Characterization of *N*-acetylneuraminic acid synthase isoenzyme 1 from *Campylobacter jejuni*

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Escherichia coli NeuNAc (*N*-acetylneuraminic acid) synthase catalyses the condensation of PEP (phosphoenolpyruvate) and ManNAc (*N*-acetylmannosamine) to form NeuNAc and is encoded by the *neuB* gene. *Campylobacter jejuni* has three *neuB* genes, one of which is very similar to the *E. coli neuB* gene. We have characterized the *C. jejuni* neuraminic acid synthase with respect to acylamino sugar specificity and stereochemistry of the PEP condensation. We determined the specificity of *C. jejuni* NeuNAc synthase for *N*-acetylmannosamine, *N*-butanoylmannosamine, *N*-propionoylmannosamine and *N*-pentanoylmannosamine. We find that, although this enzyme exhibits similar K_m values for *N*-acylmannosamine molecules with different *N*-acyl groups, the k_{cat}/K_m values decreased with increasing chain length. NeuNAc synthase is a member of a PEP-utilizing family of enzymes that form oxo acids from PEP and a monosaccharide. This family includes KDO 8-P (2-keto-3-deoxy-D-manno-octulosonate 8-phosphate) synthase and DAH 7-P (2-keto-3-deoxy-

D-arabino-heptulosonate 7-phosphate) synthase. Both enzymes catalyse the condensation of the *re* face of the aldehyde group of the monosaccharide with the *si* face of the PEP molecule. The *C. jejuni* NeuNAc synthase catalysed the condensation of Z- and E-[3-²H]PEP with ManNAc, yielding (3*S*)-3-deutero-NeuNAc and (3*R*)-3-deutero-NeuNAc respectively. The condensation of Z-[3-²F]PEP and ManNAc yielded (3*S*)-3-fluoro-NeuNAc. Results of our studies suggest that the *C. jejuni* NeuNAc synthase, similar to KDO 8-P synthase and DAH 7-P synthase, catalyses the condensation of the *si* face of PEP with the aldehyde sugar. The present study is the first stereochemical analysis of the reaction catalysed by a bacterial NeuNAc synthase.

Key words: *Campylobacter jejuni*, ManNAc analogue, *N*-acetyl-3-fluoroneuraminic acid, *N*-acetylneuraminic acid synthase, stereochemical analysis.

INTRODUCTION

Sialylated glycoconjugates play a significant role in a number of biological processes [1,2]. In microorganisms, NeuNAc is formed by the condensation of PEP (phosphoenolpyruvate) and ManNAc (*N*-acetylmannosamine; Scheme 1), a reaction catalysed by NeuNAc (*N*-acetylneuraminic acid) synthase [3,4]. The enzyme catalysing this reaction in *Escherichia coli* is encoded by the *neuB* gene as part of a polysialic acid gene cluster. Formation of NeuNAc in vertebrates is accomplished in two steps. First, NeuNAc 9-P (9-phosphate) synthase catalyses the condensation of PEP and ManNAc 6-P to form NeuNAc 9-P. NeuNAc 9-P phosphatase catalyses the removal of the phosphate group to form NeuNAc [5]. In vertebrates as well as in microbes, NeuNAc is activated with CTP by CMP-NeuNAc synthase to form CMP-NeuNAc, which serves as a substrate for sialyl transferases [6,7].

Campylobacter jejuni is a Gram-negative bacterium, the infection of which causes gastroenteritis and leads to neurological disorders such as the Guillain–Barre and Miller–Fisher syndromes [8]. The *Campylobacter* lipooligosaccharides on the surface of the bacteria are believed to induce antibodies that cross-react with the host gangliosides in the peripheral nervous system, thus leading to the above-mentioned neurological conditions. Recently, three open reading frames homologous with *E. coli* NeuB have been identified from the genome sequencing project of *C. jejuni* NCTC 11168. One of the three open reading frames with greatest

similarity with *E. coli* NeuB has been found to be involved in lipooligosaccharide biosynthesis. The other two have been suggested to be required for the modification of flagellum with pseudomonic acid [9,10]. In the present study, we report the characterization of the recombinant *C. jejuni* NeuNAc synthase isoenzyme 1 with respect to substrate specificity and the stereochemical course of the condensation reaction.

