pH 8.0, 5 mM β-mercaptoethanol) with 1 μg mL^{-1} pepstatin A, and 1 μg mL^{-1} aprotinin added. Cells were lysed at 20,000 psi in an ice-cooled French pressure cell. The cell lysate was clarified by centrifugation at 6000 rpm for 40 min and filtered through a 0.4 μM membrane prior to affinity chromatography.

The following purification procedure was performed at 4 °C. A 10 mL column containing Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 2 column volumes (CV) of 100 mM $NiSO_4$, followed by washing with 2 CV of distilled H_2O and 3 CV of running buffer (lysis buffer minus aprotinin and pepstatin plus 500 mM NaCl) containing 5 mM imidazole. The filtered cell lysate was loaded at 2 mL min^{-1}, and start buffer (∼8 CV) was passed through the column at 3 mL min^{-1} until no more flow-through protein eluted, as determined by UV observation at 280 nm. A wash with running buffer containing 125 mM imidazole was used to remove non-specifically bound proteins (∼4 CV used). Histidine-tagged protein was finally eluted with 3–4 CV of running buffer containing 500 mM imidazole. Fractions containing the enzyme were pooled and dialyzed overnight against a 1:100 volume

of dialysis buffer. Typical yields of purified protein were ~20 mg L^{-1} of cell culture. Both PseB and PglF could be stored frozen in the storage buffer (25 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol) at −80 °C for several months without loss in activity.

2.4. Preparation of UDP-4-keto-6-deoxy-GlcNAc with PglF in H_2O

UDP-GlcNAc (3 mg, 4.6 μmol) was dissolved in 400 μL 10 mM potassium phosphate buffer (pH 7.0), to which was added 50 μL of a 40 mg mL^{-1} solution of PglF (50 μmol), which had previously been exchanged into 10 mM potassium phosphate buffer (pH 7.0) by centrifugal filtration. After 12 h incubation at RT the enzyme was removed by centrifugal filtration and the filtrate was frozen and lyophilized to dryness. ^1H and ^{31}P NMR spectroscopic analysis of the residue revealed that UDP-4-keto-6-deoxy-GlcNAc was the only sugar nucleotide present in the crude product, by comparison with the reported spectrum of UDP-4-keto-6-deoxy-GlcNAc [8]. No further purification was of UDP-4-keto-6-deoxy-GlcNAc was performed. UDP-4-keto-6-deoxy-GlcNAc was stored at −20 °C in solution for several weeks, or as a lyophilized powder for up to six months with no apparent decomposition.

2.5. In situ oxidation of the tightly bound NADPH cofactor of PseB

Prior to enzyme incubations, mutants or wild-type PseB (~30 μM) were incubated with an excess of UDP-4-keto-6-deoxy-GlcNAc (250 μM, prepared as described in Section 2.4) in storage buffer (25 mM Tris–HCl, pH 8.0, containing 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol) for 12 h at RT. The sugar nucleotide solution was then removed by successive centrifugal filtration of affect a 1000-fold dilution.

2.6. Preparation of UDP-4-keto-6-deoxy-L-IdoNAc with PseB in H_2O

UDP-4-keto-6-deoxy-L-IdoNAc was found to partially decompose upon lyophilization from potassium phosphate buffered solutions, but not from pure distilled water. Thus the preparation of UDP-4-keto-6-deoxy-L-IdoNAc was carried out in unbuffered water. PseB (3.8 μmol in 140 μL), which had been exchanged into distilled water by centrifugal filtration, was added to a 325 μL solution of UDP-GlcNAc (15.4 mg, 23.6 μmol) that had been neutralized with NaOH. The RT incubation was monitored by ^1H NMR spectroscopy, using an insert containing D_2O to allow for locking of the spectrometer. After 22 h all the UDP-GlcNAc had been consumed and the enzyme was removed by centrifugal filtration, and the filtrate was lyophilized to dryness. The mixture of UDP-4-keto-6-deoxy-L-IdoNAc (56%) and UDP-4-keto-6-deoxy-GlcNAc (44%) was stored as a dry solid at −80 °C for up to several weeks with no apparent decomposition.

2.7. Monitoring enzyme incubations by NMR spectroscopy

2.7.1. General procedure

Following the in situ oxidation of the tightly bound NADPH as described above, PseB was exchanged into deuterated buffer of the appropriate concentration (pD 7.4 potassium phosphate) by successive centrifugal filtration of affect a 1000-fold dilution of the storage buffer. The enzyme solution was stored at 4 °C or on ice and used within a few hours. The substrate was dissolved with the same deuterated phosphate buffer and concentrations were measured by UV spectroscopy at 262 nm (molar absorptivity = 9890 M^{-1} cm^{-1} in 100 mM potassium phosphate, pH 7.0). Before the addition of enzyme, a spectrum of substrate alone was measured; this served as the zero time point. Incubations were initiated by addition of the enzyme to the NMR tube. Spectra were taken, initially every 5 min, then after progressively longer time intervals. The specifics of each incubation experiment are described in the following sub-sections.

2.7.2. PseB-dependence of C-5″ epimerization of UDP-4-keto-6-deoxy-L-IdoNAc to UDP-4-keto-6-deoxy-GlcNAc

A 1.00 mL solution of 5 mM UDP-GlcNAc, 32 μM PseB, and 10 mM potassium phosphate (pD 7.4, D_2O) was incubated for 1 h, at which time the enzyme was removed by centrifugal filtration. Two 400 μL aliquots of the filtrate were placed into two different NMR tubes. By ^1H NMR spectroscopy, it was determined that a mixture of 14% UDP-GlcNAc, 82% UDP-4-keto-6-deoxy-L-IdoNAc, and 4% UDP-4-keto-6-deoxy-GlcNAc (= UDP-sugar mixture) was present in the filtrate. PseB was added to one NMR tube to give 483 μL of a solution containing 91 μM PseB, 4.1 mM UDP-sugar mixture, and 10 mM potassium phosphate (pD 7.4). An 83 μL aliquot of 10 mM potassium phosphate (pD 7.4) buffer alone was added to the control. Both tubes were incubated at 23 °C.

2.7.3. Monitoring dehydratase activity

Dehydratase activities were monitored with 10 mM UDP-GlcNAc in 12.5 mM potassium phosphate (pD 7.4, D_2O) at 23 °C. The solution volume of wild-type and mutant incubations were all 400 μL, although the concentration of enzyme was 13 μM in the case of wild-type PseB, and 584 μM in the case of D126N, K127A, and Y135F.

2.7.4. Monitoring solvent isotopic exchange activity

The activities of solvent isotope exchange into the C-5″ position of UDP-4-keto-6-deoxy-L-IdoNAc for wild-type and mutants were monitored using the sample of UDP-4-keto-6-deoxy-L-IdoNAc prepared as described in Section 2.6, which contained 56% of UDP-4-keto-6-deoxy-L-IdoNAc and 44% of UDP-4-keto-6-deoxy-GlcNAc (= UDP-sugar mixture). Each NMR tube contained a 450 μL solution of 10.5 mM of the UDP-sugar mixture, 10 mM potassium phosphate (pD 7.4), and 11.5 μM of either wild-type, D126N, K127A, and Y135F. The temperature during this experiment was 23 °C. The C-5″ epimerase activities were also monitored in this experiment.

