Elimination of 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid 9-phosphate synthase activity from human *N*-acetylneuraminic acid 9-phosphate synthase by a single mutation

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The most commonly occurring sialic acid Neu5Ac (*N*-acetylneuraminic acid) and its deaminated form, KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid), participate in many biological functions. The human Neu5Ac-9-P (Neu5Ac 9-phosphate) synthase has the unique ability to catalyse the synthesis of not only Neu5Ac-9-P but also KDN-9-P (KDN 9-phosphate). Both reactions are catalysed by the mechanism of aldol condensation of PEP (phosphoenolpyruvate) with sugar substrates, ManNAc-6-P (*N*-acetylmannosamine 6-phosphate) or Man-6-P (mannose 6-phosphate). Mouse and putative rat Neu5Ac-9-P synthases, however, do not show KDN-9-P synthase activity, despite sharing high sequence identity (> 95 %) with the human enzyme. Here, we demonstrate that a single mutation, M42T, in human Neu5Ac-9-P synthase can abolish the KDN-9-P synthase activity completely without compromising the Neu5Ac-9-P synthase ac-

tivity. Saturation mutagenesis of Met⁴² of the human Neu5Ac-9-P synthase showed that the substitution with all amino acids except leucine retains only the Neu5Ac-9-P synthase activity at levels comparable with the wild-type enzyme. The M42L mutant, like the wild-type enzyme, showed the additional KDN-9-P synthase activity. In the homology model of human Neu5Ac-9-P synthase, Met⁴² is located 22 Å (1 Å = 0.1 nm) away from the substrate-binding site and the impact of this distant residue on the enzyme functions is discussed.

Key words: bifunctional enzyme, 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid 9-phosphate (KDN-9-P) synthase, *N*-acetylneuraminic acid 9-phosphate (Neu5Ac-9-P) synthase, saturation mutagenesis, sialic acid, steady-state kinetics.

INTRODUCTION

Sialic acids are a family of unique nine-carbon 2-oxo acids mainly found in higher animals including humans and certain pathogenic bacteria [1,2]. They are generally present in the terminal position of glycosyl chains of cell surface glycoproteins and glycolipids of animals and hence influence several biological properties involving cell–cell interactions. The spatial and temporal expression of sialic acids is directly linked to the development of neuronal tissues during embryogenesis. Elevated level of sialic acid expression in tumour cells suggests their association with tumorigenesis and cancer metastasis [3,4]. In certain pathogenic bacteria such as *Neisseria meningitidis* and *Escherichia coli* K1, sialic acids in their capsular polysaccharides serve as molecular masks and help the microbes to evade the immune system in the host [5].

There are more than fifty forms of sialic acid and Neu5Ac (*N*-acetylneuraminic acid) is the most common form occurring in Nature [1,2]. The deaminated sialic acid KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid) is a distinct form in which the acylamino group at C-5 position of Neu5Ac is replaced with a hydroxy group (Figure 1). Even though KDN shares several properties with Neu5Ac, it has unique biological functions such as providing resistance to cleavage of ketosidic linkage by bacterial and viral sialidases, and inhibiting the elongation of polysialyl chains by serving as the terminal sugar residue [6,7]. The levels of KDN in human fetal cord red blood cells and ovarian cancer

cells are elevated and hence free KDN and glycoconjugates containing KDN may play significant roles in normal development and malignant cell development [8]. KDN was first identified in rainbow trout egg polysialoglycoprotein [6] and is recognized to have wider distribution from bacteria to mammals [6,9–15].

The biosynthesis of Neu5Ac in animals is well understood and involves three enzymatic steps: (i) phosphorylation of ManNAc (*N*-acetylmannosamine) to produce ManNAc-6-P (ManNAc 6-phosphate) [16,17], which is catalysed by a bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase [18]; (ii) condensation of ManNAc-6-P with PEP (phosphoenolpyruvate) to form Neu5Ac-9-P (Neu5Ac 9-phosphate) by Neu5Ac-9-P synthase [19] (Scheme 1); and (iii) finally, dephosphorylation of Neu5Ac-9-P to produce Neu5Ac by a specific phosphatase [20,21]. The KDN biosynthesis in animals is less well understood but is believed to involve similar enzymatic steps as in the case of Neu5Ac biosynthesis. The first evidence for the existence of a distinct KDN biosynthetic pathway came from the identification and characterization of KDN-9-P (KDN 9-phosphate) synthase activity from trout testis [15]. This discovery indicated that KDN-9-P synthase is distinct from Neu5Ac-9-P synthase and is involved in the condensation of PEP and Man-6-P (mannose 6-phosphate) to produce KDN-9-P. While Neu5Ac-9-P synthase has been isolated from several animals [19,22,23] and its gene has been cloned from human and mouse [24,25], no distinct KDN-9-P synthase gene has been cloned to date. However, the expression

