

The role of sialic acid synthases in sialic acid biosynthesis

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Introduction

The sialic acids comprise a diverse family of nine-carbon keto-sugars, the majority of which are structural derivatives of neuraminic acid (Fig. 1). Neuraminic acid has an amino moiety at the C-5 position, and carboxylate at C-2 which confers negative charge at physiological pH. Whereas unsubstituted neuraminic acid is not known to exist in nature, over 40 substituted variations (classified as sialic acids) have now been described.¹ Common modifications to the core neuraminic acid framework include *O*-acetylation and esterification of the hydroxyl moieties, namely at positions C-7, C-8 and C-9 (Fig. 1).² Multiple substitutions at these positions allow further diversification of sialic acid structure. Other modifications include glycolation (*N*-glycolylneuraminic acid) and deamination (2-keto-3-deoxy-*D*-glycero-*D*-galacto-nononic acid) of the C-5 amino group. *N*-acetylneuraminic acid (NeuNAc), *N*-acetylated at position C-5, is the most prevalent sialic acid in nature and is often synonymous with the term sialic acid.¹

NeuNAc forms the terminal residue of cell surface glycoconjugates in mammals and therefore is critically involved in cellular recognition, signalling and cell adhesion processes.¹ Sialic acids are largely absent in plants and prokaryotes, however, several species of pathogenic bacteria incorporate sialic acid residues into their own cell wall glycoconjugates, allowing them to mimic the physiology of mammalian cells and evade detection by their mammalian host's immune system.³

Additionally, sialic acids expressed on mammalian cell surfaces facilitate the recognition and binding of certain viruses to the host cells prior to internalisation.⁴ For this reason, structural analogues of sialic acids have applications as anti-viral therapeutics, including the frontline influenza drugs Zanamavir (Relenza™) and Oseltamivir (Tamiflu®). The role of sialic acids in human disease emphasises the importance of understanding how they are biosynthesised, and how they function within biological systems.

The biosynthetic pathways of only a small number of these sugar molecules have been described. These include NeuNAc⁵, 2-keto-3-deoxy-*D*-glycero-*D*-galacto-nononic acid (KDN)⁶, and the sialic acid-like compounds; 5,7-diacetamido-3,5,7,9-tetra-deoxy-*L*-glycero-*L*-manno-nonulosonic acid (pseudaminic acid)⁷ and 5,7-diacetamido-3,5,7,9-tetra-deoxy-*D*-glycero-*D*-galacto-nonulosonic acid (legionaminic acid).⁸

The structural variation of these sialic acids allows them to fulfil different roles within living systems. NeuNAc and structurally similar KDN are components of both vertebral and bacterial cell surface glycoconjugates, mediating signalling and recognition processes.⁶ Conversely,

pseudaminic and legionaminic acid are apparently unique to bacteria and microorganisms, forming components of cell surface lipopolysaccharides, and other glycoconjugates.^{8,9} Additionally, pseudaminic acid has been shown to be critical for the proper development of flagella in certain gram-negative bacteria.⁷

The biosynthetic pathways of the aforementioned sialic acids involve the aldol-like condensation of phosphoenolpyruvate (PEP) with respective sialic acid precursors, a reaction catalysed by an evolutionarily related family of enzymes known collectively as the sialic acid synthases.¹⁰ A final step in the biosynthesis of all sialic acids is the activation of these molecules for incorporation into cell surface glycoconjugates. Condensation with cytidine triphosphate (CTP) yields the activated sialic acid, cytidine monophosphate-sialic acid (CMP-sialic acid), which acts as the glycosyl donor and can be transferred to the growing terminus of carbohydrate structures.¹¹ This activation step is catalysed by a family of enzymes known as the CMP-sialic acid synthetases, which are reviewed elsewhere.¹² This review provides a summary of the current understanding of sialic acid biosynthesis, with an emphasis on the structure and function of the enzyme that catalyses the central aldol-like condensation reaction of NeuNAc biosynthesis: NeuNAc synthase.

