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Pasteurella multocida sialic acid aldolase: a promising biocatalyst

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Abstract

Sialic acid aldolases or *N*-acetylneuraminate lyases (NanAs) catalyze the reversible aldol cleavage of *N*-acetylneuraminic acid (Neu5Ac) to form pyruvate and *N*-acetyl-D-mannosamine (ManNAc). A capillary electrophoresis (CE) assay was developed to directly characterize the activities of NanAs in both Neu5Ac cleavage and Neu5Ac synthesis directions. The assay was used to obtain the pH profile and the kinetic data of a NanA cloned from *Pasteurella multocida* P-1059 (PmNanA) and a previously reported recombinant *Escherichia coli* K12 NanA (EcNanA). Both enzymes are active in a broad pH range of 6.0–9.0 in both reaction directions and have similar kinetic parameters. Substrates specificity studies showed that 5-*O*-methyl-ManNAc, a ManNAc derivative, can be used efficiently as a substrate by PmNanA, but not efficiently by EcNanA, for the synthesis of 8-*O*-methyl Neu5Ac. In addition, PmNanA (250 mg per liter culture) has a higher expression level (2.5 fold) than EcNanA (94 mg per liter culture). The higher expression level and a broader substrate tolerance make PmNanA a better catalyst than EcNanA for the chemoenzymatic synthesis of sialic acids and their derivatives.

Keywords

aldolase; capillary electrophoresis; *Escherichia coli*; lyase; NanA; *Pasteurella multocida*

Introduction

Sialic acid aldolases or *N*-acetylneuraminate lyases (EC 4.1.3.3, NanA) are type I aldolases that catalyze the reversible aldol cleavage of *N*-acetylneuraminic acid (Neu5Ac) to form pyruvate and *N*-acetyl-D-mannosamine (ManNAc) with the equilibrium favoring the Neu5Ac cleavage (Reaction 1) (Barbosa et al. 2000; Comb and Roseman 1960; Deijl and Vliegenthart 1983; Wong and Whitesides 1994).

$$\text{Neu5Ac} \rightleftarrows \text{ManNAc} + \text{pyruvate} \qquad \text{Reaction 1}$$

NanA is important for sialic acid catabolism and has been found in mammalian and some pathogenic and non-pathogenic bacteria (Aisaka et al. 1991). It regulates the intracellular sialic acid concentration in mammalian cells and in *E. coli* K12 (Schauer 1982; Traving et al. 1997; Vimr and Troy 1985). By coupling with lactate dehydrogenase (Donald and Comb 1962; Paolo Brunetti and Roseman 1963) or pyruvate oxidase (Sugahara et al. 1980), NanA has been used for determining sialic acid concentrations (Ohta et al. 1985; Sugahara et al. 1980). It can also be used with sialidase to determine the total amount of sialic acid in glycoproteins, glycolipids, polysialic acids, and cell surfaces (Kolisis 1986). Synthetically, NanA has been broadly used in the aldol addition direction for the synthesis of Neu5Ac and

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its analogs (Huang et al. 2007; Wong and Whitesides 1994; Yu and Chen 2006; Yu et al. 2004).

NanA activity is generally characterized in the Neu5Ac cleavage direction by measuring the amount of pyruvate formed using lactate dehydrogenase and the reduced form of β-nicotinamide adenine dinucleotide (NADH) which can be spectrophotometrically quantified at A_{340nm} (Donald and Comb 1962; Paolo Brunetti and Roseman 1963). The pyruvate formed can also be measured using pyruvate oxidase to produce hydrogen peroxide which is quantified colorimetrically (Sugahara et al. 1980). Alternatively, the ManNAc formed can be quantified after fluorescent labeling via reductive amination (Chen et al. 1998). The sialic acid formed in the Neu5Ac synthesis direction has been analyzed using periodate-thiobarbituric acid, radioactive, and colorimetric resorcinol-hydrochloric acid methods (Ferrero et al. 1996; Pan et al. 2004). All of these assays are indirect and require chemical labeling or chemical/enzymatic conversion of the product for quantification.