2.7.5. HF Elimination from UDP-6-deoxy-6-fluoro-GlcNAc

The PseB-catalyzed elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc was monitored in a 400 μL solution containing 5 mM UDP-6-deoxy-6-fluoro-GlcNAc, 25 μM wild-type PseB and 50 mM potassium phosphate (pD 7.4) at 23 °C. A control sample of UDP-6-deoxy-6-fluoro-GlcNAc under identical conditions, only lacking PseB, was also monitored. No spectral changes were observed in either tube after 30 min, therefore the incubation temperature was increased to 37 °C. After 18 h at 37 °C, precipitate was observed in the PseB-containing sample.

2.8. Synthesis of uridine 5′-(2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl) diphosphate, disodium salt (UDP-6-deoxy-6-fluoro-GlcNAc)

Introduction of the fluorine at C-6 was achieved by treatment of the known compound 1,3,4-tri-*O*-acetyl-2-deoxy-D-glucopyranose with DAST, which has been described previously [19]. Purification by column chromatography (1:1 CH_2Cl_2/ethyl acetate to 100% ethyl acetate) gave the known compound 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose [20]. The anomeric position of 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose, **1**, was then selectively deacetylated using dimethylamine as follows. The fully acetylated sugar (456 mg, 1.31 mmol) was dissolved in 7 mL acetonitrile and dimethyl amine was added (6.5 mmol, 5 M solution in ethanol, Fluka). The solution was

allowed to stir at RT for 90 min, and then the solvent was removed by rotary evaporation. The crude mixture was separated by column chromatography (3:1 ethyl acetate/petroleum ether to 100% ethyl acetate). The ^{1}H NMR spectrum of the isolated product (R_f = 0.25, ethyl acetate) is consistent with the isolation of 180 mg of 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose (0.59 mmol, 45% yield). Phosphorylation of 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose (417 mg, 1.36 mmol) was achieved using the phosphoramidite methodology [21]. Purification by column chromatography (9:1 diethyl ether/ethyl acetate to 100% ethyl acetate) yielded 150 mg (0.26 mmol, 20% yield, R_f = 0.35 in ethyl acetate) of the known compound 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl dibenzyl phosphate, **2** [22]. The benzyl protecting groups were then removed by hydrogenolysis as follows. To a solution of compound **2** (50 mg, 88 μmol) in 5 mL MeOH and 25 μL (180 μmol) triethylamine, 25 mg 5% Pd/C was added. After stirring under one atmosphere H_2 for 18 h, the mixture was filtered through celite, and the solvent was removed in vacuo. The crude product was then deacetylated with sodium methoxide by stirring in 3 mL of 100 mM sodium methoxide in methanol for 1 h. The reaction solution was neutralized by addition of Amberlite IR-120H resin (proton form). Removal of the acetyl protecting groups was confirmed by ^{1}H NMR spectroscopy. To prepare for the morpholidate coupling reaction the deprotected sugar was maintained under vacuum for 24 h. One equivalent of trioctylamine (88 μmol, 39 μL) was then added with 2 mL distilled pyridine, and the solvent was removed in vacuo. An additional 2 mL distilled pyridine was added, and then removed in vacuo. This was repeated twice more, and again after the addition of 20 mg of 1*H*-tetrazole (0.28 mmol) and 91 mg UMP morpholidate (0.13 mmol). Finally 0.75 mL distilled pyridine was added and the solution was stirred for 3 days under argon at RT. The pyridine was then removed in vacuo and the residue was dissolved in 10 mL water, and then washed with 2 × 10 mL diethyl ether. The aqueous layer was then separated by anion exchange chromatography (DE-52 resin, linear gradient from 0.1 to 0.5 M of triethylammonium bicarbonate buffer, pH 7.5). Fractions containing product were flash frozen and lyophilized, pooled, and passed through a column of Amberlite IR-120(+) resin (sodium form). Pooled fractions were dried in vacuo, and the known compound uridine 5′-(2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl) diphosphate, disodium salt (UDP-6-deoxy-6-fluoro-GlcNAc) was identified [22] and characterized by NMR spectroscopy and mass spectrometry, which is fully summarized here. UV measurement (molar absorptivity = 9890 M^{-1} cm^{-1} at 262 nm, 100 mM potassium phosphate, pH 7.0) determined that 20 μmol of UDP-6-deoxy-6-fluoro-GlcNAc was isolated (23% yield). ^{1}H NMR (D_2O): δ 7.93 (d, 1H, $J_{5,6}$ = 7.9 Hz, H6), 5.92–5.98 (m, 2H, H1′, H5), 5.51 (dd, 1H, $J_{1'',P\beta}$ = 6.98 Hz, $J_{1'',2''}$ = 3.05 Hz, H1″), 4.59–4.85 (obscured by solvent peak, H6″a and H6″b), 4.31-4.37 (m, 2H, H5′a, H5′b), 4.13–4.28 (m, 3H, H2′, H3′, H4′), 4.03 (dd, 1H, $J_{5'',F}$ = 30.5 Hz, $J_{4'',5''}$ = 9.6 Hz, H-5″), 3.99 (d, 1H, $J_{2'',3''}$ = 10.5 Hz, H2″), 3.81 (dd, 1H, $J_{2'',3''} = J_{3'',4''}$ = 9.6 Hz, H3″), 3.63 (dd, 1H, $J_{3'',4''} = J_{4'',5''}$ = 9.8 Hz, H4″), 2.05 (s, 3H, $COCH_3$); ^{31}P NMR (D_2O): δ −8.96 (d, 1P, $J_{P\alpha,P\beta}$ = 21.7 Hz, Pα),−10.83 (d, 1P, $J_{P\alpha,P\beta}$ = 21.8 Hz, Pβ); ^{19}F NMR (D_2O): δ −236.88 (s); ESIMS: *m/z* 608.1 $[M - 2Na + H]^-$.

2.9. Measuring fluoride release from UDP-6-deoxy-6-fluoro-GlcNAc using a fluoride electrode

Following the in situ oxidation of the tightly bound NADPH as described above, wild-type and mutant dehydratases were exchanged into 100 mM potassium phosphate buffer by centrifugal filtration. Enzyme solutions were diluted appropriately to allow for a 300 μL solution with 44 μM of enzyme and incubated for 15 min at 30 °C with the electrode inserted to allow for equilibration. Reactions were initiated by adding 6.0 μL of a 50 mM solution of UDP-6-deoxy-6-fluoro-GlcNAc (to give a 1.0 mM solution) and the incubation was followed for 100 min.