EXPERIMENTAL

Plasmids and bacterial strains

Expression plasmid pTrc99A/cjneub1 (containing *neuB1* gene from *C. jejuni*, cloned at the *Nco*I site in the polylinker region of the expression plasmid pTrc99A) was obtained from Dr Dennis Linton (Department of Neurology, United Medical and Dental School, Guy's Hospital, London, U.K.). An expression plasmid, pWVCjSAS, was constructed as follows. The *C. jejuni neuB1* gene was amplified by PCR with the forward primer GACGACGACAAGATGCAAATAAAAATAGATAAATTAA (CjLicFor) and the reverse primer GAGGAGAAGCCCGGTTTCATTCAAAATCATCCCATGTTAGT (CjsasLicRev). The high-fidelity polymerase KOD XL (Novagen, Madison, WI, U.S.A.) was used with genomic DNA isolated from *C. jejuni* strain PG836 (kindly provided by Dr Patricia Guerry, Naval Medical Research, Silver Spring, MD, U.S.A.) as a template. The PCR fragment was ligated

Abbreviations used: DAH 7-P, 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate; DPA, dipicolinic acid; KDO 8-P, 2-keto-3-deoxy-D-manno-octulosonate 8-phosphate; LB, Luria–Bertani; PEP, phosphoenolpyruvate.

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Scheme 1 Reaction catalysed by *C. jejuni* NeuNAc synthase

into the plasmid vector pET30 E/K Lic from Novagen according to the manufacturer's instructions. Chemically competent *E. coli* TOP10 and BL21 Star (DE3) cells were purchased from Invitrogen. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

^1H -NMR spectra were recorded on a Bruker Avance DRX 300 (operating at 300.13 MHz for ^1H) using $^2\text{H}_2\text{O}$ for lock. ^{19}F -NMR spectra were recorded on a Bruker Avance DRX 300.13 (operating at 282.37 MHz for ^{19}F) using $^2\text{H}_2\text{O}$ for lock.

Expression and purification of recombinant *C. jejuni* NeuNAc synthase

Chemically competent *E. coli* TOP10 cells were transformed with plasmid pTrc99A/cjneuB1. A 1.5 litre culture of TOP10 cells harbouring plasmid pTrc99A/cjneuB1 were grown in Luria broth at 37 °C in a shaker at 150 rev./min. Cell cultures were induced at mid-exponential phase (absorbance A_{600} 0.6) with 1 mM isopropyl β -D-thiogalactoside and shaken for 3 h. The cell pellet obtained was harvested by centrifugation at 5400 g for 15 min, resuspended in 10 ml of buffer A (50 mM Bicine/10 mM MgCl_2 , pH 8.0) and lysed at 4 °C by passing through a cold French press at 20000 p.s.i. (1 p.s.i. = 6.9 kPa). A clarified lysate was prepared by centrifuging at 10000 g for 15 min to remove the cell debris (pellet) and ultracentrifuged at 145000 g for 1 h to remove the membrane fractions. The clarified lysate was applied to a column of Q-Sepharose FastFlow (20 ml) and washed with 50 ml of buffer A at a flow rate of 1 ml/min. The enzyme was then eluted with a 0–1 M KCl gradient in buffer A. Fractions (4 ml) that had NeuNAc synthase activity (as described below) were pooled and dialysed against buffer A. The dialysate was loaded on to a Blue Sepharose CL6B column (15 ml) and washed with 50 ml of buffer A. The enzyme was eluted, using a 0–0.5 M KCl gradient in buffer A. Active fractions were pooled and dialysed against buffer A, aliquoted (1 ml fractions) and stored at –80 °C until further use. This procedure yielded a partially purified enzyme.

Homogeneous protein was obtained by expressing sialic acid synthase in the pET30 E/K Lic vector as a His₆ chimaera and purification as follows. BL21 Star (DE3) was transformed with pWVCjSAS and plated on to LB (Luria–Bertani) broth-kanomycin (50 $\mu\text{g}/\text{ml}$). Half of the cells from the overnight plate were then resuspended in 1.0 ml of LB broth-kanomycin and used to inoculate a 200 ml culture in LB broth-kanomycin (50 $\mu\text{g}/\text{ml}$). The culture was grown and induced for 1 h 30 min as described above. The cell paste was lysed in the French pressure cell as described above and centrifuged at 10000 g for 15 min before

applying the soluble fraction to a 2 ml Ni-agarose resin equilibrated in 50 mM Tris (pH 7.5). The resin was washed with 50 mM Tris and 10 mM imidazole (pH 7.5) until the protein was no longer detected in the effluent. The enzyme was then eluted with 50 mM Tris, 0.25 M imidazole and 0.2 M NaCl (pH 7.5). The pooled fractions containing enzyme activity were stored at –80 °C.