NanA has been cloned from *E. coli* (Aisaka and Uwajima 1986; Ohta et al. 1986; Ohta et al. 1985), *Clostridium perfringens* A99 (Traving et al. 1997), *Haemophilus influenzae* (HI0142) (Lilley et al. 1998), and *Trichomonas vaginalis* (Meysick et al. 1996). The X-ray structures of NanA from *E. coli* and *Haemophilus influenzae* have been solved (Barbosa et al. 2000; Izard et al. 1994; Lawrence et al. 1997). NanA activity has been found in *Pasteurella multocida* (Drzeniek et al. 1972) and a hypothetic gene sequence encoding NanA (Pm1715, GenBank accession no. Q9CKB0) has been identified (Steenbergen et al. 2005). Previously, we reported the cloning of a NanA from *E. coli* K-12 substrain MG1655 (EcNanA) (Yu et al. 2004). The EcNanA has been used in one-pot multiple-enzyme systems to produce CMP-sialic acids, sialosides, and their analogs (Yu et al. 2005; Yu et al. 2006; Yu et al. 2004). It has also been used in the synthesis of disaccharides containing a sialic acid at the reducing end (Huang et al. 2007; Yu and Chen 2006). Here, we report the cloning and the detailed characterization of NanA from *Pasteurella multocida* P-1059 (ATCC#15742) (PmNanA) by a capillary electrophoresis (CE) assay which allows a direct analysis of the enzyme in both Neu5Ac cleavage and synthesis directions. The pH profile, kinetic parameters, and substrate specificity of PmNanA and EcNanA obtained by the CE assays are compared.

Materials and methods

Bacterial strains, plasmids, and materials

E. coli electrocompetent DH5α and chemically competent BL21 (DE3) cells were from Invitrogen (Carlsbad, CA). *P. multocida* P-1059 (ATCC#15742) was from American Type Culture Collection (ATCC, Manassas, VA). Vector plasmid pET22b(+) was from Novagen (EMD Biosciences Inc. Madison, WI). Ni^{2+}-NTA agarose (nickel–nitrilotriacetic acid agarose), QIAprep spin miniprep kit, and QIAEX II gel extraction kit were from Qiagen (Valencia, CA). Herculase-enhanced DNA polymerase was from Stratagene (La Jolla, CA). T4 DNA ligase and 1 kb DNA ladder were from Promega (Madison, WI). *Nde*I and *Xho*I restriction enzymes were from New England Biolabs Inc. (Beverly, MA). ManNAc and pyruvate were from Sigma (St. Louis, MO). C-His_6-tagged EcNanA was cloned previously (Yu et al. 2004). ManNAc/mannose derivatives were synthesized according to reported procedures (Augé et al. 1988; Yu et al. 2005;Yu et al. 2006). NMR spectra were recorded on a Varian Inova-600 spectrometer. Chemical shifts are reported in ppm using ^{13}C and residual ^{1}H signals from deuterated solvents as references.

Cloning

PmNanA was cloned as a C-His_6-tagged fusion protein in pET22b(+) vector using genomic DNA of *Pasteurella multocida* P-1059 (ATCC#15742) as the template for polymerase chain

reactions (PCR). The primers used were: forward primer 5′-GATCCATATGAAAAACTTAAAAGGTATCTTCAG-3′ (*Nde*I restriction site is underlined) and reverse primer 5′-CCGCTCGAGAGAAAGGTATTTGGCTTT TAATTCTTTC-3′(*Xho*I restriction site is underlined). PCR was performed in a 50 μL reaction mixture containing genomic DNA (1 μg), forward and reverse primers (1 μM each), 10 × Herculase buffer (5 μL), dNTP mixture (1 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification with an annealing temperature of 55 °C. The resulting PCR product was purified and digested with *Nde*I and *Xho*I restriction enzymes. The purified and digested PCR product was ligated with predigested pET22b(+) vector and transformed into electrocompetent *E. coli* DH5αcells. Selected clones were grown for minipreps and characterization by restriction mapping and DNA sequencing performed by Davis Sequencing Facility at the University of California-Davis.

Expression and purification

His_6-tagged PmNanA was expressed and purified from cell lysate using an ÄKTA FPLC system (GE Healthcare) equipped with a HisTrap_FF 5 mL column as described previously (Sun et al. 2007).

Quantification of purified protein

Protein concentration was determined in a 96-well plate using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a protein standard. The absorbance of each sample was measured at 562 nm by a BioTek Synergy™ HT Multi-Mode Microplate Reader.

pH Profile by capillary electrophoresis (CE)