The pathways of sialic acid biosynthesis

NeuNAc biosynthesis

It was not until the 1960s that the first enzymes involved in sialic acid biosynthesis were identified. Comb and Roseman¹³ initially discovered an enzyme from mammalian tissue which could catalyse the cleavage of NeuNAc to pyruvic acid and *N*-acetylmannosamine (ManNAc). The reverse reaction of this 'NeuNAc lyase' enzyme was thought to be a potential biosynthetic source of NeuNAc. Further investigation uncovered an independent enzyme that almost exclusively catalysed the forward condensation reaction of PEP and *N*-acyl mannosamine 6-phosphate.¹⁴ The enzyme was tentatively named *N*-acyl neuraminic acid synthetase but was renamed shortly after to *N*-acetylneuraminic acid synthetase upon the discovery of its preference for acetylated mannosamine-6-phosphate (ManNAc-6-P) as a substrate.¹⁵ This enzyme is now known to be the primary facilitator of NeuNAc synthesis in mammalian systems. In the same year, an additional two steps of the mammalian NeuNAc biosynthesis pathway were described, the initial phosphorylation of ManNAc to ManNAc-6-P, and the subsequent dephosphorylation of NeuNAc-9-P.¹⁵ The first description of a NeuNAc biosynthetic enzyme from a bacterial source was reported in 1962.¹⁶ An enzyme isolated from *Neisseria meningitidis* was shown to be capable of catalysing the condensation of ManNAc and PEP to produce NeuNAc directly.

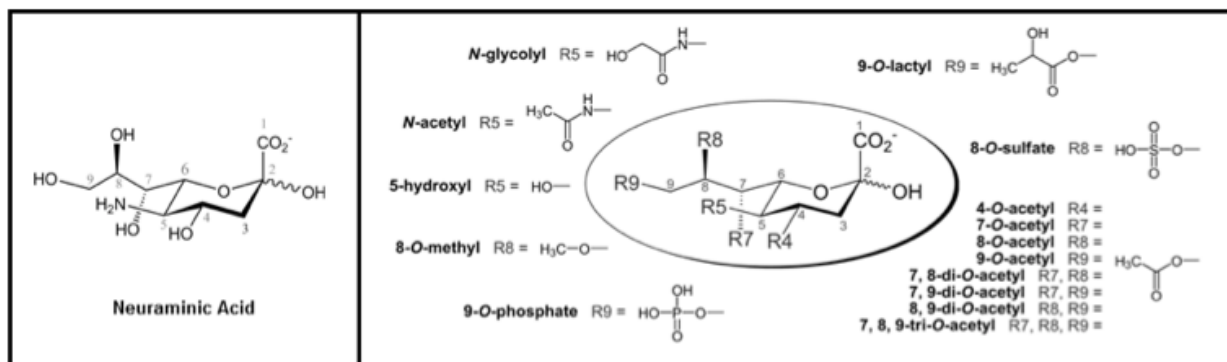


Fig. 1. Left: Unsubstituted neuraminic acid. Right: The family of naturally occurring sialic acids. Displayed in the centre is the sialic acid template architecture. Figure modified from Schauer (2004).²