3. Results and discussion

3.1. Dehydratase and epimerase activities of PseB

The recombinant PseB used in this work is derived from *C. jejuni* (NCTC 11168), and contains a C-terminal hexahistidine tag. Previous studies indicate that PseB from *H. pylori* is isolated with a tightly bound mixture of $NADP^+$ and NADPH [17], and that PseB from *C. jejuni* does not require exogenous cofactor for activity [8]. To determine if the recombinant PseB used in this work is purified with a tightly bound nicotinamide cofactor, its UV spectrum was measured. No significant absorbance was observed at wavelengths >310 nm in the spectrum of PseB alone, however; upon addition of sodium borohydride absorbance bands at 344 and 425 nm were observed, consistent with the generation of tightly bound NADPH. The 344 nm band is assigned to the biologically relevant 1,4 reduction product, and the 425 nm shoulder to 1,2 and 1,6 reduction products, which are known to form upon the reduction of NAD^+ with sodium borohydride [23]. Similar observations have been made upon sodium borohydride reduction of the tightly bound NAD^+ of dTDP-glucose 4,6-dehydratase [24]. The observations reported here are consistent with the isolation of wild-type PseB with tightly bound cofactor mostly (>90%) in the oxidized form. Thus, no exogenous cofactor was added in the experiments described below.

As a first step in characterizing the mechanism of the PseB-catalyzed dehydration, the reaction was run in a buffer prepared from D_2O and monitored by ^{1}H NMR spectroscopy. This experiment probes whether solvent-derived deuterium becomes incorporated into product during catalysis and if so, at what position. Previous studies on the non-inverting dehydratases have shown that solvent isotope is incorporated at the C-5″ position and it was anticipated that this would also be the case with PseB [10,11]. The ^{1}H NMR spectrum of UDP-4-keto-6-deoxy-L-IdoNAc product has been assigned previously [8], and it is evident that spectral overlap of the H-5″ signal with other signals would make it difficult to monitor directly. However, the H6″ methyl signal of the product is well separated, allowing it to be monitored directly and unambiguously. Before the addition of enzyme, the upfield region of the ^{1}H NMR spectrum shows only a singlet corresponding to the *N*-acetyl group of UDP-GlcNAc (Fig. 2A). Following the addition of enzyme, two singlets corresponding to the H6″ methyl protons of hydrated and unhydrated UDP-4-keto-6-deoxy-L-IdoNAc appear at 1.41 and 1.59 ppm, respectively (Fig. 2B and C). The fact that these signals appear as singlets demonstrates that a solvent-derived deuterium has been incorporated at C-5″ during catalysis (Fig. 3). A control sample of undeuterated product taken in the same deuterated buffer showed that the coupling between H-5″ and H-6″ is readily observed ($J_{5'',6''}$ = 7.6 Hz) and that deuterium exchange at C-5″ does not occur non-enzymatically on this time scale. Running the PseB reaction in D_2O did not result in deuterium incorporation at C-6″ since the methyl signal integrated to three protons and mass spectral analysis showed that only a single deuterium was incorporated into product.

These observations are consistent with a mechanism for the PseB reaction that bears similarities to those of the non-inverting dehydratases (Fig. 4) [10,11]. It is proposed that PseB first uses its tightly bound $NADP^+$ cofactor to oxidize UDP-GlcNAc at C-4″, generating a ketone intermediate. The presence of the C-4″ ketone enables dehydration that produces an enone intermediate. In the

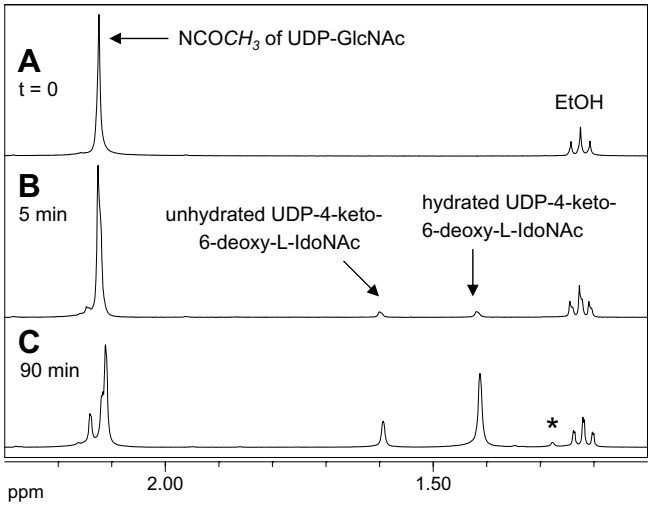

Fig. 2. Partial ^{1}H NMR spectra during dehydration of 12.1 mM UDP-GlcNAc to UDP-4-keto-6-deoxy-L-IdoNAc as catalyzed by 18 μM PseB at (400 MHz, D_2O, RT). (A) UDP-GlcNAc in 10 mM potassium phosphate pD 7.4. (B) Five minutes after the addition of PseB (9.7% conversion). (C) Ninety minutes after the addition of PseB (74% conversion). The small signal marked with an asterisk (*) indicates the appearance of a new product after 90 min.

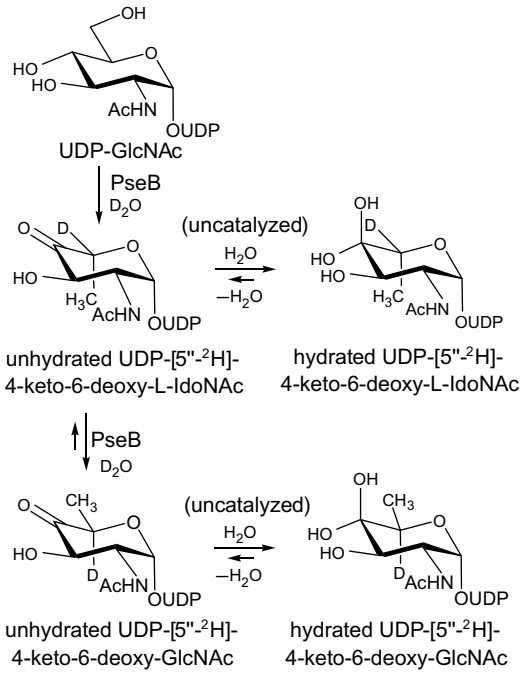

Fig. 3. PseB-catalyzed dehydration of UDP-GlcNAc in D_2O produces UDP-[5″-^{2}H]-4-keto-6-deoxy-L-IdoNAc, which exists in the unhydrated and hydrated forms in aqueous solution. In addition, PseB catalyzes epimerization of UDP-4-keto-6-deoxy-L-IdoNAc to UDP-4-keto-6-deoxy-GlcNAc, which also exists in the unhydrated and hydrated forms in aqueous solution.

final reduction step, a hydride is delivered to the C-6″ position of the enone intermediate, and a protonation occurs at C-5″. This mechanism differs from that of the non-inverting dehydratases in that C-5″ protonation occurs on the opposite face of the double bond, to generate a C-5″ inverted product. The formation of UDP-[5″-^{2}H]-4-keto-6-deoxy-L-IdoNAc during catalysis in D_2O supports this mechanism.