Typical enzymatic assays were performed in duplicate in a total volume of 20 μL in a buffer with pH varying from 5.0–11.0. For Neu5Ac cleavage activity, the assays were performed in a buffer (100 mM) containing Neu5Ac (10 mM) and the recombinant enzymes (0.3 μg). For Neu5Ac synthesis activity, a buffer (200 mM) containing ManNAc (10 mM), Pyruvate (100 mM), and the recombinant enzymes (4 μg) was used. The buffers used were: Na_2HPO_4-KH_2PO_4, pH 5.0–8.0; Tris-HCl, pH 8.5; Na_2CO_3-$NaHCO_3$, pH 9.0–10.0; and Na_2HPO_4-NaOH, pH 11.0. Reactions were allowed to proceed for 15 min at 37 °C and quenched by adding HCl (1 M) to change the pH to 2–3. The samples were analyzed by a P/ACE™Capillary Electrophoresis system equipped with a UV detector (Beckman Coulter, Inc., Fullerton, CA). CE conditions were as follows: 75 μm i.d. capillary, 25 KV/80 μÅ, 5 s vacuum injections, monitored at 200 nm, running buffer: 25 mM sodium tetraborate, pH 9.4.

Kinetics by CE assays

The enzymatic assays were carried out in duplicate in a total volume of 20 μL in Na_2HPO_4-KH_2PO_4 buffer (100 mM, pH 7.5). Reactions were allowed to proceed for 15 min at 37 °C. For the Neu5Ac cleavage activity, 100 ng of enzyme and varied concentrations (0.2, 0.25, 0.4, 1, 2, 4, 5, 10, and 20 mM) of Neu5Ac were used. For the Neu5Ac synthesis activity assay, 2 μg of enzyme, a fixed concentration of pyruvate (20 mM) and varied concentrations (0.4, 0.5, 1, 2, 4, 5, 10, 20, 40, and 50 mM) of ManNAc; or a fixed concentration of ManNAc (20 mM) and varied concentrations (0.4, 0.5, 1, 2, 4, 5, 10, 20, 40, and 50 mM) of pyruvate were used. Apparent kinetic parameters were obtained by fitting the averages to the Michaelis-Menten equation using Grafit 5.0.

Substrates specificity assays

ManNAc and its derivatives (10 mM) were used as substrates with sodium pyruvate (100 mM) in Na_2HPO_4-KH_2PO_4 buffer (pH 7.5, 200 mM) to analyze the substrate specificity of the

Neu5Ac synthesis activity of PmNanA and EcNanA (4 μg). Reactions were carried out in duplicate and allowed to proceed for 5 min at 37 °C.

Preparative synthesis of *N*-acetyl-8-*O*-methyl-neuraminate (Neu5Ac8OMe)

The reaction was carried out in a 50 mL centrifuge tube in 20 mL Tris-HCl buffer (100 mM, pH 7.5) containing ManNAc5OMe (100 mg), sodium pyruvate (5 equiv.), and PmNanA (2 mg) by incubating at 37 °C for 12 h with agitation (140 rpm). The product formation was monitored by thin-layer chromatography (TLC) with *i*-PrOH:H_2O:HOAc = 7:3:1.5 (by volume) as the developing solvent and stained with *p*-anisaldehyde sugar stain. When no additional product formation was observed, the reaction was quenched by adding an equal volume of cold 95 % EtOH and incubating on ice for 30 min to precipitate the protein followed by centrifugation to remove the precipitates. The supernatant was concentrated by rotary evaporation and the product was purified by a Bio-Gel P-2 gel filtration column.

Results

Cloning, expression, and purification of PmNanA

NanA from *Pasteurella multocida* strain P-1059 (PmNanA) was cloned as a C-His_6-tagged recombinant protein in a pET22b(+) vector. Compared to the *Pm1715* gene sequence reported for *Pasteurella multocida* genomic strain PM70 (GenBank accession no. Q9CKB0), the DNA sequence of the cloned gene differs on six bases: T228C, T303C, C624T, G777A, C786T, and C832T (the numbers indicate the positions in the gene; the bases listed after the numbers are the ones in the strain P-1059). The last base difference leads to an amino acid difference P278S (the number indicates the position in the protein; the amino acid listed after the number is the one in the strain P-1059). This strain-based sequence variation is similar to that observed for a multifunctional sialyltransferase from *Pasteurella multocida* (Yu et al. 2005). Sequence alignment indicates that the cloned PmNanA has 90%, 80%, 70%, and 37% amino acid sequence identity, respectively, with the NanAs from *Haemophilus influenzae* (HI0142), *Trichomonas vaginalis*, *Clostridium perfringens*, and *E. coli* K-12 (GenBank accession nos. P44539, AAB42182, Q9S4K9, and AAC76257).

