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Minireview

The enzymes of sialic acid biosynthesis

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Abstract

The sialic acids are a family of nine carbon α-keto acids that play a wide variety of biological roles in nature. In mammals, they are found at the distal ends of cell surface glycoconjugates, and thus are major determinants of cellular recognition and adhesion events. In certain strains of pathogenic bacteria, they are found in capsular polysaccharides that mask the organism from the immune system by mimicking the exterior of a mammalian cell. This review outlines recent developments in the understanding of the two main enzymes responsible for the biosynthesis of the sialic acid, *N*-acetylneuraminic acid. The first, a hydrolyzing UDP-*N*-acetylglucosamine 2-epimerase, generates *N*-acetylmannosamine and UDP from UDP-*N*-acetylglucosamine. The second, sialic acid synthase, generates either *N*-acetylneuraminic acid (bacteria) or *N*-acetylneuraminic acid 9-phosphate (mammals) in a condensation reaction with phosphoenolpyruvate. An emphasis is placed on an understanding of the mechanistic and structural features of these enzymes.

Keywords: *N*-Acetylneuraminic acid; Sialic acid synthase; UDP-GlcNAc 2-epimerase; *N*-Acetylmannosamine; Phosphoenolpyruvate; Biosynthesis; Mechanism

1. Introduction

The sialic acids are a large family of nine carbon polyhydroxylated α-keto acids that play a wide variety of roles in nature [1]. Most are derivatives of the common

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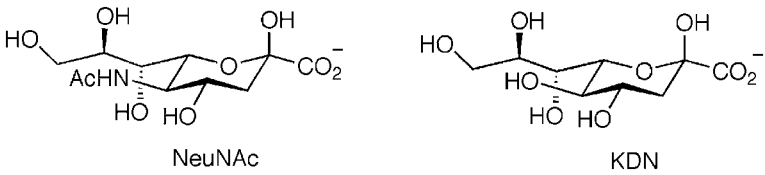

Fig. 1. The structures of NeuNAc and KDN.

core structures *N*-acetylneuraminic acid (NeuNAc) or 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (KDN) (Fig. 1). Commonly found modifications include decoration of the hydroxyl groups with acetates, sulfates, phosphates, and methyl ethers. In addition, lactonization of the carboxylic acid and hydroxylation of the acetamido group of NeuNAc are observed.

In mammals, the sialic acids are usually found as terminal residues on the outermost cell surface glycoconjugates. As a result of their location, and their negative carboxylate functionality, the sialic acids play important roles in mediating cellular recognition and adhesion processes [2,3]. Some notable examples include the sialylation of neural cell adhesion molecules that is responsible for the proper establishment of the vertebrate embryonic nervous system [4], and the necessity for recognition of sialic acids by the lectins of the inflammatory and immune response pathways [5]. In addition, the extent of cell surface sialylation has been correlated with tumorogenesis and metastasis in certain cancers [6,7]. Although most bacteria do not produce sialic acids, several pathogenic strains can biosynthesize them and display them on their cell surface to mimic mammalian cells and evade the host's immune system [8]. These include the neuroinvasive organisms *Escherichia coli* K1 and *Neisseria meningitidis* that cause meningitis [9–11], and *Campylobacter jejuni* that causes food-borne gastroenteritis [12].

The biosynthesis of the most common sialic acid, NeuNAc, begins with the formation of *N*-acetylmannosamine (ManNAc) from UDP-*N*-acetylglucosamine (UDP-GlcNAc) (Fig. 2) [1]. In mammals, the ManNAc is then phosphorylated to give ManNAc 6-phosphate (ManNAc-6P). The second step involves the condensation of either ManNAc or MacNAc-6P with phosphoenolpyruvate to give NeuNAc or NeuNAc-9P, respectively. In mammals, NeuNAc-9P is then dephosphorylated to