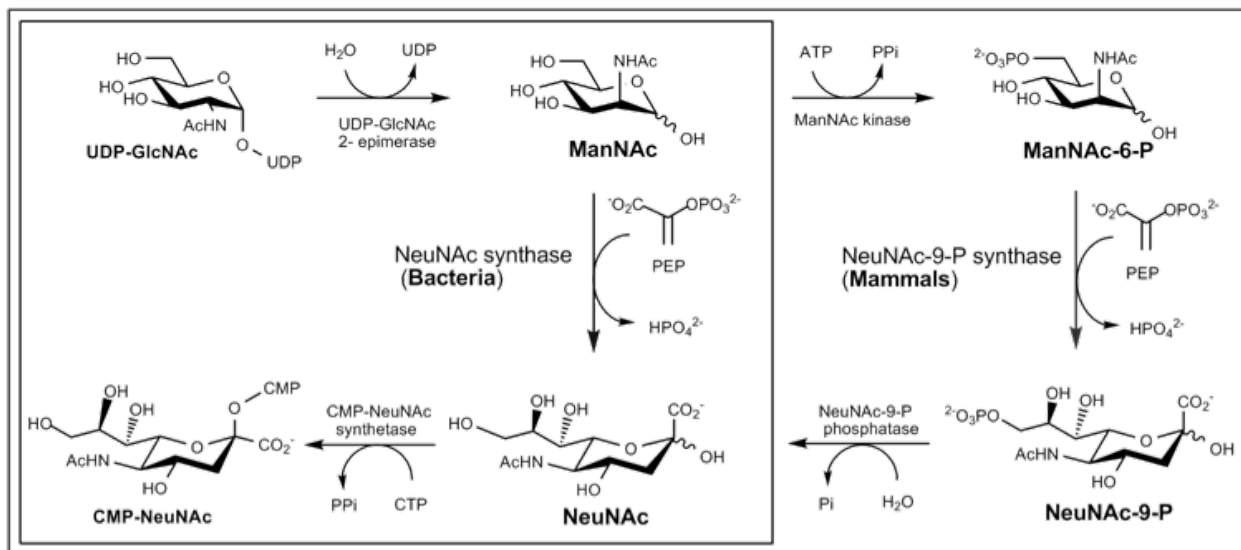


Fig. 2. The pathways of bacterial and mammalian NeuNAc biosynthesis. In bacterial systems (inner box), ManNAc condenses with PEP to give NeuNAc directly. The mammalian pathway (outer box) involves an additional two steps; phosphorylation of ManNAc to ManNAc-6-P, which then condenses with PEP to give NeuNAc-9-P, and then dephosphorylation of NeuNAc-9-P to NeuNAc. In both systems, a final step activates NeuNAc via condensation with cytidine triphosphate (CTP).

This early work provided the basis for the understanding of NeuNAc biosynthesis that we have today.

The initial epimerisation and hydrolysis of uridine diphosphate (UDP)-*N*-acetylglucosamine (UDP-GlcNAc), catalysed by UDP-GlcNAc 2-epimerase, provides the substrate ManNAc for both the bacterial and mammalian biosynthetic pathways of NeuNAc (Fig. 2).¹⁷ The bacterial pathway utilises this substrate directly in an aldol-like condensation reaction with PEP, which is catalysed by the enzyme NeuNAc synthase. The mammalian pathway involves an extra step in which ManNAc is phosphorylated via ManNAc kinase activity to ManNAc-6-P.¹⁵ This is the substrate for mammalian NeuNAc-9-P synthase which catalyses the aldol-like condensation of PEP with ManNAc-6-P yielding NeuNAc-9-P which can be subsequently dephosphorylated to form NeuNAc (Fig. 2).¹⁵ A final step in both bacterial and mammalian systems generates activated NeuNAc via conjugation to cytidine monophosphate, ready for incorporation into cell surface glycoconjugates.³

KDN biosynthesis

Almost 40 years following elucidation of the NeuNAc biosynthetic pathway, biosynthesis of a second sialic acid, KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonon-

ic acid), was described. Angata *et al*⁶ demonstrated that purified soluble fractions from trout testis were able to catalyse the formation of KDN, from adenosine triphosphate (ATP), PEP and mannose-6-phosphate (Man-6-P). The suggested biosynthetic pathway is a three step process which begins with the ATP-dependent phosphorylation of mannose to yield Man-6-P, catalysed by the enzyme hexokinase (Fig. 3). Man-6-P then undergoes condensation with PEP to produce KDN-9-P and free phosphate. This central step is catalysed by KDN-9-phosphate synthase of the sialic acid synthase family.

Interestingly, human NeuNAc-9-P synthase is also capable of utilising Man-6-P as a viable substrate and thus exhibits secondary KDN-9-P synthase activity.¹⁸ KDN-9-P phosphatase then catalyses the dephosphorylation of KDN-9-P to give KDN as the product.⁶ Glycoconjugates containing KDN are often over-expressed in ovarian cancer cells, and postulated to contribute to the metastatic potential of these cells.¹⁹ A detailed review of KDN biosynthesis is available elsewhere.²⁰

Pseudaminic acid biosynthesis

More recently the pathway for the bacterial sialic acid, pseudaminic acid (5,7-diacetamido-3,5,7,9 tetra-deoxy-L-glycero-L-manno-nonulosonic acid), has been described

(Fig. 3).⁷ This sialic acid is required for flagella development and therefore motility in certain gram-negative bacteria such as the gastrointestinal pathogens *Helicobacter pylori* and *Campylobacter jejuni*.⁹ The pathway proceeds in the same way as both NeuNAc and KDN synthesis as previously described. Condensation of the pseudaminic acid precursor 2,4-diacetamido-2,4,6-trideoxy-L-altrose with PEP is catalysed by pseudaminic acid synthase (PseI), a sialic acid synthase initially isolated from *C. jejuni*.⁹ As for NeuNAc and KDN, pseudaminic acid is