The ^{1}H NMR experiment also shows that the unhydrated form of UDP-4-keto-6-deoxy-L-IdoNAc is the first formed product [25]. This is evident upon examination of the ratio of the signals due

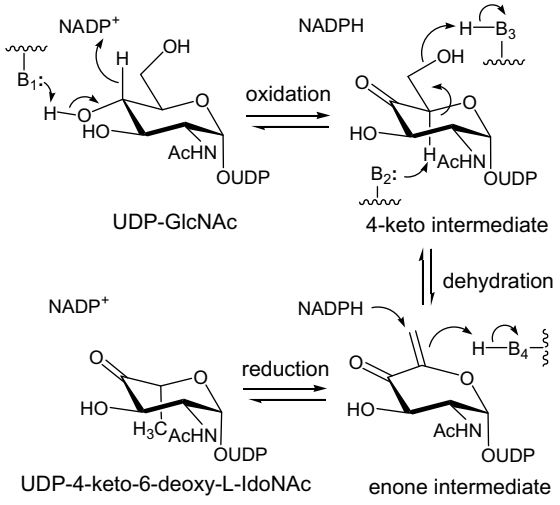

Fig. 4. Proposed mechanism of the reaction catalyzed by PseB.

to the hydrated (1.41 ppm) and unhydrated (1.59 ppm) products in the early time points during the PseB-catalyzed reaction (Fig. 2B and C). After 5 min the ratio is one to one, while at 90 min the ratio approaches four to one, the observed equilibrium ratio for hydrated to unhydrated UDP-4-keto-6-deoxy-L-IdoNAc, respectively. As expected, in incubations containing lower enzyme concentrations the hydrated to unhydrated product ratio was at the equilibrium ratio of four to one throughout the reaction. This is consistent with release of unhydrated UDP-4-keto-6-deoxy-L-IdoNAc from PseB, and hydration occurring slowly and non-enzymatically over the course of minutes (Fig. 3).

At high conversion new signals are observed in the ^{1}H NMR spectrum (one signal is indicated by an asterisk in Fig. 2C). These signals correspond to the unhydrated and hydrated signals of UDP-4-keto-6-deoxy-GlcNAc, the C-5″ epimer of UDP-4-keto-6-deoxy-L-IdoNAc (Fig. 3) [25]. The epimerization of UDP-4-keto-6-deoxy-L-IdoNAc to UDP-4-keto-6-deoxy-GlcNAc has been observed previously [8], however, it was not clear if this epimerization is catalyzed by PseB or if it occurs non-enzymatically (see also [25]). This question was addressed in a second experiment, in which a mixture of UDP-GlcNAc (14%), UDP-4-keto-6-deoxy-L-IdoNAc (82%), and UDP-4-keto-6-deoxy-GlcNAc (4%) was divided in two, and PseB was added to one sample while an equal volume of buffer was added to the control. The mixtures were monitored by ^{1}H NMR spectroscopy (Fig. 5), and epimerization was observed in the PseB-containing sample, such that UDP-4-keto-6-deoxy-GlcNAc became the major product (>90%) after 15 h of incubation (Fig. 5D). This experiment indicates that the C-5″ epimerization of UDP-4-keto-6-deoxy-L-IdoNAc to UDP-4-keto-6-deoxy-GlcNAc is PseB-catalyzed, and that the C-5″ epimerase activity of PseB is ~50-fold lower than the dehydratase activity.

3.2. Solvent isotope exchange into UDP-4-keto-6-deoxy-L-IdoNAc at H-5″

Additional studies probed the ability of PseB to catalyze solvent-derived isotope exchange into the H-5″ position of UDP-4-keto-6-deoxy-L-IdoNAc. It was anticipated that this would allow identification of the catalytic residue that protonates C-5″ during the reduction step of the dehydration (B_4 in Fig. 4). The analogous solvent isotope exchange activity of dTDP-glucose 4,6-dehydratase has been observed and used to identify the analogous catalytic acid/base residue in that enzyme [26]. UDP-4-keto-6-deoxy-L-IdoNAc was incubated with PseB in D_2O, and the wash-in of deute-

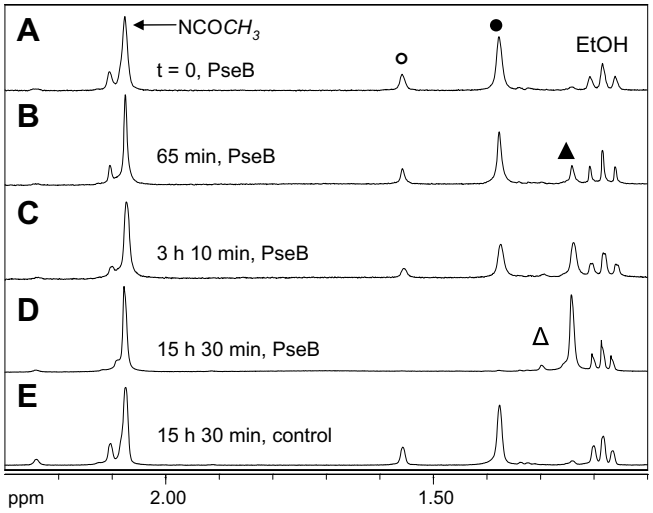

Fig. 5. Partial ^{1}H NMR spectra demonstrating the PseB-catalyzed epimerization of UDP-4-keto-6-deoxy-L-IdoNAc (○, unhydrated; ●, hydrated) to UDP-4-keto-6-deoxy-GlcNAc (Δ, unhydrated; ▲, hydrated) (total UDP-sugar concentration = 4.1 mM, pD 7.4 potassium phosphate, D_2O, RT), in which spectra A–D) document the incubation with 91 μM PseB and spectrum E) is the control with no enzyme.

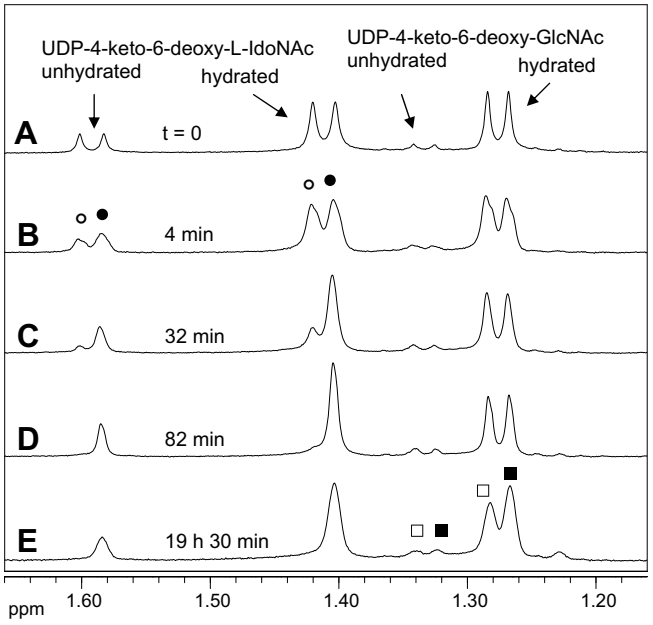

Fig. 6. Partial ^{1}H NMR spectra monitoring solvent isotopic exchange into UDP-4-keto-6-deoxy-L-IdoNAc catalyzed by 11.5 μM PseB (○ and □ are downfield H6″ signals of non-deuterated sugars, ● and ■ are upfield H6″ signals of non-deuterated sugars and overlapping H6″ signals of [5″-^{2}H] sugars)(Total concentration of UDP-sugars = 10.5 mM, 56% UDP-4-keto-6-deoxy-L-IdoNAc and 44% UDP-4-keto-6-deoxy-GlcNAc at t = 0, 10 mM pD 7.4 potassium phosphate, D_2O, 400 MHz).