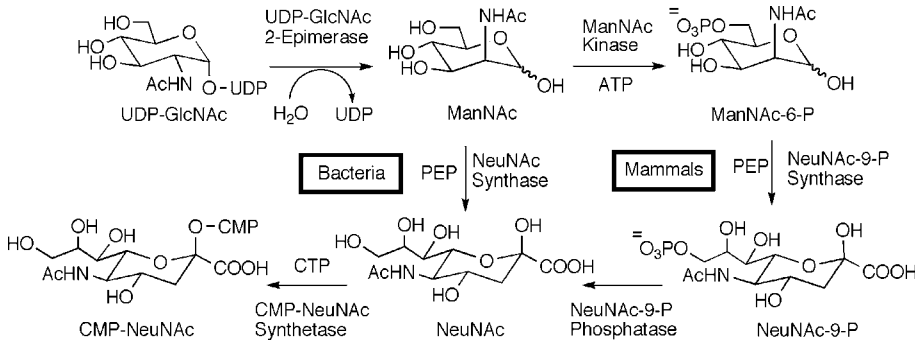

Fig. 2. The biosynthesis of CMP-NeuNAc in bacteria and mammals.

generate NeuNAc. Finally, the activated form of sialic acid, CMP-sialic acid, is generated with the use of cytosine triphosphate, CTP. CMP-sialic acid is the substrate for all the sialyltransferases that incorporate the keto acid into glycoproteins and glycolipids. This review will focus on the enzymology of the first two steps of NeuNAc biosynthesis with a particular emphasis on an understanding of the catalytic mechanism and protein structure. A recent review describing the final enzyme, CMP-sialic acid synthetase, is available elsewhere [13].

2. The hydrolyzing UDP-GlcNAc 2-epimerase

The first step of sialic acid biosynthesis is catalyzed by the enzyme UDP-*N*-acetylglucosamine 2-epimerase (hydrolyzing) (Fig. 3) [14–16]. This enzyme catalyzes both an inversion of stereochemistry at C-2 of the sugar as well as hydrolysis of the glycosidic phosphate bond to give free *N*-acetylmannosamine and UDP. As this is an irreversible reaction, the enzyme is technically not an epimerase. This enzyme is both evolutionarily and mechanistically related to the bacterial UDP-GlcNAc 2-epimerase (non-hydrolyzing) that catalyzes the interconversion of UDP-GlcNAc and UDP-ManNAc in a freely reversible reaction (Fig. 3) [17–20]. The non-hydrolyzing enzyme provides bacteria with a source of activated ManNAc residues that are used in the biosynthesis of a variety of cell surface polysaccharides, but is not involved is the sialic acid pathway.

The mammalian hydrolyzing UDP-GlcNAc 2-epimerase is a bifunctional enzyme that also possesses the ManNAc kinase activity necessary to generate ManNAc 6-P [15,16]. This enzyme catalyzes the rate-limiting steps in sialic acid biosynthesis and therefore serves as a key regulator of cell surface sialylation [21]. It is known to be essential during the embryonic development of mice [22] and point mutations in this enzyme are known to cause hereditary inclusion body myopathy in humans [23]. The

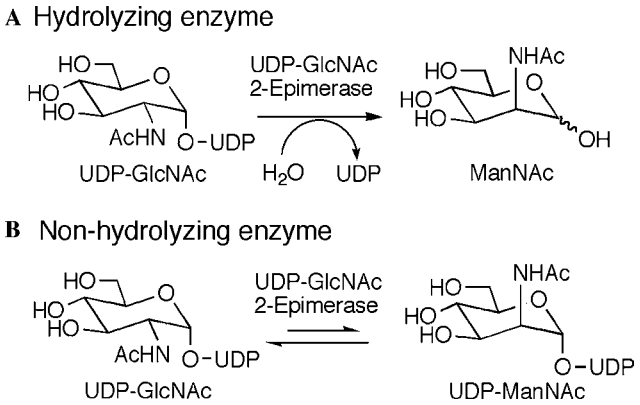

Fig. 3. The reactions catalyzed by the (A) hydrolyzing and (B) non-hydrolyzing UDP-GlcNAc 2-epimerases.

purified enzyme can exist as either a dimer or a hexamer; however, the latter appears to be the physiologically relevant species as the former lacks the epimerase activity [15]. Kinetic analysis of the epimerase activity shows strong negative cooperativity in substrate binding as well as feedback inhibition from CMP-NeuNAc with high positive cooperativity. A mutational study of the rat enzyme showed that the N-terminal domain bears the epimerase activity whereas the C-terminal domain bears the kinase activity [24]. This is consistent with the observed sequence homology that the domains share with the bacterial UDP-GlcNAc 2-epimerases (both hydrolyzing and non-hydrolyzing) and the sugar kinase superfamily, respectively. Since mutations in one domain do not dramatically appear to affect the activity of the other, they appear to be functioning independently.