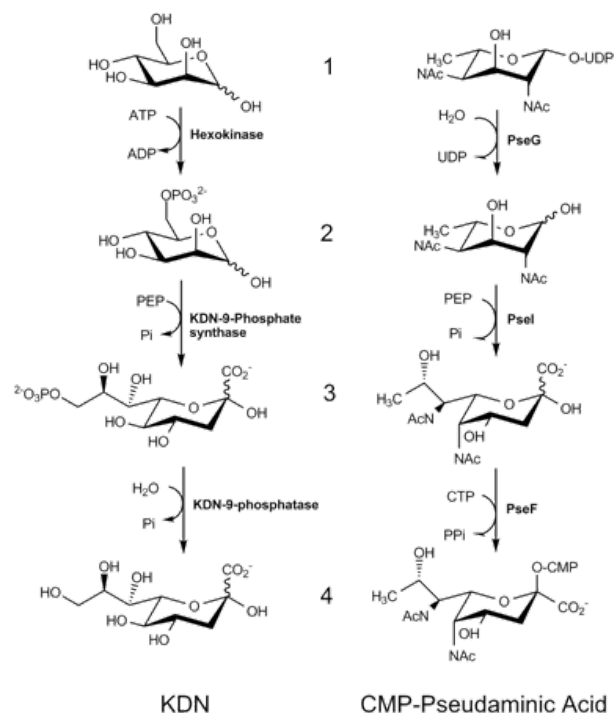


Fig. 3. The biosynthetic pathways of KDN and pseudaminic acid. **Left:** KDN (4) is synthesised via a three step process from mannose (1). A final step (not shown) conjugates KDN to CTP, forming CMP-KDN. **Right:** CMP-pseudaminic acid (4) synthesis proceeds via hydrolysis of UDP-2,4-diacetamido-2,4,6-trideoxy- β -L-altropyranose (1) derived from UDP-*N*-acetylglucosamine, releasing UDP and forming 2,4-diacetamido-2,4,6-trideoxy- β -L-altropyranose (2). Pseudaminic acid synthase (PseI) catalyses the condensation of PEP and 2,4-diacetamido-2,4,6-trideoxy- β -L-altropyranose to give pseudaminic acid (3) as the product.

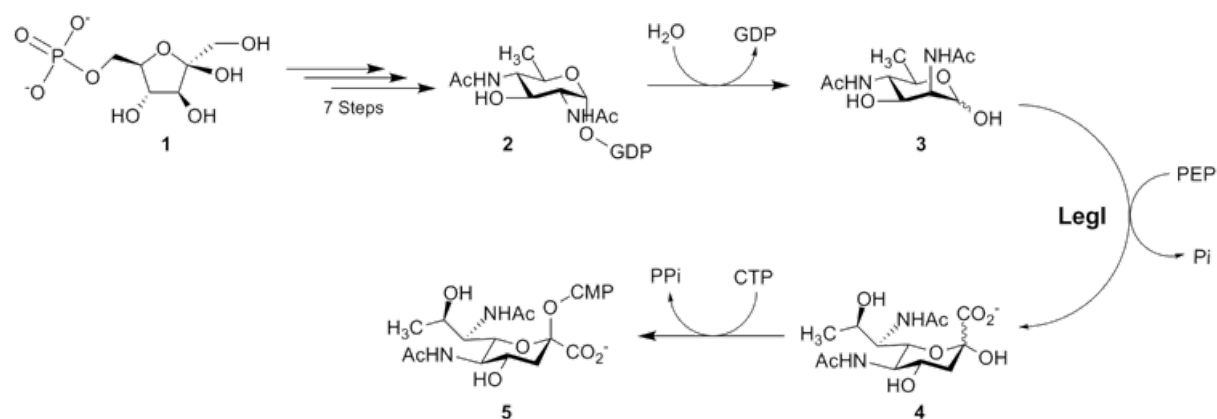


Fig. 4. The biosynthetic pathway of legionaminic acid in *C. jejuni*. Biosynthesis of legionaminic acid begins with the glycolytic intermediate fructose 6-phosphate (1). Hydrolysis of the pathway intermediate GDP-2,4-diacetamido-2,4,6-trideoxy- α -D-glucopyranose (2) yields 2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose (3). Legionaminic acid synthase (LegI) catalyses the condensation of this metabolite with PEP to form legionaminic acid (4). In the final step, CMP-legionaminic acid synthetase conjugates cytidine-triphosphate to legionaminic acid, liberating pyrophosphate and CMP-legionaminic acid (5).

also activated for sialyltransferase activity via condensation with cytidine triphosphate. CMP-pseudaminic acid can then be exported to the cell surface for sialylation of flagellin, the major glycoprotein component of flagella.²¹