Abbreviations used: AFP, antifreeze protein; IPTG, isopropyl β-D-thiogalactoside; KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid; KDN-9-P, KDN 9-phosphate; Man-6-P, mannose 6-phosphate; ManNAc, *N*-acetylmannosamine; ManNAc-6-P, ManNAc 6-phosphate; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac-9-P, Neu5Ac 9-phosphate; PEP, phosphoenolpyruvate; TBA, thiobarbituric acid; TIM, triosephosphate isomerase.

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Figure 1 Molecular structures of Neu5Ac and KDN

of human Neu5Ac-9-P synthase gene has been shown to produce both Neu5Ac and KDN *in vivo* in insect cells [24]. This bifunctional character is not displayed by the respective mouse and rat enzymes although Neu5Ac-9-P synthases from human and both mouse [25] and rat (GenBank® accession number XM-216398) show over 95% sequence identity. Thus the human enzyme is expected to contain unique amino acid residues that confer the additional function. In the present study, we identified Met⁴² in human Neu5Ac-9-P synthase essential for the secondary KDN-9-P synthase activity using site-directed mutagenesis. Additionally, saturation mutagenesis approach was used to identify other amino acid substitutions in human Neu5Ac-9-P synthase that can retain or enhance the bifunctional nature of the enzyme utilizing both ManNAc-6-P and Man-6-P sugars as substrates.

MATERIALS AND METHODS

Materials

Taq DNA polymerase was obtained from Applied Biosciences (Branchburg, NJ, U.S.A.). Oligonucleotides were from the Operon Biotechnologies (Alameda, CA, U.S.A.). The restriction endonucleases and T₄ DNA ligase were from New England Biolabs (Beverly, MA, U.S.A.), QuikChange® mutagenesis kit was purchased from Stratagene (La Jolla, CA, U.S.A.) and DNA extraction kit (QIA, Mini-preps) was from Qiagen (Valencia, CA, U.S.A.). Chelating Sepharose metal affinity resin was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). ManNAc-6-P was synthesized according to a published procedure [26]. Man-6-P, PEP, and all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Bacterial strains and plasmid

E. coli BL21 (DE3) Gold cells were from Stratagene and Epicurian Coli™ XL1-Blue supercompetent cells were from Invitrogen (Carlsbad, CA, U.S.A.). The expression vector pET15b was purchased from Novagen (Madison, WI, U.S.A.).

Protein expression and purification

Cloning, expression and purification of the wild-type human Neu5Ac-9-P synthase has been described elsewhere [27]. Briefly, the full-length gene encoding Neu5Ac-9-P synthase was cloned from adult human brain cDNA library and fused in pET15b expression vector (pET15/hSAS) for purification. The recombinant protein was expressed by growing *E. coli* BL21 cells harbouring pET15/hSAS plasmid at 37 °C to A₆₀₀ (absorbance) of 0.7 and inducing with 0.3 mM IPTG (isopropyl β-D-thiogalactoside) at 25 °C for 20 h. The expressed protein was purified using Ni²⁺ affinity column at 4 °C and stored in 20 mM Tris/HCl buffer (pH 7.9) containing 10 mM Mg²⁺ and 100 mM NaCl at –80 °C.

Enzyme assays

Activity of human Neu5Ac-9-P synthase was assayed using TBA (thiobarbituric acid) assay first developed by Warren [28] and modified by Chen et al. [23]. Reaction mixtures contained 50 mM bicine (pH 7.5), 12.5 mM MgCl₂, 8.3 mM PEP, 3.3 mM Man-6-P or ManNAc-6-P, and an aliquot of enzyme in a final volume of 125 μl. The reactions were carried out at 37 °C and stopped by heating at 100 °C for 3 min. After centrifugation for 3 min at 17900 g, 137 μl of periodic acid solution (2.5 mg/ml in 57 mM H₂SO₄) was added and incubated for 15 min at 37 °C. Then 50 μl of sodium arsenite solution (25 mg/ml in 0.5 M HCl) was added, and the tubes were shaken vigorously to ensure complete elimination of the yellow–brown colour. After this step, 100 μl of 2-TBA solution (71 mg/ml adjusted to pH 9.0 with NaOH) was added, and the samples were heated to 100 °C for 7.5 min. The solution was extracted with 1 ml of n-butanol containing 5% (v/v) 2 M HCl, and the phases were separated by centrifugation. The absorbance of the organic phase was measured at 549 nm and the molar absorption coefficient (ε) of 57000 M^{–1}·cm^{–1} at 549 nm was used [28].