Until recently, little was known about the bacterial hydrolyzing UDP-GlcNAc 2-epimerase. The NeuC protein of *E. coli* K1 was known to be required for sialic acid biosynthesis and bore sequence similarity to the non-hydrolyzing epimerases as well as the N-terminal region of the mammalian enzyme [25]. The purified recombinant protein did show a slow consumption of UDP-GlcNAc upon extended incubation; however, product analysis indicated that the major products formed were UDP and 2-acetamidoglucal, a putative intermediate in the normal reaction mechanism (vide infra). Only traces of ManNAc could be detected using radiolabeled substrates. While this provided initial evidence that bacteria generate ManNAc in a similar fashion to mammals, it appeared that the enzyme was catalyzing an abortive reaction in vitro. Subsequent studies on the homologous protein, SiaA, from *N. meningitidis* led to more conclusive results [26]. The purified recombinant version of this protein showed a clean production of UDP and ManNAc from UDP-GlcNAc without any observable 2-acetamidoglucal. This finding unambiguously demonstrated that the first step of sialic acid biosynthesis is shared by bacteria and mammals. No kinase activity was observed as would be expected given the lack of any C-terminal kinase domain. The SiaA protein was found to display strong positive cooperativity in substrate concentration, which differs distinctly from the mammalian enzyme. Instead, this resembles the situation observed with the non-hydrolyzing *E. coli* UDP-GlcNAc 2-epimerase that is thought to function as a homodimer in which one subunit must bind substrate to activate the other subunit [18].

Early mechanistic studies on the hydrolyzing UDP-GlcNAc 2-epimerase were aimed at distinguishing between three basic mechanisms (Fig. 4). In Path A, the first step is epimerization to generate UDP-ManNAc, and the second is hydrolysis of the glycosidic bond to give the products. In Path B, hydrolysis takes place first to give GlcNAc, and epimerization occurs subsequently, presumably through the open chain form of the sugar that would bear a relatively acidic C-2 proton. In Path C, an anti-elimination of UDP generates the intermediate, 2-acetamidoglucal. Hydration of this intermediate gives the product ManNAc. This mechanism is similar to that currently favored for the non-hydrolyzing epimerase except that the second step involves a *syn*-addition of UDP in the latter case [19]. The finding that tritium is incorporated into the C-2 position of the product when the reaction is run in tritiated water is consistent with all three mechanisms and indicates that the inversion of stereochemistry is ultimately brought about by removal and replacement of a proton at this position

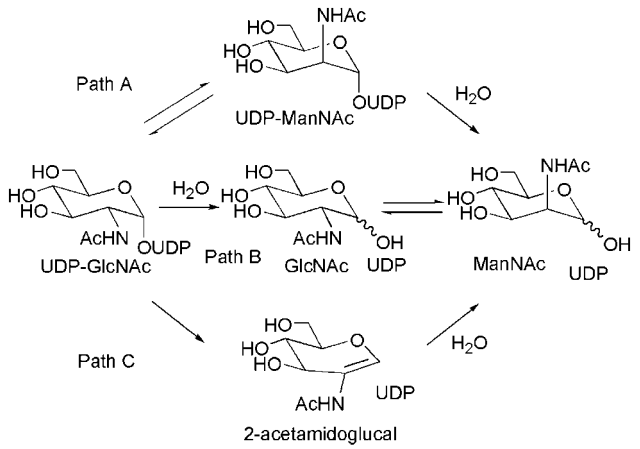

Fig. 4. Three potential mechanisms for the reaction catalyzed by the hydrolyzing UDP-GlcNAc 2-epimerase. In Path A, epimerization precedes hydrolysis. In Path B, hydrolysis precedes epimerization. In Path C, elimination precedes hydration.

[27]. Further studies focused on incubating the enzyme with the putative reaction intermediates to test for kinetic competence. Incubation of the enzyme with UDP-ManNAc did lead to the formation of ManNAc and UDP as would be expected if Path A were operative [28]. However, when the reaction was carried out in tritiated water the ManNAc produced contained only 87% of the tritium that was incorporated when UDP-GlcNAc was used as the substrate. Furthermore, no tritium was incorporated into the recovered starting material and no UDP-GlcNAc could be detected. Ultimately, it was concluded that UDP-ManNAc was simply serving as an alternate substrate and not as a true reaction intermediate. Incubation of the intermediate in Path B, GlcNAc, did not lead to any detectable formation of ManNAc [29]. A brief report of the incubation of the Path C intermediate, 2-acetamidoglucal, was made stating that a compound that co-eluted with ManNAc was detected by high voltage paper electrophoresis [30]. This experiment, along with kinetic evidence indicating an initial release of UDP, followed by an irreversible formation of ManNAc, were forwarded as evidence in support of Path C.