Legionaminic acid biosynthesis

Another sialic acid associated with the virulence of bacteria, notably *Legionella pneumophila* and *Campylobacter coli*, is legionaminic acid (5,7-diacetamido-3,5,7,9 tetra-deoxy-D-glycero-D-galacto-nonulosonic acid). These pathogenic bacteria are common causative agents of the acute respiratory infection known as legionnaires disease.²² The complete biosynthetic pathway of this sialic acid from *C. jejuni* was reported in 2009 by Schoenhofen *et al.*,⁸ and was shown to proceed via unique guanidine diphosphate (GDP)-linked intermediates, differing from the previously described sialic acid biosynthetic pathways which utilise UDP-linked intermediates. The legionaminic acid biosynthetic pathway is considerably longer than that of other sialic acids, and begins with the conversion of the glycolytic intermediate fructose 6-phosphate to glucosamine 6-phosphate. Seven subsequent reactions yield the sugar substrate 2,4-diacetamido-2,4,6-trideoxy-D-mannopyranose, which undergoes condensation with PEP to form legionaminic acid, a reaction catalysed by the sialic acid synthase denoted LegI (Fig. 4).⁸

N-Acetyl Neuraminic Acid (NeuNAc) Synthase

The enzymes responsible for catalysis of the central aldol-like condensation of PEP with sialic acid precursor sugars are collectively referred to as the sialic acid synthases. The most well understood of these enzymes is NeuNAc synthase, which catalyses the condensation of PEP with ManNAc (or ManNAc-6-P in mammalian cells), generating NeuNAc, the most abundant sialic acid in nature.

NmeNeuNAcS Structure

The first solved crystallographic structure of a sialic acid synthase was that of NeuNAc synthase from *N. meningitidis* (*NmeNeuNAcS*).²³ While several structures of *NmeNeuNAcS* are now available, no complete sialic acid synthase structure from any other species has been pub-

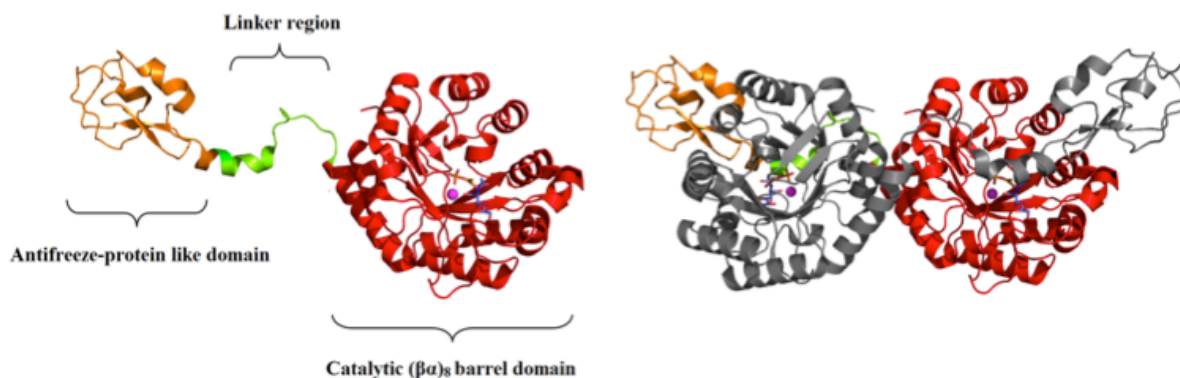


Fig. 5. Crystal structure of NeuNAc synthase from *N. meningitidis* in complex with *N*-acetylmannosaminitol, PEP and Mn^{2+} . The NeuNAc synthase monomer (left) consists of an N-terminal $(\beta\alpha)_8$ barrel domain (red) and a much smaller C-terminal AFP domain (orange). Note the presence of the substrate analogue rManNAc (blue), PEP (orange) and Mn^{2+} (magenta sphere) bound in the $(\beta\alpha)_8$ barrel active site. NeuNAc synthase from *N. meningitidis* forms a domain-swapped homo-dimer (right) in which the AFP-like domain of one monomer interacts with the $(\beta\alpha)_8$ barrel domain of the second monomer. Protein Data Bank (PDB) entry: 1XUZ.

lished to date. The initial crystal structure of *Nme*NeuNAcS revealed a unique domain-swapped homo-dimer (Fig. 5), with each monomer consisting of an N-terminal $(\beta\alpha)_8$ catalytic barrel domain and a C-terminal antifreeze protein-like (AFP) domain connected via an extended linker.²³ The presence of the AFP-like domain had been previously identified by a structural homology search against type III antifreeze proteins and was hypothesised to play a role in binding the sugar substrate ManNAc.²⁴