NeuNAc synthase assay and kinetic studies

A typical NeuNAc synthase reaction mixture contains 12.5 mM PEP, 12.5 mM ManNAc and 10 mM MnCl_2 in 100 μl of 0.1 M Bicine buffer (pH 8.0) and NeuNAc synthase in a total volume of 200 μl . The reaction was started by the addition of enzyme to the reaction mixture and incubated for 30 min at 37 °C. The reaction was quenched by adding 50 μl of 6.67 M phosphoric acid. Enzyme-catalysed formation of NeuNAc was measured by the thiobarbituric acid assay [4,7].

For kinetic studies, reactions were quenched after 5 min incubation at 37 °C and the amount of NeuNAc formed was determined by the thiobarbiturate assay. Steady-state kinetic constants were determined by fitting the reaction rates versus substrate concentrations to the Michaelis–Menton equation using the software package GraphPad Prism.

Metal requirements of NeuNAc synthase

The NeuNAc synthase used for metal studies was purified from BL21 Star (DE3):pWVCjSAS by the same method as described above, except that the cells were suspended in 50 mM Tris buffer (pH 7.5) instead of 50 mM Tris buffer (pH 8.0) containing 25 mM MgCl_2 . The pooled fractions from an IMAC (immobilized metal-affinity chromatography) column were concentrated by ultrafiltration (Centriprep YM-10 concentrator) to 5 mg/ml and used immediately in metal requirement studies.

A reaction mixture containing 12.5 mM PEP and 12.5 mM ManNAc in 20 mM Tris/HCl (pH 7.5) with either metal chelators [10 mM EDTA and 1 mM DPA (dipicolinic acid)] or various bivalent metal ions (100 μM) was preincubated at 37 °C for 3 min. The reaction was initiated by the addition of the enzyme (1 μM) and monitored for 5 min. The amount of sialic acid formed was then measured by the thiobarbiturate assay [4,7].

Synthesis of ManNAc and PEP analogues

ManNAc analogues with different N-acyl groups were prepared from mannosamine and the respective acid anhydrides and purified as reported previously [11,12].

Figure 1 SDS/PAGE of purified *C. jejuni* NeuNAc synthase (Cjsas)

MM STD, molecular-mass standards (kDa).

PEP analogues *Z*- and *E*-[3-²H]PEP as well as *Z*-[3-F]PEP were prepared by the procedure described in [13]. Irradiation of a 0.4 M solution of *Z*-[3-F]PEP in ²H₂O at 254 nm for 24 h yielded a 3:2 mixture of *E*- and *Z*-[3-F]PEP as determined by the ¹⁹F-NMR spectrum [14,15].

NeuNAc synthase-catalysed synthesis of NeuNAc

To a reaction mixture containing 37.5 mM PEP, 45 mM ManNAc and 10 mM MnCl₂ in 1 ml of 100 mM Bicine buffer (pH 8.0), 500 µl of *C. jejuni* NeuNAc synthase (0.5 mg) was added and the reaction mixture was incubated at 37 °C overnight. The reaction mixture was then treated with 1.67 M phosphoric acid and the protein was removed by centrifugation. The supernatant containing the product was loaded on to an anion-exchange column (AG 1 × 8, 10 ml) and washed with 30 ml of water at a flow rate of 1 ml/min. The product was eluted with 60 ml of 0–1 M sodium bicarbonate gradient for 1 h. Fractions containing the product were identified by the thiobarbituric acid assay, pooled together and freeze-dried. The ¹H-NMR spectrum obtained for the product was identical with that recorded for standard NeuNAc.