More recent work was greatly aided by the ability to produce the recombinant rat enzyme in insect cells using a baculovirus expression system [24]. In addition, the *N. meningitidis* enzyme was readily available by overexpression in *E. coli* [26]. In both cases, the overall stereochemistry of the reaction was determined by incubating the substrate in D_2O containing high levels of the epimerase and rapidly monitoring the reaction using ^1H NMR spectroscopy [14,26]. Under these conditions it was possible to determine that the first formed product was the α-anomer, and that rapid non-enzymatic mutarotation subsequently scrambled the stereochemistry at C-1. Overall, the reaction proceeds with inversion of stereochemistry at C-2 and retention of stereochemistry at C-1, in a similar fashion to the non-hydrolyzing enzyme (Fig. 5). This experiment also confirmed that the reaction proceeds with complete incorporation of

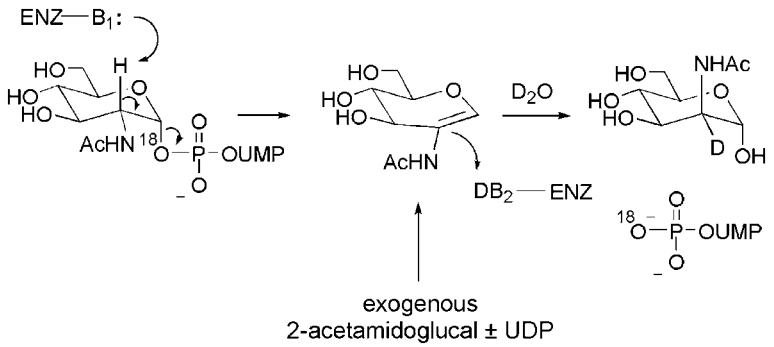

Fig. 5. Summary of mechanistic studies on the hydrolyzing UDP-GlcNAc 2-epimerase showing that the *syn*-hydration of 2-acetamidoglucal in D_2O gives α-[2-^{2}H]ManNAc, the C–O bond cleavage mechanism generates ^{18}O-labeled UDP, and 2-acetamidoglucal is catalytically competent. B_1 and B_2 represent active site acid/base residues that promote proton transfer at carbon.

solvent derived deuterium at C-2 of the product and showed that no significant solvent derived isotope was incorporated into starting material during catalysis. This is consistent with a "two-base" mechanism in which different residues are responsible for deprotonation and reprotonation at C-2 and therefore solvent derived isotope is quantitatively incorporated into product. When either enzyme was incubated with [2″-^{2}H]UDP-GlcNAc and the reaction kinetics were monitored, no significant isotope effect was observed, indicating that the deprotonation of the substrate was not a rate-determining step of the reaction. Additional work involved the use of UDP-GlcNAc that was ^{18}O-labeled at the anomeric position to distinguish between C–O and P–O bond cleavage in the hydrolysis step of the reaction [14,26]. Product analysis by both mass spectrometry and ^{31}P NMR spectroscopy confirmed that the label resided in the UDP portion, indicating that C–O bond cleavage had occurred (Fig. 5). C–O bond cleavage is a requirement of Path C since it occurs in the formation of the 2-acetamidoglucal intermediate. It was interesting to note that no positional isotope exchange (PIX) was observed in the recovered starting material indicating that either there is a barrier to rotation of the phosphate in the active site, or that the first step is effectively irreversible in this reaction. To firmly establish the previous report of 2-acetamidoglucal hydration, both enzymes were incubated with the putative reaction intermediate in the presence and absence of added UDP [14,26]. In the case of the mammalian enzyme, a very slow hydration reaction was observed by ^{1}H NMR spectroscopy (Fig. 5) [14]. The sole product of the reaction was ManNAc and the rate of ManNAc formation doubled in the presence of 5 mM UDP. In the case of the *N. mengitidis* enzyme, no reaction was observed in the absence of UDP, but a reasonable rate of ManNAc formation (approx. 1% of V_{max} for the epimerization reaction) was observed in the presence of 5 mM UDP [26]. This observation proves that the epimerase is able to catalyze the second step of Path C and strongly implicates 2-acetamidoglucal as an intermediate in the reaction. The requirement that UDP be present for the efficient hydration of the glycal suggests that the second step of the overall reaction takes place before UDP dissociates from the active site. Recent NMR

spectroscopic studies have shown that the UDP functionality of the substrate, as well as UDP alone, is tightly bound to the enzyme, and that the binding affinity for the sugar moiety is low [31].