The optimal expression condition for PmNanA was incubation at 20 °C for 24 h with vigorous shaking (250 rpm) after induction with 0.1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG). Up to 250 mg of Ni^{2+}-column purified PmNanA could be obtained from one liter of *E. coli* culture. Sialic acid aldolase from *E. coli* K12 (EcNanA) was successfully expressed and purified as reported previously (Yu et al. 2004). Compared to PmNanA, the expression level of EcNanA (94 mg L^{-1} cell culture) was about 2.5-fold lower. As a negative control, *E. coli* BL21(DE3) cells transformed with vector plasmid pET22b(+) did not exhibit any sialic acid aldolase activity in Ni^{2+}-column purified fractions. The expression profiles of both PmNanA and EcNanA were analyzed by 12% SDS-PAGE as shown in Fig. 1A. Both purified proteins exhibited a molecular mass of about 33 kDa, matching well with the calculated masses of the translated His_6-tagged proteins of 33.4 and 33.7 kDa, respectively, for EcNanA and PmNanA.

Capillary electrophoresis (CE) assays

In order to directly characterize the NanA activities on both directions of Neu5Ac cleavage and synthesis, a CE method was developed by taking the advantage of the UV-absorbance property of Neu5Ac and pyruvate. Both Neu5Ac and pyruvate absorb UV at 200 nm and the signal response is 3 to 2 for Neu5Ac versus pyruvate as measured using solutions containing different ratios of Neu5Ac and pyruvate (data not shown).

pH Profile of PmNanA and EcNanA

As shown in Fig. 1B and 1C, both PmNanA and EcNanA are active in a broad pH range varying from pH 6.0 to 9.0 in both Neu5Ac cleavage and synthesis directions. The pH optimum of both enzymes was observed between pH 7.5 and 8.0 in both directions except that PmNanA has an optimal pH at pH 9.0 in the Neu5Ac synthesis direction when Na_2CO_3-$NaHCO_3$ was used as a buffer. The pH optima of these two enzymes are similar to that of NanA from *E. coli* K1 (Ferrero et al. 1996), the native and the recombinant NanA from *C. perfringens* (Kruger et al. 2001; Nees et al. 1976). The activities in both directions of both enzymes decline quickly when pH goes below 6.0 or above 9.0.

Kinetics of PmNanA and EcNanA

As shown in Table 1, the apparent kinetic parameters are similar for EcNanA and PmNanA in both Neu5Ac cleavage and synthesis directions. There are slight differences. For example, the K_M values of Neu5Ac and ManNAc for PmNanA (4.9±0.7 mM and 220±30 mM respectively) are higher than those of EcNanA (2.5±0.3 mM and 180±10 mM respectively); the corresponding k_{cat} values of Neu5Ac and ManNAc for PmNanA (16±1 and 11±1 s^{-1} respectively) are also slightly higher than those of EcNanA (10±0.4 and 9.0±0.4 s^{-1} respectively). Obviously, the binding between ManNAc or pyruvate and PmNanA or EcNanA is much weaker as compared to the binding of Neu5Ac and the enzymes, as the K_M values of ManNAc and pyruvate for the enzymes are 2 and 1 magnitude, respectively, larger than that of Neu5Ac. The Neu5Ac synthesis activities of both PmNanA and EcNanA are much less (two magnitudes lower in k_{cat}/K_M value) efficient compared to those of the Neu5Ac cleavage activities of the enzymes. The K_M values obtained for Neu5Ac in the Neu5Ac cleavage direction of both EcNanA (2.5±0.3 mM) and PmNanA (4.9±0.7 mM) are compatible to that reported for the NanA purified from *E. coli* (K_M = 3.6 mM or 4.8 mM) (Ferrero et al. 1996;Uchida et al. 1984) and purified (2.8 mM) or recombinant (3.2 mM) *C. perfringens* NanA (Kruger et al. 2001). The K_M values of ManNAc and pyruvate in the Neu5Ac synthesis direction of both EcNanA (180±10 mM, 22±1 mM) and PmNanA (220±30 mM and 23±1 mM) are more than 20- and 2.5-fold, respectively, than those obtained for the NanA purified from *E. coli* K1 (7.7 mM and 8.3 mM) (Ferrero et al. 1996).