Scheme 1 Reactions catalysed by human Neu5Ac-9-P synthase

In the first reaction (top), Neu5Ac-9-P synthase catalyses the condensation of ManNAc-6-P and PEP, and in the second reaction (bottom), the enzyme catalyses the condensation of Man-6-P with PEP. Both reactions require a bivalent metal Mg²⁺.

Table 1 Primers used in PCR for the introduction of mutations in human Neu5Ac-9-P synthase gene

The positions where mutations were introduced are shown underlined.

Mutation	Primer sequence
R96K/R100S	5'-CATGACCAGTACAAGGAGCTGCAGAGCTACGCCGAGG-3'
E103Q/V105I	5' CAGAGCTACGCCAGGAGATTGGGATCTTCTTC-3'
V195A	5'-CCTGAGGACGCCAACCTGCGG
M42T	5'-CATGATCCGCACGCCCAAGGAG-3'
M42X	5'-CATGATCCGCNNKGCCCAAGGAG-3' (N = A, T, G or C, and K = G or T)

Homology modelling

Sequences of four bacterial Neu5Ac synthases (*E. coli* K1, *Streptococcus agalactiae*, *N. meningitidis* and *Campylobacter jejuni*) and three animal Neu5Ac-9-P synthases (human, mouse and rat) were aligned using AMPS [29]. The human Neu5Ac-9-P synthase has a sequence identity of 28 % with Neu5Ac synthase of *N. meningitidis* for which crystal structure is available [30]. A homology model of human Neu5Ac-9-P synthase dimer was built using the crystal structure of *N. meningitidis* Neu5Ac synthase (PDB accession code: 1XUZ) and the comparative modelling program MODELLER [31] using default parameters. The model was checked with PROCHECK [32] for stereochemistry.

Site-directed and saturation mutagenesis of human Neu5Ac-9-P synthase

The following mutations were introduced progressively in the human Neu5Ac-9-P synthase expression plasmid pET15b/hsas using the QuikChange[®] site-directed mutagenesis kit (Stratagene): R96K/R100S, R96K/R100S/E103Q/V105I, M42T/R96K/R100S/E103Q/V105I, R96K/R100S/E103Q/V105I/V195A and M42T. Typically, 50 µl PCR was carried out with approx. 80 ng of template, 100 pM primer pair, 200 µM dNTPs and 2 units of Pfu Turbo DNA polymerase. Amplification was initiated at 94 °C for 1 min, 16 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 7 min. The PCR products were treated with restriction enzyme DpnI before an aliquot was transformed into Epicurian Coli[™] XL1-Blue supercompetent cells. Saturation mutagenesis at amino acid position 42 of human Neu5Ac-9-P synthase was also carried out in a similar way using the QuikChange[®] mutagenesis kit. Primers used in PCRs for both site-directed mutagenesis and saturation mutagenesis are given in Table 1.

Screening the library generated by saturation mutagenesis

Individual colonies from the library generated by saturation mutagenesis were picked and grown in 1 ml cultures of LB (Luria-Bertani) media with 100 µg/ml ampicillin at 37 °C until the A_{600} reached approx. 0.7. The protein expression was induced by adding 0.3 mM IPTG at 25 °C for 20 h. Cells were harvested by centrifugation, and lysed in lysozyme buffer (50 mM bicine, pH 7.5, containing 1 mg/ml lysozyme, 100 mM NaCl and 10 mM MgCl₂) by freeze/thaw cycle. Cell debris was removed by centrifugation and 20 µl of supernatant of each culture was transferred to fresh 96-well microtitre plates containing 40 µl of assay buffer (12.5 mM MgCl₂, 6.9 mM PEP and 2.7 mM sugar substrates, Man-6-P or ManNAc-6-P, in 50 mM bicine, pH 7.5) in each well for enzyme activity assay. After the plates were incubated at 37 °C for 30 min, 50 µl of periodic acid solution (2.5 mg/ml in 57 mM H₂SO₄) was added to each well and the plates were incubated for an additional 15 min at 37 °C. Then 25 µl of sodium arsenite solution (25 mg/ml in 0.5 M HCl) was added to the wells

and the plates were sealed and shaken until the yellow-brown colour had completely disappeared. Finally, 50 µl of 2-TBA solution (71 mg/ml adjusted to pH 9.0 with NaOH) was added to the mixtures in each well, and the samples were heated to 95 °C for 7.5 min in the water bath. The potential positives showing strong pink colour for Man-6-P and/or ManNAc-6-P substrate(s) were further verified using standard TBA assay.