While no structural information has been obtained on a hydrolyzing UDP-GlcNAc 2-epimerase of sialic acid biosynthesis, a structure of the *E. coli* non-hydrolyzing epimerase in complex with UDP is available [18]. This protein is a homodimer in which each monomer contains two domains with a Rossman fold topology linked by α-helices. In each dimer, the two monomers differ by an interdomain rotation that serves to either "open" or "close" the active site cleft. This observation has been used to explain the allosteric regulation of activity by substrate concentration that shows strong positive cooperativity. It is speculated that one monomer plays a regulatory role and activates the other monomer for catalysis when substrate is bound. A similar situation is likely at play with homologous *N. meningitidis* hydrolyzing epimerase that also displays strong regulation by substrate concentration [26].

A remarkable observation was made while searching for proteins that shared structural homology with the epimerase [18]. Despite low sequence identities, it is clear that the non-hydrolyzing epimerase belongs to a superfamily of glycosyltransferases that include MurG and the T4 phage β-glucosyltransferase [32,33]. The transferases catalyze substitution reactions at the anomeric position of glycosyl phosphates and sugar nucleotides and are thought to employ dissociative or "S_N1-like" mechanisms with significant oxocarbenium character in the transition state [34,35]. This may help to explain how the epimerases are able to catalyze an elimination of UDP from UDP-GlcNAc when the proton at C-2 is non-acidic. If the elimination reaction has a significant "E1-like" component and the transition state has significant oxocarbenium character, the partial positive charge should effectively acidify the proton at C-2 and facilitate the reaction. Thus, it is compelling to speculate that both the glycosyltransferases and the UDP-GlcNAc 2-epimerases evolved from a common ancestor that was able to stabilize oxocarbenium-like transition states/intermediates during catalysis. The notion of such a transition state/intermediate is also supported by a recent report of epimerase inhibition by positively charged oxocarbenium-ion mimics [36].

The structure of the non-hydrolyzing epimerase also provides significant insight into the nature of the active site residues present in the hydrolyzing epimerase since almost all of the conserved residues in the former are also conserved in the latter [18]. In particular, two glutamates and an aspartate were located in an appropriate position to serve as acid/base catalysts in the non-hydrolyzing enzyme (B_1 and B_2 in Fig. 5). When these residues were mutated to the corresponding amides, the activity dropped by about four orders of magnitude indicating that they played key roles in both catalysis and regulation [37]. When the corresponding mutants were made in the hydrolyzing *N. meningitidis* enzyme (D100N, E122Q, and D131N), similar decreases in activity were observed [26]. An interesting finding came from following the slow reactions of these crippled mutants by ^{1}H NMR spectroscopy. Two of the mutants (E122Q and D131N) were found to generate 2-acetamidoglucal as the first formed product, and ManNAc only appeared after very long incubation times. This is in sharp contrast to the wild type enzyme that does not release any detectable

2-acetamidoglucal during catalysis. This suggests that either the mutations impaired the ability of the enzyme to bind the intermediates tightly or they affected the second step of the reaction more dramatically than the first. The observation of 2-acetamidoglucal release in these mutants as well as the "crippled" *E. coli* K1 enzyme described previously [25], provides strong support to the notion that the enzymes can catalyze the anti-elimination of UDP from UDP-GlcNAc (Fig. 4, first step in Path C).

