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Purification and characterization of *N*-acetylneuraminic acid-9-phosphate synthase from rat liver

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Sialic acids are a group of carboxylated amino sugars important for a variety of cellular functions. *N*-Acetylneuraminic acid (Neu5Ac) is the predominant sialic acid in nature. Neu5Ac-9-phosphate synthase catalyzes the formation of Neu5Ac-9-phosphate from *N*-acetylmannosamine-6-phosphate and phospho*enol*pyruvate. Neu5Ac-9-phosphate synthase was purified 11,700-fold from rat liver cytosol to apparent homogeneity by ammonium sulfate precipitation, chromatography on hydroxylapatite, phenyl-Sepharose, MonoQ, and finally gel filtration. SDS–PAGE and gel filtration chromatography indicated that the enzyme is a dimer composed of 37-kDa subunits. Analysis of trypic peptides by MALDI-TOF MS verified a high sequence similarity to the corresponding murine enzyme. The K_m values of Neu5Ac-9-phosphate synthase were 35 μM for *N*-acetylmannosamine-6-phosphate and 100 μM for phospho*enol*pyruvate. The enzyme displayed an absolute requirement for divalent cations, Mn^{2+}, Fe^{2+}, and Mg^{2+} being the most effective. In contrast to human Neu5Ac-9-phosphate synthase, the rat enzyme did not utilize mannose-6-phosphate in the synthesis of 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid 9-phosphate. Neu5Ac-9-phosphate synthase was inactivated by the sulfhydryl modifying reagents, 5,5′-dithio-bis (2-nitrobenzoic acid) and *N*-ethylmaleimide, and protected from inactivation by the presence of the substrate phospho*enol*pyruvate, but not by the presence of *N*-acetylmannosamine-6-phosphate, showing that at least one cysteine residue is located in the active site of the enzyme.

Key words: N-acetylmannosamine-6-phosphate/ *N*-acetylneuraminic acid-9-phosphate synthase/ phospho*enol*pyruvate/sialic acid

Introduction

Sialic acids are terminal nonreducing units of oligosaccharide chains of many glycoconjugates. They belong to a family of 9-carbon carboxylated sugars found in viruses, bacteria, and many higher animals (Schauer *et al.*, 1995). Their carboxyl group at carbon atom 1 is typically ionized at physiological pH. Due to their terminal localization and negative charge, sialic acids are involved in cellular adhesion (Yang *et al.*, 1994), and play important roles as recognition determinants (Varki, 1992). Sialic acid residues can also mask recognition sites (Schauer, 1985), for example, they protect serum glycoproteins *in vivo* against removal from circulation by the asialoglycoprotein receptor (Mortensen and Huseby, 1997). Overexpression of membrane-bound sialic acids is frequently correlated with the metastatic and invasive potential of tumor cells (Fukuda, 1996).

N-Acetylneuraminic acid (Neu5Ac), the predominant sialic acid in nature, is synthesized *in vivo* by a multistep cytosolic pathway. In mammals this starts with the formation of *N*-acetylmannosamine (ManNAc) from UDP-*N*-acetylglucosamine (UDP-GlcNAc) by the action of UDP-GlcNAc 2-epimerase. ManNAc is then phosphorylated at C-6 by a specific kinase. Recently it was found that these two steps are catalyzed by one bifunctional enzyme (Hinderlich *et al.*, 1997; Stäsche *et al.*, 1997). ManNAc-6-phosphate is then converted to Neu5Ac-9-phosphate by condensation with phospho*enol*pyruvate (PEP) catalyzed by Neu5Ac-9-phosphate synthase (Watson *et al.*, 1966). The phosphate is released, probably by a specific phosphatase (Jourdian *et al.*, 1964), and finally CMP-Neu5Ac, the activated Neu5Ac donor for glycolipid and glycoprotein oligosaccharide biosynthesis, is formed in the nucleus (Kean, 1970).

Neu5Ac-9-phosphate synthase was first described in hog submaxillary glands (Roseman *et al.*, 1961) and rat liver (Warren and Felsenfeld, 1961). The specifity of the enzyme for ManNAc-6-phosphate and PEP was shown for the purified hog enzyme (Watson *et al.*, 1966). Watson *et al.* (1966) and Van Rinsum *et al.* (1984) detected the enzyme in all rat tissues investigated with the highest activity in salivary glands. In contrast to the mammalian enzyme, the bacterial enzyme catalyzes the condensation of ManNAc and PEP in the synthesis of Neu5Ac (Vann *et al.*, 1997). It was also suggested that the bacterial Neu5Ac lyase synthesizes sialic acid by condensation of ManNAc and pyruvate (Ferrero *et al.*, 1995; Rodriguez-Aparicio *et al.*, 1995). Nevertheless it was possible to clone the human (Lawrence *et al.*, 2000) and murine (Nakata *et al.*, 2000) cDNA of Neu5Ac-9-phosphate synthase based on homology to the *Escherichia coli* Neu5Ac synthase gene (*neuB*). In this article we report the purification of Neu5Ac-9-phosphate synthase to homogeneity and the characterization of its biochemical properties.