Substrate specificity

Using the CE method described above, the substrate specificities of PmNanA and EcNanA were examined using pyruvate and ManNAc or its derivatives. As shown in Fig. 2, both enzymes can accept a broad array of ManNAc/mannose derivatives as substrates. Most of the ManNAc/mannose derivatives tested were equally well accepted by both enzymes. Among these, ManNCPg **7** is a worse substrate than ManNAc **1** for both PmNanA and EcNanA. Other ManNAc/mannose analogs with a substitution at C-2 or C-6 (e.g. $Man2N_3$ **2**, ManNGc **3**, ManNGcMe **4**, ManNAcF **5**, ManNGcAc **6**, $ManNAcN_3$ **8**, $ManNAc6N_3$ **9**, ManNCbzGly **10**) are similarly good or better substrates compared to ManNAc for both enzymes. Interestingly, ManNCbzGly **10** with a large benzoxycarbonylamido group linked to the *N*-acetyl of the ManNAc **1** is a better substrate than ManNAc **1** for both PmNanA and EcNanA. In addition, it can be converted to the corresponding sialic acid analog Neu5CbzGly more (at least 2-fold more) efficiently by PmNanA. Significantly, under the assay conditions described in the experimental section for the substrate specificity assays (2 μg enzyme, 37 °C, 5 min), a C-5 derivative of ManNAc, ManNAc5OMe **11**, can be used only by PmNanA, but not EcNanA, for the synthesis of the corresponding sialic acid Neu5Ac8OMe. When 10-fold more (40 μg) EcNanA was used in a longer reaction period (1 hr), 10 % product conversion was observed. The samer product conversion can be achieved by using 2 μg of PmNanA in a 45 min-reaction. This indicates that the activity of EcNanA is at least 13 times lower than that of PmNanA when ManNAc5OMe **11** is used as the substrate.

Enzymatic synthesis and characterization of *N*-acetyl-8-*O*-methyl-neuraminate (Neu5Ac8OMe)

In order to confirm that ManNAc5OMe is indeed a substrate for PmNanA, a preparative (100 mg) scale synthesis of *N*-acetyl-8-*O*-methyl-neuraminate (Neu5Ac8OMe) was carried out using PmNanA, ManNAc5OMe, and 5 equivalent of pyruvate. The aldol addition product Neu5Ac8OMe was obtained in high yield (93 %) after gel filtration chromatographic purification. The purified product was characterized by NMR spectrometry and chemical shifts are listed in Table 2. Comparing the NMR data of β-D-Neu5Ac and Neu5Ac8OMe shows that the chemical shift of the proton at C-8 of Neu5Ac8OMe (3.85 ppm) is 0.31 ppm downfield to that in Neu5Ac, and the chemical shift of C-8 of Neu5Ac8OMe (80.12 ppm) is 9.90 ppm downfield to that observed in Neu5Ac in ^{13}C NMR spectrum. These data confirm that the *O*-methyl modification is on C-8 of Neu5Ac.

Discussion

NanA is an important enzyme that has been broadly applied for the chemoenzymatic synthesis of sialic acids and their analogs (Ferrero et al. 1996; Yu et al. 2004). The NanA activity, however, has been commonly assayed in the sialic acid cleavage direction (DeVries 1972; Nees et al. 1976). Indirect assay methods such as those involve periodate-thiobarbituric acid, radioactive, and colorimetric resorcinol-hydrochloric acid approaches have been used to characterize the sialic acid formed in the Neu5Ac synthesis direction (Ferrero et al. 1996; Pan et al. 2004). A direct assay method has been lacking. The direct capillary electrophoresis assay described here has significant advantages. Other than the capability of direct measuring the formation or the decrease of Neu5Ac in the Neu5Ac synthesis or the Neu5Ac cleavage directions respectively, the CE method has the advantages of high separation efficiency, low sample consumption, and short time of analysis. It is readily applicable for clinical research, food science, and basic research including oligosaccharide, glycoprotein, and polysaccharide analysis (Chen et al. 1998; Cheng et al. 1998; Hempel 2003; Watanabe and Terabe 2000). The data obtained by the CE method are comparable to those reported previously, confirming the reliability of the method. Nevertheless, the CE approach described here has some limitations. Since the NanA activity was characterized by measuring the absorbance of Neu5Ac and pyruvate at 200 nm, buffers that give signals in the same position or near those of Neu5Ac or pyruvate (e.g. MES buffer and HEPES buffers) can not be used under the assay conditions described. On the other hand, sialic acid analogs that do not give significant absorbance signal at 200 nm (e.g. ketodeoxynonulosonic acid or KDN) are also not suitable for the CE method.

In an attempt to find aldolases having broad substrate specificity for the synthesis of sialic acid analogs, NanA was cloned from *Pasteurella multocida* and its properties was compared to a previously reported EcNanA (Ferrero et al. 1996; Yu et al. 2004). The newly cloned PmNanA has a high expression level (250 mg per liter of culture) and is superior in tolerating substrate modifications. The PmNanA reported here, therefore, is a promising biocatalyst for biotechnology applications.