rium at C-5″ was monitored by observing changes in the coupling pattern of the H6″ ^{1}H NMR signal. UDP-4-keto-6-deoxy-L-IdoNAc was first prepared by running the PseB reaction in H_2O until UDP-GlcNAc was quantitatively consumed. UDP-4-keto-6-deoxy-L-IdoNAc prepared in this manner contained 44% UDP-4-keto-6-deoxy-GlcNAc due to the aforementioned epimerase activity of PseB (Fig. 6A). Upon addition of wild-type PseB to this sample in D_2O the H6″ doublet of UDP-4-keto-6-deoxy-L-IdoNAc is observed to collapse into a singlet, indicating that solvent deuterium washes into the C-5″ position (Fig. 6B–E). The H6″ singlet of the UDP-[5″-^{2}H]-4-keto-6-deoxy-L-IdoNAc is slightly shifted upfield, such that it overlaps with the upfield peak of the H6″ doublet of the non-deuterated UDP-4-keto-6-deoxy-L-IdoNAc. The rate of deuterium wash-in was estimated by monitoring peak height ratios. In this way it was determined that deuterium wash-in at C-5″ to UDP-4-keto-6-deoxy-L-IdoNAc occurs with the same approximate activity as the normal dehydration reaction. These observations are fully consistent with the proposed mechanism of PseB.

3.3. Elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc

To expand the repertoire of tools available to probe the mechanism of PseB, UDP-6-deoxy-6-fluoro-GlcNAc was prepared. It was anticipated that UDP-6-deoxy-6-fluoro-GlcNAc would undergo enzyme-catalyzed elimination of HF, generating the normal UDP-4-keto-6-deoxy-L-IdoNAc product. This activity would enable identification of the catalytic acid residue that protonates the C-6″ hydroxyl during the elimination step (B_3 in Fig. 4). A mutant lacking this residue should still accept the 6-fluoro substrate since acid catalysis would not be required for the loss of fluoride. An analogous approach was used to identify the corresponding residue of dTDP-glucose 4,6-dehydratase [26]. UDP-6-deoxy-6-fluoro-GlcNAc has been previously prepared via an oxazoline intermediate [22]; however, we prepared UDP-6-deoxy-6-fluoro-GlcNAc via a different synthetic route using a phosphoramidite reagent, as described in Fig. 7. The known compound 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose, **1**, [20] was anomerically deprotected with dimethyl amine to afforded 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-6-fluoro-D-glucopyranose, which was then phosphorylated using the phosphoramidite methodology to give compound **2** [21]. Following deprotection by hydrogenation and transesterification, a morpholidate coupling reaction generated the desired UDP-6-deoxy-6-fluoro-GlcNAc product. Incubation of UDP-6-deoxy-6-fluoro-GlcNAc with PseB in D_2O was monitored by ^{1}H and ^{19}F NMR spectroscopy (Fig. 8). After extended incubation with large amounts of enzyme (1 mg mL^{-1}) a new signal at −122.8 ppm slowly appeared in the ^{19}F NMR spectrum. Addition of sodium fluoride enhanced this signal, identifying *fluoride* as a product released from UDP-6-deoxy-6-fluoro-GlcNAc. In the ^{1}H NMR spectrum signals of a similar intensity corresponding to UDP-[5″-^{2}H]-4-keto-6-deoxy-GlcNAc were also observed. No deuterium wash-in at H-5″ of UDP-6-deoxy-6-fluoro-GlcNAc was detected and no spectral changes were observed in a control sample lacking PseB. It is likely that UDP-4-keto-6-deoxy-L-IdoNAc is the first formed product and that after extensive incubation the C-5″ epimerase activity of PseB converts it into UDP-4-keto-6-deoxy-GlcNAc. These observations indicate that PseB has a very low, yet measurable activity with UDP-6-deoxy-6-fluoro-GlcNAc

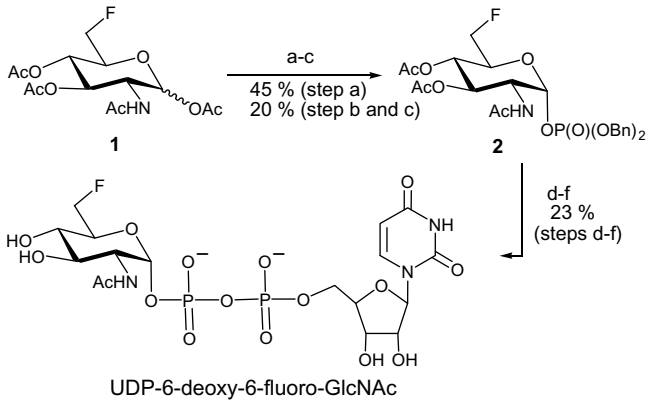

Fig. 7. Synthesis of UDP-6-deoxy-6-fluoro-GlcNAc. (a) Me_2NH. (b) $Et_2NP(OBn)_2$, triazole. (c) H_2O_2. (d) H_2, Pd/C. (e) $NaOCH_3$. (f) UMP morpholidate, tetrazole.

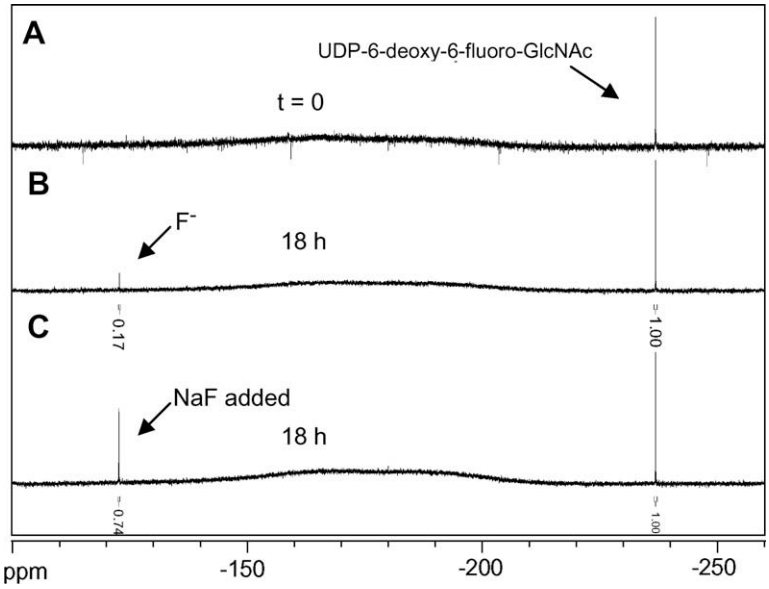

Fig. 8. ^{19}F NMR spectra during the incubation of 5 mM UDP-6-deoxy-6-fluoro-GlcNAc with 25 μM PseB in 50 mM potassium phosphate pD 7.4 (282.4 MHz, D_2O). (A) Spectrum immediately after addition of PseB ($t = 0$). B) After 18 h incubation at 37 °C. (C) After addition of 2.4 μmol NaF to incubated sample.

as an alternate substrate, and that HF elimination occurs as the proposed mechanism would predict.

