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Probing the determinants of phosphorylated sugar-substrate binding for human sialic acid synthase

Thomas R. Cotton, Dmitri D.A. Joseph, Wanting Jiao, Emily J. Parker *

Biomolecular Interaction Centre, Department of Chemistry, University of Canterbury, Christchurch, New Zealand

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ABSTRACT

N-acetylneuraminic acid (NeuNAc), the most naturally abundant sialic acid, is incorporated as the terminal residue of mammalian cell surface glycoconjugates and acts as a key facilitator of cellular recognition, adhesion and signalling. Several pathogenic bacteria similarly express NeuNAc on their cell surfaces, allowing evasion of their host's immune system. Prokaryotic NeuNAc biosynthesis proceeds via condensation of phosphoenolpyruvate (PEP) with *N*-acetylmannosamine (ManNAc), a reaction catalysed by the domain-swapped homodimeric enzyme, *N*-acetylneuraminic acid synthase (NeuNAcS). Conversely, the mammalian orthologue, *N*-acetylneuraminic acid 9-phosphate synthase (NeuNAc 9-PS) utilises the phosphorylated substrate *N*-acetylmannosamine 6-phosphate (ManNAc 6-P) in catalysis. Here we report an investigation into the determinants of substrate specificity of human NeuNAc 9-PS, using model-guided mutagenesis to delineate binding interactions with ManNAc 6-P. Modelling predicts the formation of a domain-swapped homodimer as observed for bacterial variants, which was supported by experimental small angle X-ray scattering. A number of conserved residues which may play key roles in the selection of ManNAc 6-P were identified and substituted for alanine to assess their function. Lys290 and Thr80 were identified as a putative phosphate binding pair, with the cationic lysine residue extending into the active site from the adjacent chain of the dimeric enzyme. Substitution of these residues results in a significant loss of activity and reduced affinity for ManNAc 6-P. These residues, along with the electropositive $\beta_2\alpha_2$ loop, are likely to facilitate the PEP dependent binding and stabilisation of ManNAc 6-P. By utilising a phosphorylated sugar-substrate, the mammalian enzyme gains considerable catalytic affinity advantage over its bacterial counterpart.

1. Introduction

N-acetylneuraminic acid (NeuNAc) is the most naturally abundant member of the diverse family of nine carbon keto-acids known as the sialic acids [1]. These compounds, being structural derivatives of neuraminic acid, share a keto group at C-2, and a carboxylate at C-1, conferring negative charge at physiological pH [2]. NeuNAc is the terminal residue of mammalian cell surface glycoconjugates, and therefore plays an intrinsic role in cellular signalling, recognition and adhesion processes [2–5]. Unlike mammals, plants and prokaryotes typically lack NeuNAc expression, however a number of pathogenic Gram-negative bacteria including *Neisseria meningitidis* and *Campylobacter jejuni* have evolved to exploit the expression of NeuNAc [5,6]. By presenting NeuNAc on their own cell surfaces, these pathogens are able to mimic the physiology of the mammalian cell and effectively disguise themselves from their mammalian host's immune system [5–8].

The pathways of NeuNAc biosynthesis are well documented, and differ notably between bacterial and mammalian systems (Fig. 1) [1,3]. The amino-sugar *N*-acetylmannosamine (ManNAc) is the precursory metabolite for both pathways, and undergoes an aldol-like condensation with phosphoenolpyruvate (PEP) in the bacterial pathway to yield NeuNAc directly [9]. In mammalian systems (Fig. 1), ManNAc is first phosphorylated at C-6 to *N*-acetylmannosamine 6-phosphate (ManNAc 6-P) via kinase activity [1,10]. This phosphorylated intermediate then undergoes condensation with PEP to yield *N*-acetylneuraminic acid 9-phosphate (NeuNAc 9-P) which is subsequently dephosphorylated to form the end product NeuNAc [11]. A final step common to both bacterial and mammalian systems conjugates NeuNAc to cytidine monophosphate (CMP) forming CMP-NeuNAc which acts as the glycosyl donor for sialyltransferases for the extension of cell surface glycoconjugates [12] or sialylation of galactose.