PmNanA, but not EcNanA, can be efficiently used in the chemoenzymatic synthesis of Neu5Ac8OMe, a natural occurring sialic acid that has been found in the gangliosides from starfish and the sperm and eggs of teleost fish (Varki, A. 1992). This compound is important for understanding the significance of 8-OMe modification of Neu5Ac in nature and the biological functions of sialosides containing Neu5Ac8OMe. The applicability of PmNanA in the efficient synthesis of Neu5Ac8OMe is very significant since the chemical synthesis of this compound is tedious and low yielding (Dufner et al. 2000; Khorlin and Privalova 1970), and previous aldolase-catalyzed enzymatic synthesis was either not successful (Augé et al. 1988) or only produced trace amount of product (Augé et al. 1990). Also as demonstrated in this

study, a much larger amount of the enzyme and a longer incubation time will be required to produce a similar amount of the Neu5Ac8OMe product using EcNanA.

PmNanA has 90% amino acid sequence identity with NanA from *Haemophilus influenzae* (HiNanA) and only 37% sequence identity with EcNanA. We anticipate that the three-dimensional structure of PmNanA will be closely similar to that of HiNanA. The X-ray crystal structures of HiNanA in complex with three substrate analogues (Barbosa et al. 2000) allow the identification of amino acid residues in the substrate binding picket. Only one out of nine amino acid residues in the substrate binding site (Ser47, Thr48, Tyr136, Lys164, Gly188, Asp190, Glu191, Ser207, and Tyr251) is different in PmNanA (Tyr251) and EcNanA (Phe252). In the co-crystal structure of HiNanA and Neu5Ac2ol, Tyr251 is involved in hydrogen-bonding with an ordered water molecule which is hydrogen bonded with the carbonyl oxygen of the *N*-acetyl group in substrate analog Neu5Ac2ol. It is not directly involved in the interaction of the hydroxyl group at C8 of the Neu5Ac analog and the protein. In the crystal structures of HiNanA, Asp190, Glu191, and Ser207 define the binding pocket of the hydroxyl group at C8 of Neu5Ac analog. The small difference of the spatial orientations of the corresponding residues in EcNanA (Asp191, Glu192, and Ser208) may be partially responsible for the difference of efficiency of PmNanA and EcNanA in using ManNAc5OMe as a substrate for the aldol addition reaction. We are currently carrying out crystal structure studies to better understand the structure-based differentiation of substrate specificity of PmNanA and EcNanA.

With a high expression level and a relaxed substrate specificity, the newly characterized PmNanA can be used an ideal candidate for further mutagenesis studies, such as error-prone PCR, saturation mutagenesis, site-directed mutagenesis, and directed evolution, to generate efficient biocatalysts for the synthesis of biologically important sialic acid analogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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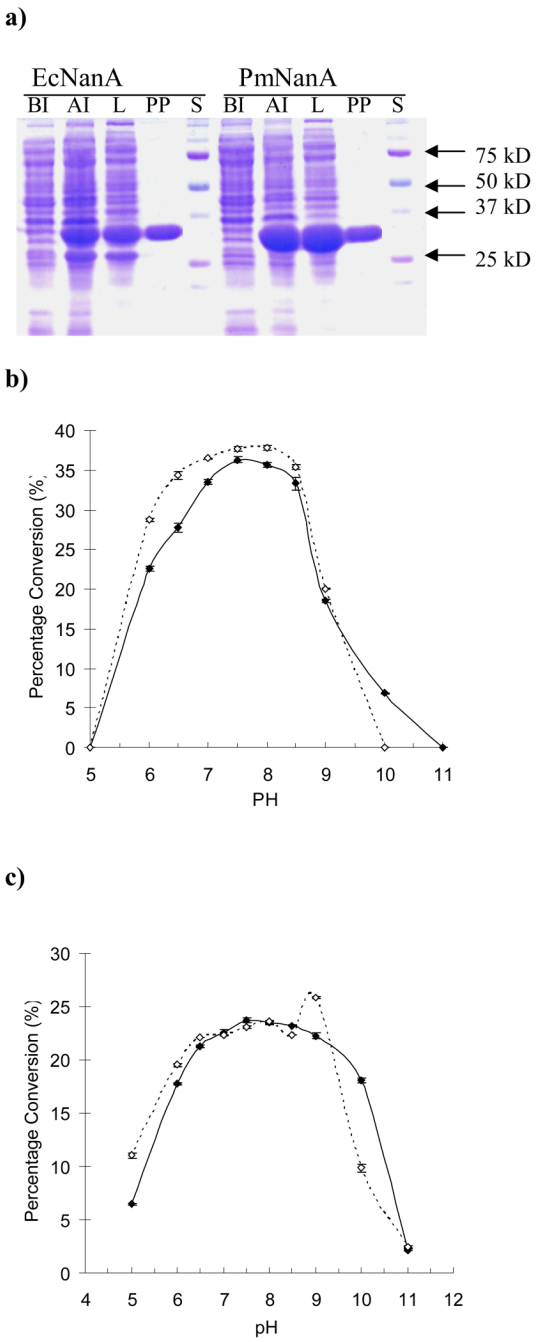

Fig. 1.