Enzymic synthesis of [3-²H]NeuNAc analogues

The reaction mixture contained 23 mM ManNAc, 10 mM MnCl₂ and 18 mM *E*- or *Z*-[3-²H]PEP in 1 ml of 50 mM Bicine buffer (pH 8.0). After the addition of *C. jejuni* NeuNAc synthase, the reaction was treated in the same way as described above for the NeuNAc synthesis. The [3-²H]NeuNAc analogues were purified by the procedure described above and characterized by ¹H-NMR spectroscopy.

Enzymic synthesis of [3-F]NeuNAc

For the synthesis of [3-F]NeuNAc, a reaction mixture containing 18 mM *Z*-[3-F]PEP, 25 mM ManNAc and 3 mM MnCl₂ in 400 µl of 150 mM Bicine buffer (pH 8.0) was taken in a 5 mm NMR tube and the reaction was initiated by the addition of 0.1 mg of NeuNAc synthase in 100 µl. The reaction mixture contained 10% ²H₂O for lock. The reaction was followed by monitoring the disappearance of ¹⁹F-NMR signals for PEP and the appearance of ¹⁹F-NMR signals for the products during 24 h. The reaction products were purified as mentioned above for NeuNAc. ¹H- and ¹⁹F-NMR spectra were recorded for the products.

A similar experiment was performed using a reaction mixture containing *E*- and *Z*-[3-F]PEP (3:2 mixture, 18 mM), 25 mM ManNAc and 3 mM MnCl₂ in 400 µl of 150 mM Bicine buffer (pH 8.0). The reaction mixture contained 10% ²H₂O for lock. After the addition of NeuNAc synthase, the reaction was followed by monitoring the disappearance of ¹⁹F-NMR signals for PEP and the appearance of ¹⁹F-NMR signals for the products during 24 h. After 24 h, an additional 10 mM ManNAc was added to the reaction mixture to exhaust all the available [3-F]PEP completely. Even after prolonged incubation, only the *Z*-isomer appeared to have completely reacted, whereas the *E*-isomer remained unchanged. The reaction products were purified as mentioned above for NeuNAc. ¹H and ¹⁹F-NMR spectra were recorded for the products.

RESULTS

Purification of NeuNAc synthase

C. jejuni NeuNAc synthase was partially purified by ion-exchange and dye-binding chromatography [4] from a lysate of *E. coli* TOP10 cells harbouring plasmid pTrc99A/cjneub1. The enzyme was purified to homogeneity as a His₆-S-tag chimera by Ni-agarose (IMAC) chromatography (Figure 1). The molecular mass of the isolated chimera was confirmed to be 43.3 kDa by matrix-assisted laser desorption ionization–time-of-flight MS, which agrees with the expected value calculated from the amino acid sequence. As shown in Table 1, the pure enzyme was not very stable. Most of the experiments below, unless otherwise indicated, were performed with the more stable, partially purified enzyme.

Metal requirements

To determine whether *C. jejuni* NeuNAc synthase requires a metal cofactor for activity, the enzyme was first assayed in the presence

Table 1 Purification of CjSAS from BI21 Star (DE3):pWVCjSAS lysate

	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol · min ⁻¹ · mg ⁻¹)	Purification (fold)	Recovery (%)
Soluble lysate	212.67	13873	65.23	1.00	100.00
Ni-agarose pool (same day)	3.06	9977	3258.74	49.96	71.91
Ni-agarose pool (next day)	3.06	3116	1017.83	15.60	22.46

Table 2 Effects of metal chelators and bivalent metal ions on *C. jejuni* NeuNAc synthase activity

The activity of NeuNAc synthase was determined at 12.5 mM PEP and 12.5 mM ManNAc in 20 mM Tris/HCl (pH 7.5) in the presence of either metal chelators or bivalent metal ions.

Addition(s)	Relative activity (%)
None*	100
10 mM EDTA + 1 mM DPA	NA†
100 μ M Mn ²⁺	150
100 μ M Co ²⁺	122
100 μ M Mg ²⁺	101
100 μ M Zn ²⁺	68

* The enzyme as isolated.

† NA, no activity.