While extensive mechanistic and structural studies on the ManNAc kinase activity of the mammalian epimerase/kinase are lacking, sequence homology comparisons suggest the kinase domain belongs to a kinase superfamily that includes the yeast hexokinase and the rat glucokinase [24]. In yeast hexokinase, an aspartate is thought to interact with the ATP-complexed Mg^{2+}, and an arginine is thought to interact with the α- and β-phosphate oxygens of ATP. When the corresponding mutations were made in the kinase domain of the rat epimerase/kinase, the kinase activity was dramatically affected whereas the epimerase activity was not. This suggests that the enzymes may be evolutionarily related. Recent NMR spectroscopic studies have shown that the enzyme requires the correct configuration at C1 and C4 of the substrate for binding, but tolerates modifications and inversions of stereochemistry at C-2 [38]. Despite this tolerance, *N*-acylmannosamines with side chains of varying length showed differing binding modes. In addition, the researchers found that Mg^{2+} was not an absolute requirement for activity, but likely aids catalysis by promoting the binding of ATP to the enzyme.

3. Sialic acid (*N*-acetylneuraminic acid) synthase

Sialic acid synthase catalyzes the condensation of phosphoenolpyruvate (PEP) with either ManNAc (bacteria) or ManNAc-6P (mammals) to give NeuNAc or NeuNAc-9P, respectively (Fig. 2). The eukaryotic enzyme has been purified or produced in recombinant form from several sources including human [39], *Drosophila melanogaster* [40], and rat [41], although structural and mechanistic studies on these enzymes is limited. One notable observation is that the human and *D. melanogaster* enzymes will also accept mannose 6-phosphate as an alternate substrate and thereby produce KDN-6P, whereas the rat enzyme will not. The prokaryotic sources of recombinant enzyme that have been studied include *E. coli* K1 [42,43], *Streptococcus agalactiae* [44], *C. jejuni* [45], and *N. meningitidis* [46,47]. These enzymes do not accept mannose as an alternate substrate, and the majority of the structural and mechanistic studies have been carried out on the latter two enzymes. All of the sialic acid synthases studied to date appear to be metalloenzymes that require a divalent cation for activity and most show the highest activity in the presence of Mn^{2+}.

Two potential mechanisms have been considered for enzymes such as sialic acid synthase that catalyze the condensation of PEP with an aldehyde. In the first mechanism, the initial step involves an attack by the C-3 of PEP onto the carbonyl carbon of ManNAc (Fig. 6A). This would generate an oxocarbenium ion that would readily be attacked by water to give a tetrahedral intermediate. A concerted process could also be envisioned to form this tetrahedral intermediate (not shown). The tetrahedral

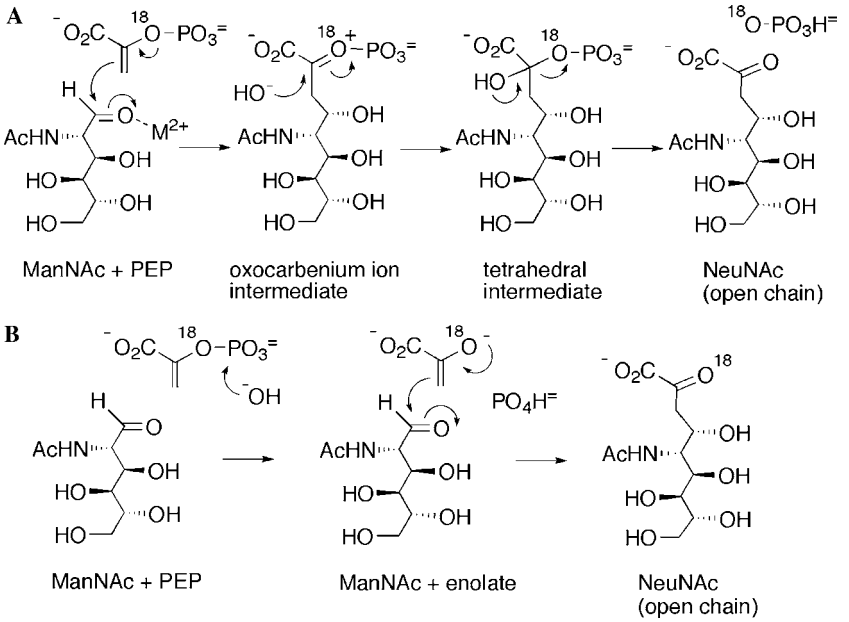

Fig. 6. Two potential mechanisms for the reaction catalyzed by sialic acid synthase. (A) A C–O bond cleavage mechanism. (B) A P–O bond cleavage mechanism.