Light scattering experiments show *Nme*NeuNAcS exists primarily as a native dimer in solution, consistent with the asymmetric unit of the crystal structure.²³ Upon dimer assembly, the AFP-like domain of one monomer interacts with the $(\beta\alpha)_8$ barrel domain of the adjacent monomer, capping the active site and forming the domain swapped homo-dimer arrangement which has since been shown to be critical for catalysis.²⁵

This initial *Nme*NeuNAcS structure was obtained from crystals soaked with an excess of PEP and the substrate analogue *N*-acetylmannosaminitol (rManNAc). As can be seen in Fig. 5, the bound location of these molecules identifies the active site of NeuNAc synthase at the C-terminal end of the $(\beta\alpha)_8$ barrel domain, as is typical for enzymes of the $(\beta\alpha)_8$ barrel fold.²⁶ An interesting structural point to note is that the AFP-like domain from the adjacent monomer effectively seals the active site from bulk solvent, and contributes residues involved in substrate recognition.^{23, 25}

The active site of *Nme*NeuNAcS is defined largely by residues from β -strands 2 and 4 at the C-terminal of the $(\beta\alpha)_8$ barrel, however the linker and AFP-like domain also contribute residues to the active site. NeuNAc synthases from various bacterial species are known to be highly dependent on the presence of divalent metal ions.^{10, 27} A single manganese ion is present within the active site of the *Nme*NeuNAcS structure, and is coordinated with octahedral geometry by the imidazole moieties of histidine (His)-215 and His-236, O2P of PEP, O-1 of rManNAc and two water molecules (Fig. 6). The C-1 hydroxyl group of the substrate analogue rManNAc is replaced by an aldehyde in the native substrate ManNAc. O-1 (rManNAc) coordination of the metal suggests that the native substrate aldehyde may similarly coordinate the metal thus allowing activation of the carbonyl centre for nucleophilic attack by PEP.²³

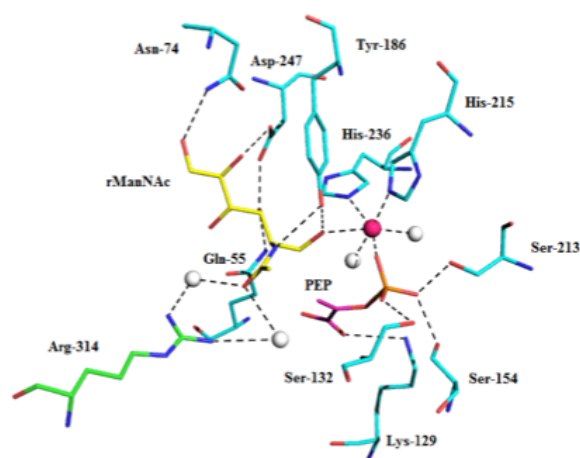


Fig. 6. The active site of NeuNAc synthase from *N. meningitidis*. The active site manganese ion (magenta sphere) has an octahedral coordination sphere, in which His-215, His-236, O2P of PEP (orange) and water (white spheres) fill the equatorial positions. An additional water molecule and O-1 from rManNAc (yellow) occupy the axial Mn^{2+} coordination sites. Multiple active site residues (cyan) form hydrogen bonds and/or salt bridges with the substrate molecules, stabilising catalytic orientations within the active site. Arg-314 (green/blue) from the AFP-like domain of the adjacent monomer extends into the active site and interacts with the *N*-acetyl group of rManNAc via active site water molecules. PDB entry: 1XUZ.

The substrate analogue rManNAc forms interactions with the highly conserved active site residues Asp-247, Gln-55 and Tyr-186 of *Nme*NeuNAcS. Interestingly, Arg-314 from the AFP-like domain of the adjacent monomer extends into the active site and forms hydrogen bonding interactions with the *N*-acetyl group of rManNAc via active site water molecules.^{23, 28} Deletion of this highly conserved residue renders *Nme*NeuNAcS catalytically inert.²⁵ PEP also makes a number of interactions within the active site. The phosphate group of PEP forms hydrogen bonds with three conserved serine residues (Ser-132, Ser-154 and Ser-213) and the asparagine (Asn) residue Asn-184, while the PEP carboxylate is shown to form electrostatic interactions with two nearby lysine residues (Lys-129 and Lys-53).