SDS-PAGE analysis and pH profiles of EcNanA and PmNanA. **a)** SDS-Page. Lanes: BI, whole cell extract before induction; AI, whole cell extract after induction; L, lysate; PP, purified protein; S, Bio-Rad Precision Plus Protein Standards (10–250 kDa). **b)** pH Profile for the Neu5Ac cleavage activity of EcNanA (◆, filled diamond) and PmNanA (◇, unfilled diamond) **c)** pH Profile for the Neu5Ac synthesis activity of EcNanA (◆, filled diamond) and PmNanA (◇, unfilled diamond). Buffers used: Na_2HPO_4-KH_2PO_4, pH 5.0–8.0; Tris-HCl, pH 8.5; Na_2CO_3-$NaHCO_3$, pH 9.0–10.0; and Na_2HPO_4-NaOH, pH 11.0.

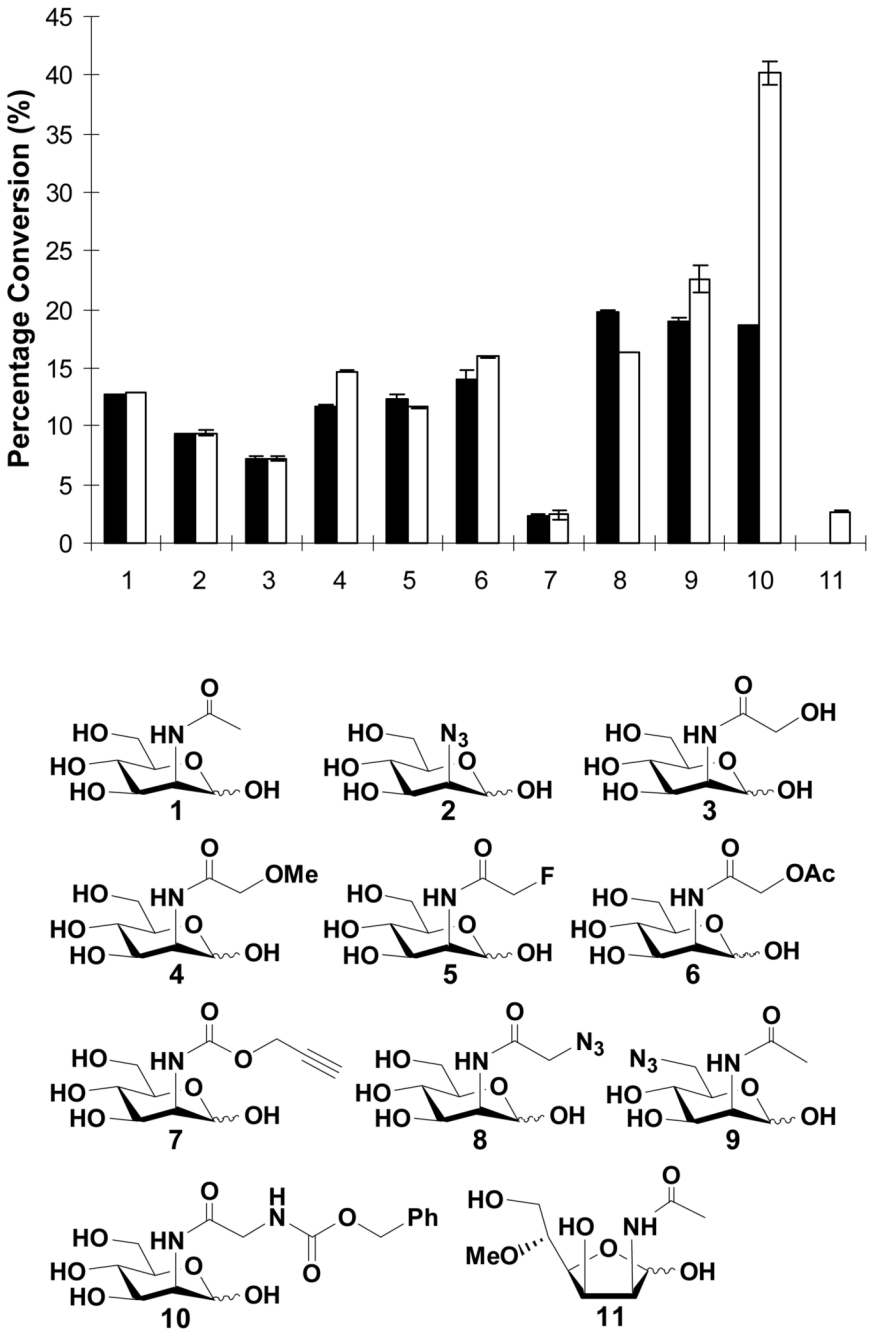

Fig. 2.

Substrate specificity assay for the Neu5Ac synthesis activity of EcNanA (black columns) and PmNanA (white columns). Compounds used: **1**, ManNAc (*N*-acetylmannosamine); **2**, Man2N_3 (2-azido-2-deoxy-D-mannose); **3**, ManNGc (*N*-glycolylmannosamine); **4**, ManNGcMe (*N*-methoxyacetyl-D-mannosamine); **5**, ManNAcF (*N*-fluoroacetyl-D-mannosamine); **6**, ManNGcAc (*N*-acetoxyacetyl-D-mannosamine); **7**, ManNCPg (*N*-propargyloxycarbonyl-D-mannosamine); **8**, ManNAcN_3 (*N*-azidoacetyl-D-mannosamine); **9**, ManNAc6N_3 (*N*-acetyl-6-azido-6-deoxy-D-mannosamine); **10**, ManNCbzGly (*N*-benzoxycarbonylamidoacetyl-D-mannosamine); **11***, ManNAc5OMe (*N*-acetyl-5-*O*-methyl-D-mannosamine). *When 10-fold more (40μg) EcNanA was used in the reaction at 37 °C for

1 hr, 10 % product conversion was observed. The same product conversion was achieved when 2 μg of PmNanA was used in a 45 min-reaction.