Table 3 Kinetic constants obtained for substrates and analogues of recombinant *C. jejuni* NeuNAc synthase

Substrate	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ · min ⁻¹)
PEP	7.3	19	2.6
ManNAc	17.6	19.9	1.08
<i>N</i> -Propanoylmannosamine	5.5	11.8	2.14
<i>N</i> -Butanoylmannosamine	15.0	3.5	0.23
<i>N</i> -Pentanoylmannosamine	31.1	0.8	0.026

of metal chelators (10 mM EDTA and 1 mM DPA). No significant activity could be detected (Table 2). The effects of bivalent metals on the enzyme as isolated were assessed by adding the bivalent metals to the assay mixture to a final concentration of 100 μ M. Mn²⁺ and Co²⁺ stimulated the enzyme activity; Mg²⁺ had no effect on the activity, whereas Zn²⁺ showed an inhibitory effect on the activity. These results suggest that *C. jejuni* NeuNAc synthase requires a metal cofactor for activity.

NeuNAc synthase activity and kinetics

Of the three putative NeuNAc synthase genes identified in the *C. jejuni* genome, *neuB1* has the greatest homology with the *E. coli* NeuNAc synthase [9]. The *C. jejuni* NeuNAc synthase has a preference for ManNAc and did not form any product when mannose or ManNAc 6-P was used as the monosaccharide substrate. ManNAc 6-P was tested over the range 0.25–2 mM. As was observed with the *E. coli* enzyme, 6-azido-NeuNAc is a substrate and thereby served as a convenient means of preparing 9-azido-NeuNAc. Kinetic constants for recombinant *C. jejuni* NeuNAc synthase were obtained using the substrates PEP and ManNAc as mentioned in the Experimental section. Recombinant *C. jejuni* NeuNAc synthase exhibited K_m values of 7.3 and 17.6 mM for PEP and ManNAc respectively (Table 3). The catalytic-centre activity (k_{cat}) for this enzyme is 19.9/min for its normal substrates ManNAc and PEP.

Substrate analogues

The mammalian sialic acid 9-P synthase has a broad specificity for *N*-acylmannosamines with modified acyl chains [12]. This property has been exploited to modify the K_m value for mammalian cell-surface sialic acid [16]. Similar *in vivo* experiments were not successful with bacteria [17]. To examine the effect of the *N*-acyl group on the reaction catalysed by the bac-

Figure 2 ManNAc analogues

terial NeuNAc synthase, we prepared ManNAc analogues (Figure 2) with different side chains by the procedure described in [12]. Kinetic studies utilizing the ManNAc analogues indicate that the rates of the reaction decreased with increase in the chain length of the *N*-acyl moiety, even though there is no correlation with the affinity of the enzyme for these molecules (Table 3). The recombinant NeuNAc synthase exhibited K_m values of 5.5, 15 and 31.1 mM for *N*-propanoylmannosamine, *N*-butanoylmannosamine and *N*-pentanoylmannosamine respectively, which is within a 3-fold difference from the natural substrate (17.6 mM). On the other hand, there was a more significant effect on k_{cat} and k_{cat}/K_m values. The catalytic-centre activity for these substrates, on the other hand, decreased significantly with increase in chain length. Although the propanoyl analogue appears to be equal to or better than the natural substrate, increase of the chain by 1–2 methylene groups results in a 5–40-fold change in k_{cat}/K_m values.

NeuNAc synthase belongs to a class of PEP-utilizing enzymes that include KDO 8-P (2-keto-3-deoxy-D-manno-octulosonate 8-phosphate) synthase and DAH 7-P (2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate) synthase. KDO 8-P synthase and DAH 7-P synthase catalyse the condensation of PEP with D-arabinose 5-P and D-erythrose 4-P respectively. Both these enzymes catalyse the *si* face addition of C-3 of PEP to the *re* face of the aldehyde group of the monosaccharides [15,18–22]. In the present study, we have prepared 3-fluoro- and 3-deutero-PEP analogues (both *E*- and *Z*-isomers) and utilized these analogues to study the stereochemical course of the reaction catalysed by NeuNAc synthase.