The central aldol-like condensation of respective sialic acid precursors with PEP is catalysed by an evolutionarily related family of enzymes broadly termed the sialic acid synthases, which include the mammalian NeuNAc 9-P synthases (NeuNAc 9-PS) as well as the bacterial NeuNAc synthases (NeuNAcS), legionaminic acid synthases (LegS) and pseudaminic acid synthases (PseS) [3,7,13,14]. These alternate

* Corresponding author at: Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand. Tel.: +64 3 364 5682; fax: +64 3 364 2110.
E-mail address: emily.parker@canterbury.ac.nz (E.J. Parker).

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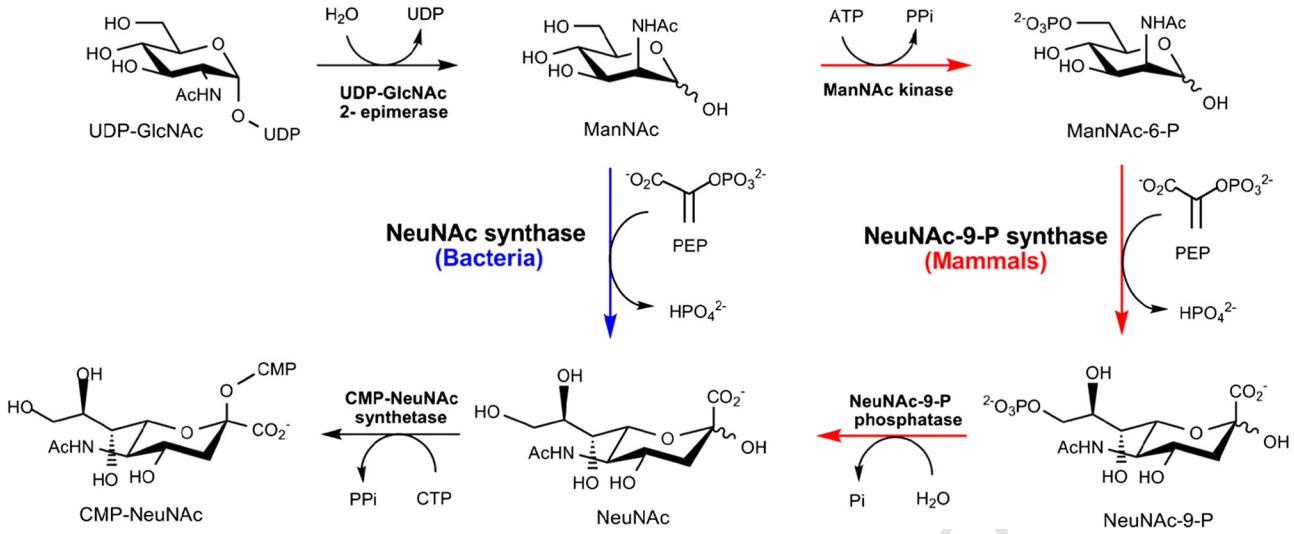

Fig. 1. The bacterial and mammalian biosynthetic pathways for NeuNAc. Figure adapted from Tanner [1].

sialic acid synthases differ primarily in the specificity for the sugar-substrates they utilise in catalysis [7,10,15,16]. Although a number of bacterial NeuNAcS have been characterised in significant detail [8,17], structural and functional data pertaining to mammalian NeuNAc 9-PS remains relatively sparse. The crystal structure of NeuNAcS from *N. meningitidis* (*Nme*NeuNAcS) has been previously solved (PDB ID: 1XUZ) in the presence of Mn^{2+}, PEP and a reduced non-reactive analogue of ManNAc (rManNAc) [8]. This structure revealed a unique domain swapped homo-dimeric arrangement, in which each monomer consists of an N-terminal $(\beta/\alpha)_8$ barrel and a C-terminal antifreeze protein-like (AFPL) domain connected via a flexible linker. Upon dimer assembly, the AFPL domain of one monomer interacts with the catalytic barrel of the other, effectively capping the active site from solvents [8]. The presence of the AFPL domain had been previously identified by homology search against type III antifreeze proteins, and was hypothesised to play a role in binding the polar sugar-substrate [18]. The AFPL domain has since been shown to be essential for catalysis in *Nme*NeuNAcS, and contributes active site residues involved in maintaining the catalytic orientation of the sugar-substrate ManNAc within the active site [19,20].