Table 1

Apparent kinetic parameters of EcNanA and PmNanA in the Neu5Ac cleavage and synthesis directions.

Enzymes	**EcNanA**			**PmNanA**		
Activities	**Cleavage**	**Neu5Ac synthesis**		**cleavage**	**Neu5Ac synthesis**	
Substrates	**Neu5Ac**	**ManNAc**	**Pyruvate**	**Neu5Ac**	**ManNAc**	**Pyruvate**
K_M (mM)	2.5±0.3	(1.8±0.1)×10^2	22±1	4.9±0.7	(2.2±0.3)×10^2	23±1
V_{max} (mM min^{-1})	(9.2±0.4)×10^{-2}	1.6±0.1	0.33±0.01	0.14±0.01	2.0±0.2	0.33±0.01
k_{cat} (s^{-1})	10±0.4	9.0±0.4	1.8±0.4	16±1	11±1	1.9±0.1
k_{cat}/K_M (mM^{-1} s^{-1})	4	0.05	0.08	3	0.05	0.08

Table 2

Carbon and proton chemical shifts (ppm) and assignments of Neu5Ac and Neu5Ac8OMe, the aldol addition product synthesized from ManNAc5OMe and pyruvate using PmNanA.[a]

Carbon Chemical shift (ppm)			Proton Chemical shift (ppm)		
Position	**Neu5Ac**	**Neu5Ac8OMe**	**Position**	**Neu5Ac**	**Neu5Ac8OMe**
1	173.28	174.93			
2	95.35	96.73	3_{ax}	1.67	1.68
3	38.91	39.72	3_{eq}	2.11	2.06
4	68.31	67.35	4	3.87	3.77
5	52.15	52.41	5	3.72	3.54
6	70.51	70.58	6	3.85	3.76
7	66.79	67.24	7	3.34	3.27
8	**70.22**	**80.12**	**8**	**3.54**	**3.85**
9	63.27	59.93	9_a	3.63	3.50
NHCO	174.94	176.66	9_b	3.42	3.48
(CO)$\underline{C}H_3$	22.19	22.25	(CO)C$\underline{H}_3$	1.84	1.88
C8-O$\underline{C}H_3$		57.98	C8-OC$\underline{H}_3$		3.26

[a] NMR spectra were recorded at 299 K in D_2O at neutral pH. Chemical shifts (ppm) are given relative to HOD (4.79 ppm) for ^1H NMR or the methyl resonance of the internal deuterated acetone (30.89 ppm) for ^{13}C NMR. The peaks were assigned using ^1H-^1H COSY and ^1H-^{13}C HSQC.