Reaction mixtures containing either *E*- or *Z*-[3-²H]PEP and ManNAc were incubated with NeuNAc synthase for 12 h. Reaction products were purified utilizing an anion-exchange column and ¹H-NMR spectra were recorded for the purified products (Figure 3). For the product from PEP condensation with ManNAc, the ¹H-NMR spectrum (Figure 3a) shows the C-3 proton signals at 1.78 p.p.m. (H_{3a} , dd, 11 and 12 Hz) and 2.2 p.p.m. (H_{3e} , dd, 12 and 5.5 Hz). The ¹H-NMR spectrum obtained for the product of NeuNAc synthase-catalysed condensation of *E*-[3-²H]PEP and ManNAc (Figure 3b) shows the disappearance of the signals at 1.8 p.p.m. for the axial proton on C-3, whereas the signal for the equatorial proton appears as a simple doublet at 2.19 p.p.m. (coupling constant $J = 5.5$ Hz). This value of $J = 5.5$ Hz between the axial proton on C-4 and the proton on C-3 suggests a *gauche* orientation between the two and, therefore, an equatorial position for the C-3 proton. The slightly up-field chemical shift of the C-3 equatorial proton in this compound is due to the shielding effect caused by the deuterium atom. These results suggest that, in the condensation product of *E*-[3-²H]PEP and ManNAc, the deuterium is incorporated into the axial position, indicating that the product is (3*R*)-[3-²H]NeuNAc. A closer look at Figure 3 reveals that a small amount of (3*S*)-3-deuterated NeuNAc is also present as a minor product (10 % of the total products). We believe that this (3*S*)-3-deuterated NeuNAc is formed from small amounts of *Z*-[3-²H]PEP present as a minor contaminant in *E*-[3-²H]PEP. Consistent with this result, the ¹H-NMR spectrum recorded for the product of NeuNAc synthase-catalysed condensation of *Z*-[3-²H]PEP and ManNAc (Figure 3c) shows that the signal

Figure 3 ^1H -NMR spectra of NeuNAc

^1H -NMR spectra of (a) NeuNAc, (b) (3*R*)-[3- ^2H]NeuNAc obtained from *E*-[3- ^2H]PEP, and (c) (3*S*)-[3- ^2H]NeuNAc obtained from *Z*-[3- ^2H]PEP. Only the regions exhibiting signals for C-3 protons are shown (the signal at 1.78 p.p.m. is from H_{ax} and the signal at 2.19 p.p.m. is from H_{eq}). The signal at 2 p.p.m. is from the methyl group of *N*-acyl side chain.

at 2.2 p.p.m. has disappeared, whereas the signal at 1.78 p.p.m. appears as a simple doublet ($J = 11$ Hz). The coupling constant of 11 Hz between the axial protons on C-4 and C-3 suggests a *trans*-diaxial relationship between the two. Therefore the C-3 proton is in an axial position in this product, whereas the deuterium must be in the equatorial position. Again, the up-field shift of the C-3 proton chemical shift in this compound is caused by the shielding effect of C-3 deuterium present in this compound. Thus NeuNAc synthase-catalysed condensation of *Z*-[3- ^2H]PEP and ManNAc leads to the formation of (3*S*)-[3- ^2H]NeuNAc. A small amount of (3*R*)-3-deuterated NeuNAc is also formed as a minor product due to contaminating *E*-[3- ^2H]PEP present in *Z*-[3- ^2H]PEP. In brief, proton NMR spectra obtained for the purified products indicate that incubation of *Z*-[3- ^2H]PEP and ManNAc with NeuNAc synthase resulted in the formation of (3*S*)-3-deuterated NeuNAc, whereas incubation of *E*-[3- ^2H]PEP and ManNAc with NeuNAc synthase resulted in the formation of (3*R*)-3-deuterated NeuNAc.

We also studied the stereochemical nature of the enzyme-catalysed reaction using *Z*-[3- ^19F]PEP. Owing to the difficulty in preparing isomerically pure *E*-isomer, a 3:2 mixture of *E*- and *Z*-[3- ^19F]PEP as PEP analogues was prepared and used to observe the reaction with the *E*-isomer. The progress of the reaction in a mixture containing ManNAc, [3- ^19F]PEP analogue, MnCl_2 and the enzyme was followed by recording the ^{19}F -NMR spectra at various time intervals. As shown in Figure 4, the NMR signal for *Z*-[3- ^19F]PEP (−141.5 p.p.m.) decreased over time with a concomitant appearance and increase in intensity of the signal at −199.4 p.p.m. for the product. The ^{19}F -NMR signal obtained for the product (−199.4 p.p.m.) exhibited coupling constant values of 40 Hz between C-3 fluorine and C-3 proton and 11.2 Hz between the C-3 fluorine and C-4 axial proton. These values are indicative of a *gauche* orientation between the C-3 fluorine and C-4 axial proton. These results suggest that the fluorine atom is in an equatorial position on C-3 of NeuNAc. The NeuNAc synthase-catalysed condensation of *Z*-[3- ^19F]PEP and ManNAc results in the formation of (3*S*)-[3- ^19F]NeuNAc. When a mixture of *E*- and *Z*-[3- ^19F]PEP was included in the reaction as PEP analogues (results are shown in Figure 5), the *Z*-isomer was utilized by the enzyme to form (3*S*)-[3- ^19F]NeuNAc (as evidenced by the disappearance of NMR signal for the *Z*-isomer at −140 p.p.m. and the appearance of new