As previously stated, only the full structure of *Nme*NeuNAcS has been solved, however the small AFP-like do-

main of the human sialic acid synthase NeuNAc-9-PS has been structurally characterised in solution by NMR.²⁸ This structure shows overall similarity to the bacterial AFP-like domain from *Nme*NeuNAcS with a backbone root-mean-square deviation (RMSD) value of 0.90 Å relative to residues 7-66 from the solved human domain (Fig. 7). Interestingly, the human AFP-like domain lacks the conserved residue Arg-314 that enters the active site in the bacterial enzyme (Fig. 7). Instead, conserved nearby lysine residues may facilitate the required electrostatic recognition and stabilisation of the mammalian substrate.^{28,29} Evolutionary analysis of the AFP-like domain of human NeuNAc-9-PS and type III antifreeze proteins from both bacteria and vertebrates suggests that the sialic acid synthase domain may be the evolutionary precursor to type III antifreeze proteins in vertebrates.²⁸ An antifreeze function of the ancestral sialic acid synthase domain may have evolved in response to changes in environmental temperature.²⁸

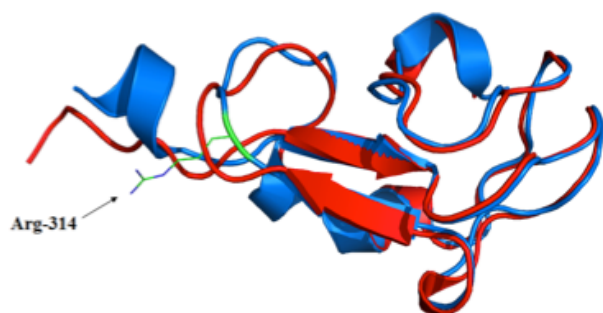


Fig. 7. Alignment of antifreeze protein-like domains from *Nme*-NeuNAcS (blue) and human NeuNAc-9-PS (red). PDB entries: 1XUZ and 1WVO.

Mechanism of the sialic acid synthase catalysed reaction

Preliminary mechanistic studies on NeuNAc synthase from *C. jejuni* using deuterated substrates demonstrated that the enzyme catalysed aldol-like condensation reaction is stereospecific for nucleophilic attack of the *si* face of PEP with the *si* face of the aldehydic (open-chain) form of ManNAc.¹⁰ Subsequent labelling studies with *Nme*-NeuNAcS showed that isotopically labelled ¹⁸O (bridging C-2 and P) from PEP is released as free phosphate through the course of the catalytic cycle.²³ This observation has important implications for the proposed reaction mechanism. The loss of labelled ¹⁸O as free phosphate suggests a mechanism which proceeds via a tetrahedral intermediate. Gunawan *et al*²³ proposed a C-O cleavage mechanism based on the results of their ¹⁸O labelling studies (Fig. 8). Initially PEP attacks the C-1 aldehyde

of ManNAc, yielding an oxocarbenium ion intermediate. Catalytic water present in the active site then attacks the electrophilic oxocarbenium carbonyl centre, generating a tetrahedral intermediate which spontaneously releases free phosphate via C-O bond cleavage. This yields open chain NeuNAc which can cyclise to its stable pyranose form in solution. This C-O bond cleavage mechanism is well documented in related PEP-utilising enzymes; 3-deoxy-D-manno-octulosonate 8-phosphate (KDO8P) synthase and 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAH7P) synthase.^{30,31}

For this reaction mechanism to be plausible, the C-1 carbonyl of ManNAc requires activation to promote nucleophilic attack by PEP in the first step. Similarly, the nucleophilicity of the catalytic water must be increased to drive its attack on the oxocarbenium ion in the subsequent step. The crystal structure of *Nme*NeuNAcS shows the active site divalent metal ion occupies a position proximal to the C-1 hydroxyl of the ManNAc analogue. It could be imagined that the active site metal may coordinate the aldehyde oxygen of the native substrate, activating the aldehydic carbon to nucleophilic attack by PEP.²³ Several suggestions for the identity of the species responsible for activating water have been made. A glutamate residue (Glu-134) within close proximity of the active site water may act as a base, deprotonating the water and thereby promoting attack on the oxocarbenium ion intermediate. In addition, water coordination to the divalent metal ion in the active site may assist its deprotonation.²³

There is overwhelming evidence that a divalent metal ion is essential for catalysis of the sialic acid synthases.^{23, 32} This metal dependency can be rationalised if the metal is involved in such roles as activation of the ManNAc aldehyde and the catalytic water. Crystallisation of *Nme*NeuNAcS in the presence of a putative tetrahedral intermediate analogue provides strong evidence for a dual role of the divalent metal ion in catalysis.³² The orientation of the tetrahedral intermediate analogue within the active site strongly implies the role of metal in both activation of the ManNAc aldehyde, and delivery of water to the oxocarbenium ion in the following step of catalysis. This inhibitor bound crystal structure also suggests a strong preference for a tetrahedral intermediate with an (*R*)-configuration at C-2 due to the selectivity of the (*2R*) inhibitor over its (*S*)-diastereomer. The formation of this tetrahedral configuration would require a reaction mechanism that proceeds via the facial attack of catalytic water onto the *si*-face of the oxocarbenium ion intermediate.³²