RESULTS

Sequence alignment and homology modelling

Amino acid sequences of four bacterial (*C. jejuni*, *E. coli* K1, *N. meningitidis* and *Strep. agalactiae*) Neu5Ac synthases and three animal (human, mouse and rat) Neu5Ac-9-P synthases were aligned (Figure 2). The animal sequences are 95–97 % identical between themselves and share 25–30 % identity with bacterial sequences (results not shown). The Neu5Ac-9-P synthase sequences of human and mouse differ by 19 amino acid residues and the difference between human enzyme and putative rat Neu5Ac-9-P synthase sequences is 18 residues. The human enzyme sequence differs by 15 residues compared with combined mouse and rat sequences. Homology model of human Neu5Ac-9-P synthase was built using the crystal structure of *N. meningitidis* Neu5Ac synthase as the template. The human protein is assumed to be a domain-swapped dimer similar to the *N. meningitidis* enzyme and a dimer model of the human protein was generated using homology modelling (Figure 3). Each monomer contains a large TIM (triosephosphate isomerase) barrel domain and a small AFP (antifreeze protein) domain linked by a small loop in the model. All 15 amino acids in the human Neu5Ac-9-P synthase that are different from both mouse and rat Neu5Ac-9-P synthase sequences map on the surface in the homology model and are far removed from the substrate-binding site. Among these 15 residues, six are located in the TIM barrel domain where the active site is located and the remaining nine amino acids are in the AFP domain. We, therefore, assumed that the six residues in the TIM barrel domain are more likely to contribute to the KDN-9-P synthase activity in human Neu5Ac-9-P synthase.

Site-directed mutagenesis to identify crucial residues for KDN-9-P synthase activity

Four of the six amino acid differences found in the TIM barrel domain of animal sequences are clustered in a small region from Arg⁹⁶ to Val¹⁰⁵. Therefore we first changed Arg⁹⁶ and Arg¹⁰⁰ in the human sequence to the corresponding residues in mouse/rat enzymes, lysine and serine respectively. The mutant enzyme was expressed in *E. coli* and the cytosolic supernatant obtained after centrifugation of the crude lysate was used for enzyme assays. The enzyme assays were performed for the double mutant R96K/R100S and the wild-type enzyme was used as the positive control. The mutant shows significant activities for both ManNAc-6-P and Man-6-P substrates, indicating that neither Arg⁹⁶ nor Arg¹⁰⁰ is responsible for the KDN-9-P synthase activity in the human Neu5Ac-9-P synthase (Figure 4). A quadruple mutant R96K/R100S/E103Q/V105I was constructed by mutating two more residues in the same region, Glu¹⁰³ and Val¹⁰⁵, on the template of the previous double mutant R96K/R100S. Enzyme assays of the quadruple mutant also showed significant KDN-9-P synthase and Neu5Ac-9-P synthase activities. A fifth mutation M42T in a different region of the TIM barrel was introduced in the quadruple mutant, which resulted in the complete loss of KDN-9-P synthase activity (Figure 4). However, this quintuple mutant M42T/R96K/R100S/E103Q/V105I retained full Neu5Ac-9-P synthase activity. This suggests that a single residue, Met⁴²,

Figure 2 Sequence alignment

Sequences of four bacterial (*C. jejuni*, *E. coli* K1, *N. meningitidis* and *Strep. agalactiae*) Neu5Ac synthases were aligned with three animal (human, mouse and rat) Neu5Ac-9-P synthases. All three animal sequences and only one bacterial sequence from *N. meningitidis* whose crystal structure was used for homology modelling are shown. Identical amino acids in all four sequences are shaded in light grey and those that are identical only in three animal sequences are shaded in dark grey. The human sequence differs at 15 positions compared with mouse and rat sequences combined and the differences are shown as white characters in the dark background.

in human Neu5Ac-9-P synthase might be essential for KDN-9-P synthase activity. The single mutant M42T, therefore, was generated and the activity assays of the expressed protein confirmed that it did eliminate KDN-9-P synthase activity while retaining significant Neu5Ac-9-P synthase activity (Figure 4). Another quintuple mutant R96K/R100S/E103Q/V105I/V195A was also generated and the activity assays showed that it had significant levels of both KDN-9-P synthase and Neu5Ac-9-P synthase activities. These results together strongly suggest that among the six amino acid differences in the TIM barrel domain, only Met⁴² is essential for the bifunctional nature of the human Neu5Ac-9-P synthase.