Figure 4 ^{19}F -NMR spectra obtained for the reaction mixture containing *Z*-[3- ^19F]PEP, ManNAc, MnCl_2 and *C. jejuni* NeuNAc synthase

Signal at −140 p.p.m. is from *Z*-[3- ^19F]PEP and the signal at −199.4 p.p.m. is from the product (3*S*)-[3- ^19F]NeuNAc.

Figure 5 ^{19}F -NMR spectra obtained for the reaction mixture containing *E*- and *Z*-[3- ^19F]PEP (3:2 mixture), ManNAc, MnCl_2 and *C. jejuni* NeuNAc synthase

The signal at −140 p.p.m. is from *Z*-[3- ^19F]PEP and the signal at −150 p.p.m. is from *E*-[3- ^19F]PEP. The signal at −199.4 p.p.m. is from the product (3*S*)-[3- ^19F]NeuNAc (formed from the *Z*-isomer).

signals over time at −199.4 p.p.m. for the product (3*S*)-[3- ^19F]NeuNAc, whereas the *E*-isomer was not utilized at all (there was no change in the NMR signals for the *E*-isomer at −153 p.p.m. over time).

DISCUSSION

Molecular mimicry is a common virulence tool of bacterial pathogens. Structural resemblance between the lipooligosaccharide of *C. jejuni* and the human gangliosides is believed to play a role in the neurological conditions, Guillain–Barre syndrome and Miller–Fisher syndrome, associated with *C. jejuni* infection [8]. A better understanding of enzymes in NeuNAc biosynthesis is of importance in the use of NeuNAc analogues as potential targets for designing drugs and other types of interventions. Results of our kinetic studies indicate that *C. jejuni* NeuNAc synthase has a similar specificity of *N*-acylmannosamine substrates as the human NeuNAc 9-P synthase. The enzyme exhibits similar affinity for ManNAc analogues with different acyl side chains. On the other hand, the rate of formation of products from these analogues decreased with increasing side-chain length. In the enzyme-bound conformation, the distance between C-1 of the monosaccharide and C-3 of PEP is expected to be approx. 1.54 Å (1 Å = 0.1 nm) for the reaction to occur. It is possible that an increase in the size (length) of the *N*-acyl side chain at C-2 of the monosaccharide may sterically interfere with the interaction between C-3 of PEP and C-1 of ManNAc during catalysis, leading to decreased reaction rates.