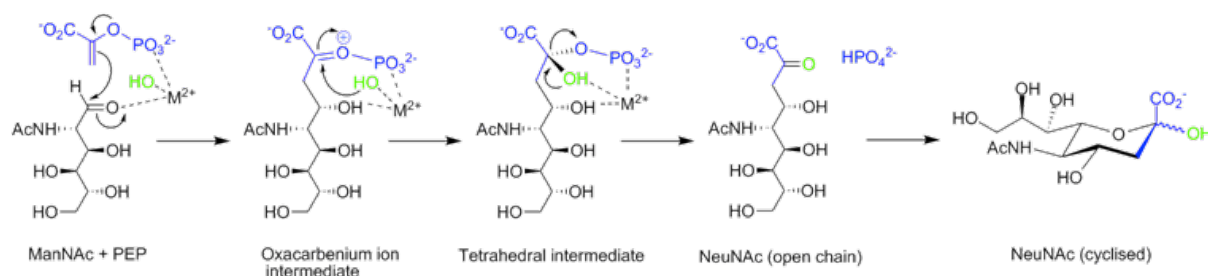


Fig. 8. Postulated mechanism of the reaction catalysed by *Nme*NeuNAcS.²³ Nucleophilic attack of PEP at the metal activated aldehyde gives rise to an oxocarbenium ion intermediate which is then attacked by water to form a tetrahedral intermediate. Decomposition of the intermediate via C-O bond cleavage releases NeuNAc and free phosphate to solution.

Decomposition of the tetrahedral intermediate in the final step of catalysis releases free phosphate and NeuNAc to solution. The driving force behind the collapse of the tetrahedral intermediate is thought to be ionisation of the C-2 hydroxyl group. Promoting deprotonation of the C-2 hydroxyl may be a third role for the coordinated metal ion, or this role may be played by a nearby basic residue.²³

There exists little mechanistic information pertaining to any of the other sialic synthases. However, sequence similarity implies that PEP and sugar substrate condensation may proceed via the same mechanism as is now established for *NmeNeuNAcS*. Glu-25 and Glu-234 are postulated catalytic residues involved in decomposition of the tetrahedral intermediate.²³ These residues, along with metal binding residues, are almost entirely conserved across phyla, suggesting a mechanistic commonality. The differential substrate specificity of the numerous sialic acid synthases would require general active site architecture modification, however the catalytic residues would be expected to remain largely unchanged if the same reaction mechanism is employed ubiquitously. A good example of this is the presence of a number of cationic residues near the human NeuNAc synthase active site that may encourage binding of the polyanionic substrate ManNAc-6-P. These charged residues are mostly absent in bacterial variants of the enzyme which utilise a neutral substrate, however other key catalytic residues are conserved. Furthermore, the metal dependency of all known sialic acid synthases suggests a common metallo-catalytic cycle. Further elucidation of the precise mechanism of the other sialic acid synthases will be made possible when high resolution crystal structures of these enzymes become available.

Conclusions

The sialic acids are a diverse family of nine-carbon keto-sugars, and the terminal residues of mammalian cell surface carbohydrate structures which facilitate cellular signalling and recognition events. Alongside their central biological function in mammals, sialic acids are involved in mediating bacterial and viral infection of mammalian hosts. The sialic acid synthases are an evolutionarily related family of enzymes which catalyse the formation of sialic acids via an aldol-like condensation of PEP with respective sialic acid precursor molecules. Biochemical and crystallographic analyses have provided insights into the structural and mechanistic detail of these enzymes. NeuNAc synthase from *N. meningitidis* exists as a unique domain swapped homo-dimer, each monomer of which contains a C-terminal ($\beta\alpha$)₈ barrel domain and an N-terminal antifreeze protein-like domain. This structure facilitates the condensation of PEP and ManNAc to form NeuNAc and free phosphate. ¹⁸O labelling studies have shown the reaction mechanism proceeds via oxocarbenium and tetrahedral intermediates, catalysis of which is highly dependent on divalent metal ions. Appreciable sequence conservation suggests that the structural and mechanistic understanding of *NmeNeuNAcS* can likely be applied to the other known sialic acid synthases for which such information is not yet available.

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