Saturation mutagenesis of Met⁴²

Next we constructed a library of M42X mutants by saturation mutagenesis to systematically analyse the effect of each of the 20 proteinogenic amino acids on the bifunctional character of the human enzyme. SDS/PAGE analysis of 20 randomly picked colonies from this library showed that all of them overexpressed proteins of similar levels and sizes corresponding to wild-type Neu5Ac-9-P synthase. Additionally, five of the clones were sequenced and corresponding residues Ser, Arg, Leu, Ile and Met were found at position 42, indicating that saturation mutagenesis was successful. Statistically, screening 150 clones from a specific position library by saturation mutagenesis will sample all 20 possible amino acid substitutions with 90% confidence [33]. Crude

lysates of approx. 160 variants from this library were screened to identify positive clones that can utilize sugar substrates Man-6-P as well as negative clones that do not catalyse the condensation of physiological substrate, ManNAc-6-P and PEP. More than 90% of the clones showed negative results in the KDN-9-P synthase activity screening and only those showing positive results were sequenced. The positive clones have either the original residue methionine or substituted with leucine at position 42. All clones except two showed positive results in the screening when ManNAc-6-P was used as the sugar substrate. DNA sequencing indicated that stop codon was introduced at position 42 in these two negative clones and hence the protein did not express.

Steady-state kinetics of the wild-type and mutant enzymes

For kinetic characterization, the wild-type enzyme and the mutants M42T and M42L of human Neu5Ac-9-P synthase were expressed in *E. coli* and purified to homogeneity using Ni²⁺ chelating Sepharose column. Steady-state kinetic experiments were performed for both mutants and their kinetic constants were determined for both ManNAc-6-P and Man-6-P substrates (Table 2). The k_{cat}/K_m of the wild-type enzyme for ManNAc-6-P is approx. 5-fold higher than that for Man-6-P and this result is consistent with the previous competitive study of crude lysates showing that the enzyme significantly favours ManNAc-6-P over Man-6-P [24]. The mutant M42T shows a similar k_{cat} value to the wild-type enzyme for sugar ManNAc-6-P with a slight increase

Figure 3 Homology model of human Neu5Ac-9-P synthase dimer (A) and close-up view of helix-loop contact (B)

(A) The model was built using the sequence alignment in Figure 2 and the crystal structure of *N. meningitidis* Neu5Ac synthase as the template. The human enzyme sequence is longer than the *N. meningitidis* enzyme sequence by ten residues. The extra seven residue C-terminal fragment is removed from the modelling as the bacterial sequence is shorter and there is no model for this fragment. Residues that are different from mouse/rat sequences are highlighted in TIM barrel domain of monomer 1 (light grey) and AFP domain of monomer 2 (dark grey). The two conserved metal-binding histidine residues His²¹⁷ and His²³⁸ are also shown as a reference to the active site pocket. Met⁴², which confers the second function, KDN-9-P synthase activity, is identified. (B) The helix containing Met⁴² contacts the loop that extends from the β -strand containing a metal ligand His²³⁸.

in K_m . The catalytic efficiency, k_{cat}/K_m , of the newly identified bifunctional mutant M42L is approximately half the value of the wild-type enzyme for ManNAc-6-P substrate. However, k_{cat}/K_m of both the wild-type and mutant M42L enzymes are about the same when sugar Man-6-P was used as the substrate.