intermediate would then lose phosphate to give the open chain keto form of NeuNAc that could spontaneously cyclize in solution. This type of mechanism is well precedented by two other systems that catalyze a very similar reaction and that have received a great deal of attention, 2-keto-3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHP synthase) and 2-keto-3-deoxy-D-*manno*-octulosonate-8-phosphate synthase (KDO-8P synthase) (Fig. 7) [48–54]. In each of these systems, a key experiment in support of this mechanism involves the use of ^{18}O-labeled PEP (see labeled atoms in Figs. 6A and 7) [55,56]. The observation that the ^{18}O-label is found in the phosphate product demonstrates that the reaction proceeds via a C–O bond cleavage process. Despite this precedence, the very low sequence homology between sialic acid synthase and these enzymes (<10% identity for the *E. coli* synthases) does not imply that a common mechanism is necessarily employed. A second possible mechanism involves an initial attack of water at the phosphorus of PEP to give free

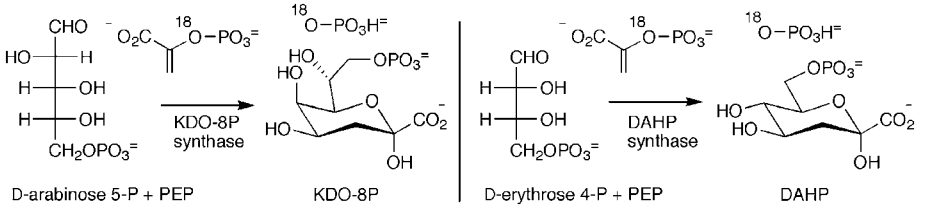

Fig. 7. The reactions catalyzed by KDO-8P synthase and DAHP synthase highlighting the fate of the ^{18}O-label in PEP.

phosphate and the enolate of pyruvate (Fig. 6B). The enolate could attack the carbonyl of ManNAc in an aldol-like fashion to generate the open chain form of NeuNAc. This mechanism operates via P–O bond cleavage and the use of ^{18}O-labeled PEP would give labeled NeuNAc (see labeled atoms in Fig. 6B). Precedence for a nucleophilic attack at the phosphate of PEP with release of the pyruvate enolate can be found in the reactions catalyzed by pyruvate kinase and PEP carboxykinase [57,58].

Recent studies on the mechanism of sialic acid synthase from *N. meningitidis* have involved the use of ^{18}O-labeled PEP to distinguish between a C–O and P–O cleavage mechanism [46]. A sample of the substrate containing 66% enrichment of the isotope at the phosphate bridging position was incubated with ManNAc and the enzyme. The resulting reaction was monitored by ^{31}P NMR spectroscopy and the characteristic upfield shift of phosphorus atoms bearing an ^{18}O-label was used to demonstrate that the label was retained in the phosphate produced (Fig. 8). This establishes that the overall mechanism involves C–O bond cleavage and is similar to that employed by either DAHP synthase or KDO-8P synthase. Additional mechanistic studies have addressed the stereospecificity of PEP addition in the reaction catalyzed by the *C. jejuni* enzyme [45]. A ^{1}H NMR spectroscopic analysis demonstrated that the use of *Z*- and *E*-[3-^{2}H]PEP led to the formation of (3*S*)-3-deutero-NeuNAc and (3*R*)-3-deutero-NeuNAc, respectively (see Fig. 8 for the *Z*-example). Similarly, a ^{19}F NMR spectroscopic study has shown that *Z*-[3-F]PEP produces (3*S*)-3-fluoro-NeuNAc; whereas, *E*-[3-F]PEP is not accepted by the enzyme. These results show that the *si* face of the PEP molecule adds to the carbonyl of the ManNAc in agreement with the results obtained for both DAHP synthase and KDO-8P synthase. Together these mechanistic studies suggest a common overall mechanism and active site architecture likely exists between all three enzymes.