Mammalian sialic acid synthases (NeuNAc 9-PS) readily utilise the phosphorylated sugar-substrate ManNAc 6-P, yet are apparently inactive with respect to the unphosphorylated bacterial substrate ManNAc [10,21]. Interestingly, human NeuNAc 9-PS (*Hsa*NeuNAc 9-PS) is also capable of catalysing the aldol-like condensation reaction of mannose 6-phosphate with PEP, giving rise to the deaminated form of NeuNAc: 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid 9-phosphate (KDN 9-P) [21]. This secondary KDN 9-P synthase activity, albeit far weaker than NeuNAc 9-PS activity, gives *Hsa*NeuNAc 9-PS a unique bifunctional character not yet observed in other mammalian homologues [10,22]. Unlike the bacterial variant, there is currently no full length crystal structure available for this enzyme, however the structure of the C-terminal AFPL domain from the human enzyme has been solved by NMR (PDB ID: 1WVO) [23].

It is likely that structural variation between the sugar-substrate binding sites of mammalian and bacterial sialic acid synthases dictates the exclusive utilisation of the phosphorylated or non-phosphorylated substrates as previously observed. The $\beta_2\alpha_2$ loop of the catalytic domain is one such region of high sequence variability between bacterial and mammalian sialic acid synthases, and is known to function as a sugar-substrate binding loop in *Nme*NeuNAcS [8]. Given that the mammalian substrate differs only by phosphorylation at C-6, the $\beta_2\alpha_2$ loop is likely to function as a sugar-substrate binding loop in mammalian orthologues also. Modelling and site-directed mutagenesis were used to examine the architecture of the sugar-substrate binding site, and the key determinants of sugar-substrate specificity of the human enzyme. Localised modifications to the protein structure facilitate the specific utilisation of a phosphorylated substrate with an inherently higher binding affinity.

2. Materials and methods

2.1. Sequence alignment

Pairwise and multiple sequence alignments of sialic acid synthases were generated using the ClustalOmega server [24,25]. Alignment outputs were visualised using JalView [26].

2.2. Molecular modelling of the interactions between ManNAc 6-P and HsaNeuNAc 9-PS

A model of *Hsa*NeuNAc-9-PS was built by homology modelling using Prime [27–29]. The model was constructed using the comparative method, using the crystal structure of *Nme*NeuNAcS as the template (PDB ID: 1XUZ). Both monomers were built at the same time to generate the dimer structure. The ligands bound in the active site of *Nme*NeuNAcS (PEP and rManNAc) were retained in the homology model of *Hsa*NeuNAc-9-PS. The dimer was then minimised with Prime, to optimise bond length and bond angles, and to remove steric clashes.

The structure of the ligand ManNAc 6-P was built and prepared in Maestro and LigPrep respectively [30,31]. The modelling of ManNAc 6-P to the active site of *Hsa*NeuNAc 9-PS was conducted with the Induced Fit Docking protocol from Schrodinger Suite 2012 [32–35]. Since the ManNAc binding loop in the homology model shows different conformations in each monomer, induced fit docking of ManNAc 6-P was conducted in each of the monomers. The centre of the grid was defined as centroid of the workspace ligand, i.e. the ManNAc molecule bound in the homology model, since ManNAc 6-P was expected to bind in the same pocket in the active site as ManNAc in *Nme*NeuNAcS. For the initial docking, the Van der Waals radii of the atoms of the ManNAc 6-P molecule and the enzyme homology model were scaled by a factor of 0.5. The 20 best solutions of the initial docking were kept. Residues 70–78 (on the ManNAc binding loop) as well as all residues on the homology model within a 5 Å distance of the respective docked pose were refined. The ligands were re-docked to the top 20 newly