DISCUSSION

In the present study, we have identified that Met⁴² of the human Neu5Ac-9-P synthase is essential for its KDN-9-P synthase activity. Mutation of Met⁴² to threonine, a residue found in the corresponding position of the mouse and rat sequences, can eliminate the KDN-9-P synthase activity in the human Neu5Ac-9-P synthase without the loss of Neu5Ac-9-P synthase activity. Saturation mutagenesis of Met⁴² showed that the enzyme can exhibit both Neu5Ac-9-P synthase and KDN-9-P synthase activities with only methionine or leucine residue at this site. In the

screening with ManNAc-6-P substrate, all the variants showed enzyme activity comparable with that of the wild-type enzyme, suggesting that the residue at position 42 does not play any role in the condensation of PEP and ManNAc-6-P. By examining the amino acid sequences of Neu5Ac-9-P synthases of other species, we found out that Met⁴² is highly conserved among chicken, frog and fish, and that only some mammals, mouse, rat and dog, contain Thr⁴² instead of Met⁴². Therefore it is likely that a common progenitor of rodents and dogs lost the ability to synthesize KDN-9-P by Neu5Ac-9-P synthase. Because KDN is still found in these species, it can be hypothesized that this event occurred simultaneously with the evolution of a new KDN-9-P-synthesizing enzyme, which has not been identified so far.

In the homology model of human Neu5Ac-9-P synthase, Met⁴² is located in the middle of a helix on the surface of the TIM barrel domain and is not a part of the dimer interface (Figure 3A). The residue corresponding to Met⁴² is alanine in *N. meningitidis* enzyme which is approx. 22 Å away from the N-acetyl group

Figure 4 Enzyme activities of human Neu5Ac-9-P synthase mutants

Wild-type enzyme Neu5Ac-9-P synthase activity is used as a reference to normalize the activities of the mutants. The Neu5Ac-9-P synthase activity is shown as black bars and the KDN-9-P synthase activity is shown as grey bars. (A) R96K/R100S; (B) R96K/R100S/E103Q/V105I; (C) M42T/R96K/R100S/E103Q/V105I; (D) R96K/R100S/E103Q/V105I/V195A; (E) M42T.

Table 2 Steady-state kinetics of the wild-type human Neu5Ac-9-P synthase and the mutants

The steady-state kinetic parameters of the wild-type and mutant enzymes were measured in 50 mM bicine (pH 7.5) buffer supplemented with 12.5 mM MgCl₂ using a TBA assay [28]. The parameters were calculated from initial velocities measured by varying the sugar concentrations at saturation level of PEP (8.3 mM). ND, not detectable.

Neu5Ac-9-P synthase	ManNAc-6-P			Man-6-P		
	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)
Wild-type	1.31	1.04	20.99	0.67	2.62	4.26
M42T	0.77	1.26	8.8	ND	ND	
M42L	0.93	1.72	9.01	0.5	2.55	3.27

of the rManNAc (reduced ManNAc) [30]. These observations suggest that a residue far away from the active site or dimer interface influences the secondary function, KDN-9-P synthase activity of human Neu5Ac-9-P synthase, without affecting its primary function. Significance of the residues farther from the active site on enzyme activity is increasingly evident in recent directed evolution studies [34–38]. For example, in vanillyl-alcohol oxidase, I238T mutation improved enzyme activity by 4.2-fold. This residue is located at 33 Å from the FAD-binding site and the 2.55 Å resolution of X-ray structure of I238T did not reveal discernible structural changes that could account for the difference in the enzyme activity [38]. All these observations imply that enzymes have at least two approaches to exert their functions, one is through chemical properties of side chains of active site residues and the other is through long range effects such as hydrogen bond network and electrostatic interactions such that the distant mutations can propagate subtle changes to the active site. In this context, amino acids can be viewed as ‘molecular shims’ and the larger the number of shim sizes introduced at a particular position, the higher chance that some optimal substrate-binding geometry will be approached [39]. It is possible that a network of hydrophobic interactions promote this effect, because in the case of Neu5Ac-9-P synthase, exchange of the hydrophobic methionine by hydrophobic leucine maintains both functions of the enzyme. The helix containing Met⁴² makes contact with the loop extending from the β -strand containing a metal ligand His²³⁸

(the actual distance between Met⁴² and His²³⁸ is ~12 Å) (Figure 3B). The enzyme requires a bivalent metal cofactor for both Neu5Ac-9-P and KDN activities. It should be borne in mind that the human Neu5Ac-9-P synthase shares only 28 % sequence identity with *N. meningitidis* Neu5Ac synthase and the exact location of Met⁴² is difficult to ascertain. However, given that the conserved residues are distributed throughout the alignment and Met⁴² is in the middle of the helix, its shift by one or two residues will still keep it as a part of the helix that is proximal to the loop close to metal-binding histidine. Thus this helix-loop packing, influenced by the type of amino acid at position 42, might determine the substrate selectivity. The picture will be clearer when the crystal structure of human Neu5Ac-9-P synthase becomes available, and efforts to solve the structure are under way in our laboratory.

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