ManNAc

Scheme 2 Condensation of PEP with ManNAc by NeuNAc synthase

Stereospecificity and regiospecificity of a number of PEP-utilizing enzymes have been studied by including various PEP analogues such as *E*- and *Z*-[3-²H]PEP and *E*- and *Z*-[3-³F]PEP as alternative substrates of these enzymes [15]. We have used such PEP analogues to study the nature of the reaction catalysed by *C. jejuni* NeuNAc synthase. When either *Z*-[3-³F]PEP or *Z*-[3-²H]PEP was included in the reaction mixture, it was found that the fluorine and the deuterium atoms were incorporated in the equatorial position on C-3 of the product (with a 3*S* configuration). Similarly, inclusion of *E*-[3-²H]PEP in the reaction mixture leads to the formation of (3*R*)-[3-²H]NeuNAc, in which the deuterium is incorporated in an axial orientation. These results show that the stereochemistry of C-3 of PEP is maintained throughout the course of the enzyme-catalysed reaction, suggesting that any anion formed at the C-3 position of PEP as an intermediate has a very short half-life (< 10⁻¹⁰ s, since formation of a stable anion probably leads to racemization at the C-3 position). These results show that the *si* face of PEP condenses with the *si* face of ManNAc to form the NeuNAc (Scheme 2). This facial selectivity with respect to PEP exhibited by NeuNAc synthase is similar to that observed for DAH 7-P synthase- and KDO 8-P synthase-catalysed condensation of PEP with D-erythrose 4-P and D-arabinose 5-P respectively. However, certain differences do exist among the three enzymes, even though their overall reaction mechanism seems to be similar. Sialic acid synthase condenses the *si* face of the aldehyde group with the *si* face of PEP, whereas DAH 7-P synthase and KDO 8-P synthase catalyse the condensation of *re* face of aldehyde groups with the *si* face of PEP. NeuNAc synthase utilizes *Z*-[3-³F]PEP and not *E*-[3-³F]PEP as a substrate, whereas KDO 8-P synthase prefers *E*-[3-³F]PEP. DAH 7-P synthase, on the other hand, utilizes both the isomers at similar rates. Therefore

the mechanism of binding of PEP molecules in the active sites of these enzymes is probably similar. Inability of NeuNAc synthase to utilize *E*-[3-³F]PEP is interesting and needs to be investigated further since this may give more information about the enzyme-bound conformation and spatial orientation of PEP and ManNAc. It is possible that when the substrates are bound to the enzyme, the electronics of the fluorine atom of *E*-[3-³F]PEP may contribute to non-productive interactions of the enzyme-bound substrates, resulting in no product being formed. On the other hand, NeuNAc synthase-bound conformation of ManNAc is probably different from the bound conformations of monosaccharides in DAH 7-P synthase and KDO 8-P synthase. Further comparison of these PEP-utilizing enzymes with respect to their three-dimensional structures will provide more insight into the mechanism of reactions catalysed by these enzymes as well as their substrate-binding motifs. Three-dimensional X-ray crystal structures are now available for both KDO 8-P synthase [23] and DAH 7-P synthase [24–26]. Interestingly, the active-site architecture of these two enzymes is very similar, even though their overall amino acid sequence similarity is less than 30%. Both DAH 7-P synthase and KDO 8-P synthase have been shown to exist as tetramers in their native form and the mechanism of binding of the substrates is believed to be similar in both cases. Hwang et al. [27] have predicted the secondary structure of *E. coli* NeuNAc synthase to be similar to that of KDO 8-P synthase. Further analysis of NeuNAc synthase in terms of its three-dimensional structure and mechanism of action will be of significant help in targeting not only this enzyme but also KDO 8-P synthase and DAH 7-P synthase for developing novel antibacterials.

The *C. jejuni* isoenzyme 1 and *E. coli* NeuNAc synthase share 34% identity and 51% sequence similarity at the amino acid level when compared using the NCBI BLAST 2 algorithm and probably have similar mechanisms. The enzyme from these two sources share similar metal ion requirements and specificity [4]. Bravo et al. [28] have recently analysed the evolutionary relationship of α -oxo acid-synthesizing enzymes. These investigators have concluded that NeuNAc synthase, D-arabino-heptulosonate 7-P synthase and KDO-8-P synthase originated from a common ancestor. There is limited sequence similarity among these enzymes. Thus, without a three-dimensional structure, it is difficult to speculate on active-site relationships of *C. jejuni* isoenzyme 1 and *E. coli* NeuNAc synthase. The substrate PEP is common to α -oxo acid synthase and reacts with the same stereochemistry as was observed in the present study. The fact that all of these enzymes catalyse the condensation of the *si* face of PEP suggests commonalities in the PEP site. The addition of an acyl group to the sugar aldehyde resulted in the divergence of mechanism for sialic acid synthases.

Although chemical methods have been developed for preparing NeuNAc analogues, an improved and efficient enzymic method for preparing NeuNAc derivatives will be of great significance in developing NeuNAc-based new drug candidates. By using the enzymic methods, NeuNAc analogues with desired stereochemical properties could be prepared directly from ManNAc and PEP analogues. The fact that *C. jejuni* NeuNAc synthase does not use the *E*-isomer of 3-fluoro-PEP facilitates the preparation of the axial stereoisomer of 3-fluoro-NeuNAc.

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