Very recently the first structural characterization of a sialic acid synthase, from *N. meningitidis*, has been reported [46]. The enzyme was crystallized in the presence of Mn^{2+}, PEP, and the unreactive substrate analog *N*-acetylmannosaminitol (rManNAc or reduced ManNAc). Each monomer of the enzyme contains two domains joined by an extended linker region. The larger N-terminal domain has the classic TIM barrel fold of an eight-stranded β-barrel enclosed by eight helices. The smaller C-terminal domain is comprised of 65 residues in a "pretzel-shaped" fold that is remarkably similar to those found in the fish type III antifreeze proteins [59]. Two such monomers arrange to form a domain-swapped homodimeric architecture in which the active

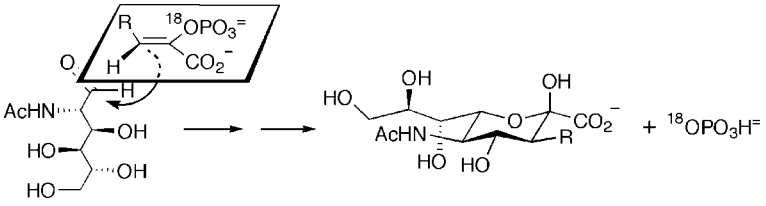

Fig. 8. Summary of mechanistic studies on sialic acid synthase showing that the C–O bond cleavage mechanism generates ^{18}O-labeled phosphate and the *si* face of PEP attacks the *si* face of ManNAc (R = ^{2}H, F).

sites are comprised of the C-terminal portion of the TIM barrel from one monomer capped by the antifreeze domain of the opposite monomer. The antifreeze domain donates one key residue, Arg-314, directly into the active site where it forms a hydrogen bond with the acetyl oxygen of rManNAc. While both DAHP synthase and KDO-8P synthase utilize a similar TIM barrel active site, these enzymes are homotetramers and completely lack the antifreeze domain of sialic acid synthase. Presumably all three enzymes have evolved from a common ancestor with a TIM barrel fold; however, the incorporation of the antifreeze domain into the bacterial sialic acid synthases allows them to discriminate between substrates bearing either an acetamido group or a hydroxyl group at the C-2 position.

An inspection of the active site clearly shows the proximity of the C-3 carbon of PEP to the C-1 carbon of rManNAc, as would be expected for the first step of the C–O bond cleavage mechanism (Fig. 9). In addition the *si* face of the PEP is directed towards the rManNAc as anticipated from the mechanistic studies [45]. The Mn^{2+} ion coordinates to both the C-1 hydroxyl group of rManNAc and to a phosphate oxygen of PEP. The former interaction strongly suggests that the metal plays the role of an electrophilic catalyst and activates the carbonyl carbon for attack by PEP since the position of the ManNAc aldehyde should be quite similar to that of the rManNAc C-1 hydroxyl. This orientation of the aldehyde is also expected since it exposes the *si* face of the carbonyl to the C-3 of PEP as is required to obtain the proper NeuNAc stereochemistry. Active site residues that may serve to stabilize an oxocarbenium intermediate or act as acid/base catalysts during the delivery of water and the breakdown of the tetrahedral intermediate include three glutamate residues (E25, E134, and E234). Two water molecules, one proximal to the *re* face of PEP and one proximal to the *si* face, are both reasonable candidates for attack in formation of

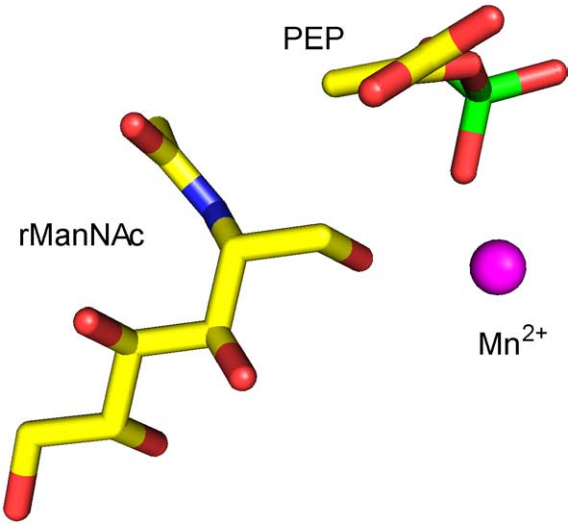

Fig. 9. The structure of bound rManNAc and PEP in the active site of the *N. meningitidis* sialic acid synthase with proximal Mn^{2+}.

the tetrahedral intermediate. A notable difference between the sialic acid synthase reaction and reactions catalyzed by DAHP synthase and KDO-8P synthase is that the PEP must attack the *re* face of the aldehyde in the latter cases. This difference may account for the findings in recent studies that have indicated the metal ion does not play a direct role in catalysis for either DAHP synthase or KDO-8P synthase [52,53]. Further work must be performed to investigate the similarities and differences between this interesting superfamily of enzymes.

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