3.4. Site-directed mutagenesis and preparation of PseB mutants

The putative catalytic acid/base residues Asp132, Lys133, and Tyr141 have been identified in the *H. pylori* version of PseB by X-ray crystallography (PDB code 2GN4) and site-directed mutagenesis (Fig. 9) [17,27]. The *C. jejuni* PseB used in this work shares 63% sequence identity with the *H. pylori* version, and most importantly, these residues are conserved. In *C. jejuni* PseB, the corresponding residues are Asp126, Lys127, and Tyr135, so the following mutants were prepared: D126N, K127A, and Y135F.

The mutant PseB proteins were overexpressed and purified in the same manner as described for the wild-type enzyme. Proper folding of the recombinant proteins was assessed by observation of tightly bound NADPH by UV/vis spectroscopy. In contrast to the wild-type dehydratase, UV spectral analysis of the three PseB mutants revealed a strong absorbance band at 344 nm. Upon addition of sodium borohydride only a small increase in absorbance was observed, indicating that ~95% of the tightly bound cofactor is in the reduced, NADPH form.

To allow a meaningful study of the mutant dehydratases, the catalytically relevant oxidized form of the tightly bound cofactor ($NADP^+$) was required. To this end a reagent that would selectively oxidize the tightly bound cofactor in situ was sought. This selective oxidizing agent proved to be UDP-4-keto-6-deoxy-GlcNAc, which is the product of the non-inverting dehydratase PglF (Fig. 1B) [8]. This compound closely resembles the 4-keto intermediate that is normally formed in the PseB reaction (Fig. 4), but as it lacks the C-6 hydroxyl it cannot proceed to product and is readily reduced by the NADPH form of the enzyme to give UDP-6-deoxy-GlcNAc. Upon addition of UDP-4-keto-6-deoxy-GlcNAc (260 μM) to either D126N or K127A (50 μM) the tightly bound NADPH was immediately oxidized, such that upon returning the cuvette to the spectrometer the 344 nm absorption band had been quantitatively bleached. With Y135F the disappearance of the 344 nm band was dramatically slower. Upon incubation of 68 μM Y135F with 560 μM UDP-4-keto-6-deoxy-GlcNAc oxidation of the tightly bound NADPH was found to take 9 h to quantitatively bleach the NADPH absorption band. The observed difference in rates of oxidation of the tightly bound NADPH in Y135F versus D126N or K127A is consistent with the anticipated role of tyrosine 135. Tyrosine 135 is part of the conserved triad found in SDR enzymes, and it is likely to be the catalytic residue involved in C-4″ oxidation (B_1 in Fig. 4) [17]. The rapid oxidation of NADPH observed with D126N or K127A indicates that these residues are not involved in the first step of catalysis. In all further studies with these mutants, treatment with UDP-4-keto-6-deoxy-GlcNAc was used as a standard step during enzyme preparation to ensure that the catalytically relevant form of the cofactor was present.

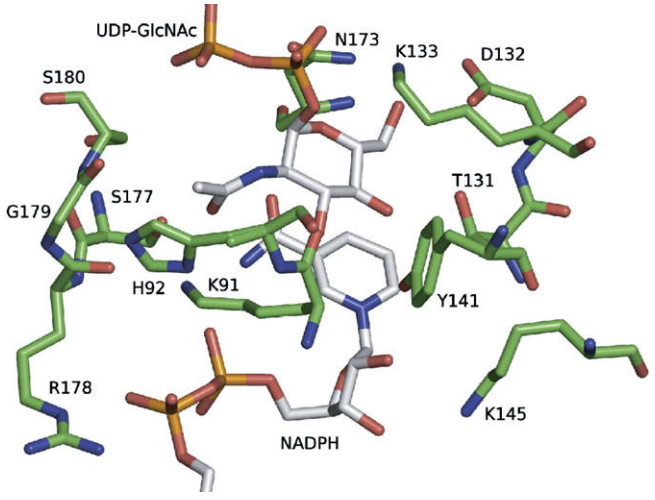

Fig. 9. The active site of the *H. pylori* UDP-GlcNAc 5-inverting 4,6-dehydratase in an abortive complex with NADPH and UDP-GlcNAc (PDB code 2GN4). The active site residues Asp132, Lys133, and Tyr141 correspond to Asp126, Lys127, and Tyr135 in the *C. jejuni* enzyme.

3.5. Evaluation of dehydratase, solvent isotope exchange, and HF elimination activities of wild-type and mutant PseB

The specific activity of the His-tagged wild-type PseB was measured using ^1H NMR spectroscopy in deuterated phosphate buffer (pD 7.4). The initial 10% of conversion to UDP-4-keto-6-

deoxy-L-IdoNAc was monitored by comparing the integrals of the H6″ signals of product to those of the combined *N*-acetyl signals. In this manner the specific catalytic activity was determined to be 0.23 ± 0.05 μmol min^{-1} mg^{-1} at 23 °C. It was not possible to measure accurate activities with the mutants since extended incubations over several days were required to obtain 5% conversion to product even with concentrated enzyme solutions. The dehydratase activities of the mutants were estimated to be 0.001–0.002% of the activity of wild-type PseB (Table 1). It should be noted that UDP-4-keto-6-deoxy-GlcNAc was the observed product in the case of the mutants. This is consistent with the observation that the C-5″ epimerase activity of the mutants was approximately equal to that of wild-type, as discussed below. As a side note, during the incubation of D126N, K127A, and Y135F with UDP-GlcNAc in D_2O, deuterium exchange into H-5″ of substrate was considered to be a possible side-reaction, however, no evidence for this was observed by ^1H NMR spectroscopy over the three days that the experiment was monitored.

The observation that these mutants all tightly bind the nicotinamide cofactor, yet are almost devoid of dehydratase activity is consistent with the proposal that they are key catalytic residues [17]. With the goal of identifying the residue that catalyzes the protonation of the C-5″ position of the α,β unsaturated intermediate during the reduction step (B_4 in Fig. 4), the activities of solvent isotope exchange into H-5″ of UDP-4-keto-6-deoxy-L-IdoNAc was monitored. It was anticipated that the mutant lacking the residue that is the acid/base catalyst for this reaction would show a severely compromised activity, whereas the other mutants would show near wild-type activity. The specific activity for deuterium wash-in to UDP-4-keto-6-deoxy-L-IdoNAc at C-5″ was found to be 0.26 ± 0.08 μmol min^{-1} mg^{-1} at 23 °C with His-tagged wild-type PseB, as determined by ^1H NMR spectroscopy (as in Section 3.2). The activities of solvent isotope exchange into UDP-4-keto-6-deoxy-L-IdoNAc for D126N, K127A and Y135F were all ~100-fold lower than that of wild-type PseB (Table 1). This is somewhat surprising, since one would not expect all three residues to be required for exchange to take place. Another surprising finding was that the C-5″ epimerase activities of the mutants, which is also observed during this experiment, are the same as observed for the wild-type PseB (0.002 ± 0.0015 μmol min^{-1} mg^{-1} at 23 °C). It would seem reasonable that the same residues involved in the inverting dehydration would also promote epimerization, but this apparently is not the case. This value is three orders of magnitude greater than that of the residual dehydratase activity in these mutants suggesting that the residues responsible for the epimerization are not also responsible for the residual dehydration activity.

With the goal of identifying the catalytic residue that protonates the C-6″ hydroxyl during dehydration (Fig. 2), the wild-type and mutant PseB were screened for their abilities to eliminate HF from UDP-6-deoxy-6-fluoro-GlcNAc. As the elimination of fluoride is not anticipated to require protonation, the mutant lacking the appropriate catalytic acid/base residue (B_3 in Fig. 4) was anticipated to turn over UDP-6-deoxy-6-fluoro-GlcNAc with near wild-type activity. The specific activity for the release of HF from UDP-6-deoxy-6-fluoro-GlcNAc was measured for His-tagged wild-type PseB using a fluoride electrode and found to be 37 ± 9 pmol min^{-1} mg^{-1} at 30 °C. The elimination of HF is four orders of magnitude slower than the normal dehydration reaction. When the concentration of UDP-6-deoxy-6-fluoro-GlcNAc was increased to 2 mM the observed activity was 44 pmol min^{-1} mg^{-1}, indicating that the concentration of UDP-6-deoxy-6-fluoro-GlcNAc was well above its K_m value. With all three mutants the fluoride electrode was unable to measure increases in fluoride concentration greater than the background noise and only an upper limit could be estimated. HF elimination activities of D126N, K127A, and Y135F are <1 ± 0.9 pmol min^{-1} mg^{-1} at 30 °C, which is <3% of the wild-type activity. As none of the mutants displayed HF elimination activity of the same magnitude as wild-type activity, it is not possible to identify the catalytic residue responsible for protonation of the C-6″ hydroxyl in this fashion.

Table 1
Summary of the relative activities of wild-type and mutant PseB for dehydration of UDP-GlcNAc (at 23 °C), H-5″ isotopic exchange into UDP-4-keto-6-deoxy-L-IdoNAc (at 23 °C), and elimination of HF from UDP-6-deoxy-6-fluoro-GlcNAc (at 30 °C)

PseB	Relative activity (%)		
	Dehydration	H-5" isotope exchange	HF elimination
Wild-type	100	100	100
D126N	0.002	1	<3
K127A	0.0015	2	<3
Y135F	0.001	1.5	<3

4. Conclusion

The sugar nucleotide 4,6-dehydratases are members of the SDR family of enzymes and are required in the biosynthesis of 6-deoxyhexoses [10,11,12]. These dehydratases have been the subject of extensive investigation and are known to utilize a tightly bound $NAD(P)^+$ cofactor in a mechanism involving transient oxidation of the substrate. The PseB enzyme is a UDP-GlcNAc 5-inverting 4,6-dehydratase and is unique in that it generates a product with inverted stereochemistry at C-5″ (UDP-4-keto-6-deoxy-L-IdoNAc) (Fig. 1A). Since PseB is also an SDR family member it is reasonable to expect that it will employ a very similar mechanism during catalysis. This mechanism involves an initial oxidation at C-4″ by the tightly bound $NADP^+$ cofactor to give a 4-keto intermediate (Fig. 4). Deprotonation of the relatively acidic proton at C-5″ then allows an elimination of water to occur that generates an enone intermediate. Finally, the conjugate addition of a hydride to the C-6″ position of the enone and protonation at C-5″ gives the final product. The only difference between the retaining and inverting dehydratases would be that in the latter case the C-5″ proton would have to be removed and replaced on opposite faces of the sugar ring.

The results described in this study are entirely consistent with such a mechanism. The observation that deuterium is incorporated into the C-5″ position during catalysis in D_2O indicates that a deprotonation has occurred at this position and that a subsequent reprotonation of opposite stereochemical outcome ultimately introduces a solvent-derived isotope. Similarly, incubation of the product with the dehydratase leads to solvent isotope incorporation at C-5″, consistent with the notion that protonation at this position is the final step in the mechanism. The concept that an enone intermediate is formed by the elimination of water across C-5″ and C-6″ is supported by the observation that the enzyme can utilize UDP-6-deoxy-6-fluoro-GlcNAc as an alternate substrate (albeit very slowly). In previous studies on *H. pylori* PseB (or FlaA1) a somewhat different mechanism was proposed in which the final reduction of the enone involved an attack of hydride at C-5″ with protonation at C-6″ [17]. This is inconsistent with these findings on *C. jejuni* PseB, those on *H. pylori* PseB [28], and studies on the non-inverting dehydratases, as such a mechanism would result in solvent-derived deuterium incorporation at C-6″, and not at C-5″. Given that *C. jejuni* and *H. pylori* PseB catalyze the same reaction and share 63% amino acid sequence identity, we expect they share a common reaction mechanism involving hydride delivery at C-6″. Hydride delivery at C-6″ is also expected from basic chemical principles as nucleophilic addition to the terminal alkene carbon of an enone would be expected to occur with a much lower energy barrier than to the internal alkene carbon.

The observation that PseB also catalyzes a slow C-5″ epimerization of the initial product UDP-4-keto-6-deoxy-L-IdoNAc into UDP-4-keto-6-deoxy-D-GlcNAc was confirmed in this study. The fact that it occurs at a fraction of the rate of the dehydratase reaction suggests it is not physiologically relevant. It was initially expected that this epimerization would be catalyzed by the same residues responsible for the normal dehydratase activity; however, mutation of the key active site acid base residues did not affect the rate of epimerization. This suggests that either the epimerization does not take place in the active site of the enzyme, or that completely different active site residues are involved in catalysis.

An initial goal of this work was to identify the key catalytic residues in PseB and compare and contrast them to those of the non-inverting dehydratases. In the latter enzymes one might expect three residues to play these roles: one to deprotonate the C-4″ hydroxyl during oxidation, a second to remove and deliver a proton to C-5″, and a third to protonate the C-6″ hydroxyl as it leaves. In all cases the first residue is provided by the tyrosine of the conserved Ser/Thr-Tyr-Lys triad that is a hallmark of the SDR enzymes. With dTDP-glucose 4,6-dehydratase, the second and third residues are provided by a glutamate and an aspartate, respectively[26,29]. In the case of CDP-glucose 4,6-dehydratase, a lysine transfers protons to and from C-5″ and an aspartate likely aids in helping the C-6″ hydroxyl to leave [30,31]. Finally, in the case of GDP-mannose 4,6-dehydratase, it has been suggested that a single glutamate plays both the second and third roles in catalysis [32,33].

With an inverting dehydratase such as PseB, one might expect that as many as four active site acid/base residues could be involved in catalysis (see B_1–B_4 in Fig. 4). When compared to the non-inverting dehydratases, an additional base may be required since the removal and replacement of the proton on opposite faces of C-5″ would likely not be facilitated by the same residue. An examination of the active site of the *H. pylori* PseB (Fig. 9), however, reveals only three reasonable candidates: Asp126, Lys127 and Tyr135 (*C. jejuni* numbering) [17]. Tyrosine 135 is clearly a member of the conserved catalytic triad and is expected to facilitate oxidation at C-4″ (B_1 in Fig. 4). Our observations that mutation of this residue dramatically affected the rate of the overall reaction, as well as the rate of reduction of UDP-4-keto-6-deoxy-GlcNAc (a mimic of the normal 4-keto intermediate), strongly support this notion. Aspartate 126 and Lysine 127 were also found to be key catalytic residues as mutations to these positions dramatically crippled the activity of the enzyme. Both D126N and K127A appear to be properly folded and were capable of catalyzing the first step of the PseB reaction, as they were isolated with tightly bound NADPH and readily reduced UDP-4-keto-6-deoxy-GlcNAc. Unfortunately, it was not possible to disentangle the individual roles of these residues through the use of alternate substrates and solvent isotope incorporation studies as had proven successful with dTDP-glucose dehydratase [26]. It is likely that mutation of either residue has a dramatic effect on the ability of the other residue to function properly. This is reasonable since both are seen to make hydrogen bonds to the C-6 hydroxyl of the bound substrate and disruption of this hydrogen-bonding network may greatly affect all of the latter steps in catalysis. Disruption of this hydrogen bonding arrangement may also explain why UDP-6-deoxy-6-fluoro-GlcNAc was such a poor alternate substrate for PseB. As suggested previously [17], Lys127 is positioned appropriately to serve as the base that deprotonates the C-5" position during the elimination of water (B_2 in Fig. 4), however, it is difficult to make further predictions on the roles of individual residues at this point.

Acknowledgments

The authors are grateful to Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding.

References

[1] P. Thibault, S.M. Logan, J.F. Kelly, J.R. Brisson, C.P. Ewing, T.J. Trust, P.J. Guerry, J. Biol. Chem. 276 (2001) 34864–34870.
[2] M. Schirm, E.C. Soo, A.J. Aubry, J. Austin, P. Thibault, S.M. Logan, Mol. Microbiol. 48 (2003) 1579–1592.
[3] D. Linton, A.V. Karlyshev, P.G. Hitchen, H.R. Morris, A. Dell, N.A. Gregson, B.W. Wren, Mol. Microbiol. 35 (2000) 1120–1134.
[4] T. Morooka, A. Umeda, K. Amako, Gen. Microbiol. 131 (1985) 1973–1980.
[5] M.B. Caldwell, P. Guerry, E.C. Lee, J.P. Burans, R.I. Walker, Infect. Immun. 50 (1985) 941–943.
[6] C. Josenhans, A. Labigne, S. Suerbaum, J. Bacteriol. 177 (1995) 3010–3020.
[7] I.C. Schoenhofen, D.J. McNally, J.-R. Brisson, S.M. Logan, Glycobiology 16 (2006) 8C–14C.
[8] I.C. Schoenhofen, D.J. McNally, E. Vinogradov, D. Whitfield, N.M. Young, S. Dick, W.W. Wakarchuk, J.-R. Brisson, S.M. Logan, J. Biol. Chem. 281 (2006) 723–732.
[9] C. Creuzenet, M.J. Schur, J. Li, W.W. Wakarchuk, J.S. Lam, J. Biol. Chem. 275 (2000) 34873–34880.
[10] P.A. Frey, in: R.P.D. Dolphin, O. Avramovic (Ed.), Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects, John Wiley and Sons, New York, 1987, pp. 461–511.
[11] X.M. He, H.-w. Liu, Annu. Rev. Biochem. 71 (2002) 701–754.
[12] R.A. Field, J.H. Naismith, Biochemistry 42 (2003) 7637–7647.
[13] N.B. Olivier, M.M. Chen, J.R. Behr, B. Imperiali, Biochemistry 45 (2006) 13659–13669.
[14] P.A. Glaze, D.C. Watson, N.M. Young, M.E. Tanner, Biochemistry 47 (2008) 3272–3282.
[15] S.T.M. Allard, M.-F. Girauda, J.H. Naismith, Cell. Mol. Life Sci. 58 (2001) 1650–1665.
[16] J. Samuel, M.E. Tanner, Nat. Prod. Rep. 19 (2002) 261–277.
[17] N. Ishiyama, C. Creuzenet, W.L. Miller, M. Demendi, E.M. Anderson, G. Harauz, J.S. Lam, A.M. Berghuis, J. Biol. Chem. 281 (2006) 24489–24495.
[18] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[19] M. Sharma, W. Korytnyk, Tetrahedron Lett. 6 (1977) 573–576.
[20] L. Hough, A.A.E. Penglis, A.C. Richardson, Can. J. Chem. 59 (1981) 396–405.
[21] M.M. Sim, H. Kondo, C.-H. Wong, J. Am. Chem. Soc. 115 (1993) 2260–2267.
[22] R.L. Thomas, S.A. Abbas, K.L. Matta, Carbohydr. Res. 184 (1988) 77–85.
[23] L.KingS. Chaykin, J.G. Watson, Biochim. Biophys. Acta 124 (1966) 13–25.
[24] A.D. Hegeman, J.W. Gross, M.M. Vestling, P.A. Frey, Biochemistry 39 (2000) 13633–13640.
[25] D.J. McNally, I.C. Schoenhofen, R.S. Houliston, E.F. Mulrooney, D.M. Whitfield, E. Vinogradov, J.S. Lam, S.M. Logan, J.-R. Brisson, ChemBioChem 7 (2006) 1865–1868.
[26] J.W. Gross, A.D. Hegeman, B. Gerratana, P.A. Frey, Biochemistry 40 (2001) 12497–12504.
[27] C. Creuzenet, R.V. Urbanic, J.S. Lam, J. Biol. Chem. 277 (2002) 26769–26778.
[28] D.J. McNally, I.C. Schoenhofen, R.S. Houliston, N.H. Khieu, D.M. Whitfield, S.M. Logan, H.C. Jarrell, J.-R. Brisson, ChemMedChem 3 (2008) 55–59.
[29] S.T.M. Allard, K. Beis, M.-F. Giraud, A.D. Hegeman, J.W. Gross, R.C. Wilmouth, C. Whitfield, M. Graninger, P. Messner, A.G. Allen, D.J. Maskell, J.H. Naismith, Structure 10 (2002) 81–92.
[30] N.M. Koropatkin, H.M. Holden, Acta Cryst. D61 (2005) 365–373.
[31] E.M. Vogan, C. Bellamacina, X. He, H.-w. Liu, D. Ringe, G.A. Petsko, Biochemistry 43 (2004) 3057–3067.
[32] A.M. Mulichak, C.P. Bonin, W.-D. Reiter, R.M. Garavito, Biochemistry 41 (2002) 15578–15589.
[33] J.R. Somoza, S. Menon, H. Schmidt, D. Joseph-McCarthy, A. Dessen, M.L. Stahl, W.S. Somers, F.X. Sullivan, Structure 8 (2000) 123–135.