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Results

Purification of Neu5Ac-9-phosphate synthase from rat liver

Neu5Ac-9-phosphate synthase was purified to homogeneity from rat liver cytosol, following the procedure outlined under *Materials and methods*. The purification scheme is summarized in Table I. The penultimate purification step, anion exchange chromatography on MonoQ, was highly effective due to an unusually weak binding of the enzyme to the column. The application buffer had a low salt concentration (10 mM sodium phosphate) and the enzyme was eluted by only 75 mM NaCl, which leaves most of the other proteins bound to the column.

Purification to homogeneity was achieved by a gel filtration chromatography. After calibration of the gel filtration column with standard proteins, the estimated molecular mass of Neu5Ac-9-phosphate synthase was 75 kDa (Figure 1). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified enzyme demonstrated one band only with a molecular mass of 37 kDa (Figure 2). From these data it can be concluded that the native enzyme forms a dimer of 37-kDa subunits.

In previous reports, the enzyme was characterized as unstable (Warren and Felsenfeld, 1961). We found that the crude fractions from $(NH_4)_2SO_4$ precipitation retained 60% of their enzyme activity when stored at 4°C for 1 week. However, the homogeneous enzyme completely lost activity after 1 day of storage at 4°C in phosphate buffer. Higher stability was achieved when the enzyme was kept in phosphate buffer containing 5 mM $MgCl_2$; under these conditions, about 50% of the enzyme activity remained after 2 days, but after 3 days enzyme activity was lost entirely.

Sequence analysis

To verify that the 37-kDa polypeptide found in SDS–PAGE is Neu5Ac-9-phosphate synthase, a part of the amino acid sequence was determined. N-Terminal sequencing was not successful, indicating a blocked N-terminus. Therefore the protein was digested by trypsin and the masses of the resulting peptides were determined by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS). Four of the peptides had the same masses as theoretical tryptic peptides derived from amino acid sequences of murine Neu5Ac-9-phosphate synthase (Table II). For control purposes peptide 1 was analyzed by the post–source decay method, and its sequence was identical to the corresponding peptide of the mouse enzyme. Therefore we conclude that the 37-kDa polypeptide represented rat Neu5Ac-9-phosphate synthase.

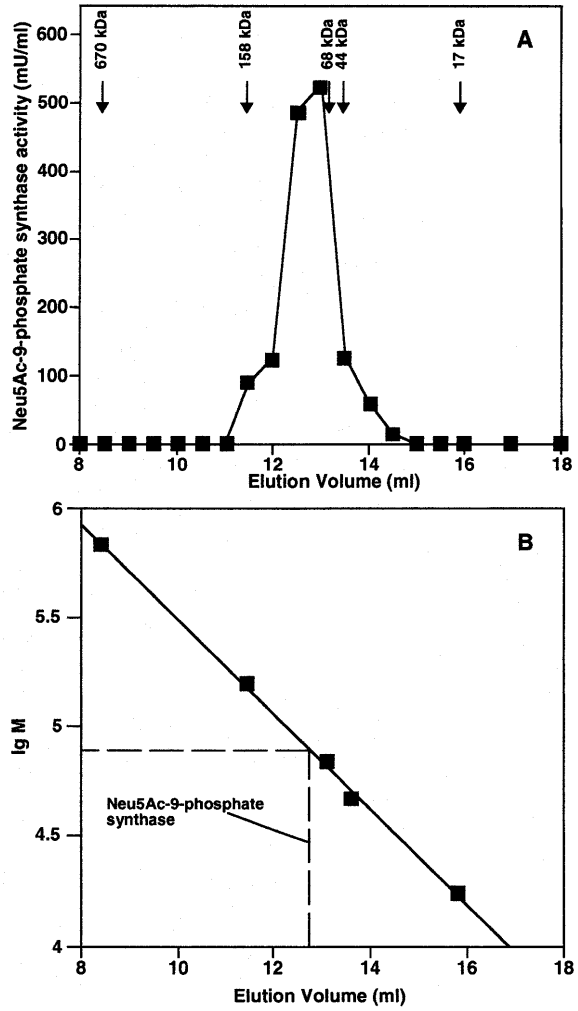

Fig. 1. (**A**) Gel filtration on Superdex 200 of a MonoQ fraction obtained during purification of Neu5Ac-9-phosphate synthase. Enzyme activity was determined as described in *Materials and methods*. Molecular weight standards were: thyroglobulin (670 kDa), γ-globulin (156 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), myoglobin (17 kDa). (**B**) Semi-logarithmic plot of elution volumes versus molecular weight standards.

Table I. Purification of Neu5Ac-9-phosphate synthase from rat liver

Purification step	Protein (mg)	Specific activity (mU/mg)	Purification factor (-fold)	Recovery (%)
Cytosol[a]	1292	0.043	1	100
$(NH_4)_2SO_4$ precipitation and Sephadex G25	958	0.20	5	350
Hydroxylapatite	119	0.44	10	94
Phenyl-Sepharose	23	0.97	23	40
MonoQ	0.20	107	2492	38
Superdex 200	0.02	505	11,736	18

[a]Cytosol was prepared from 26 g rat liver.

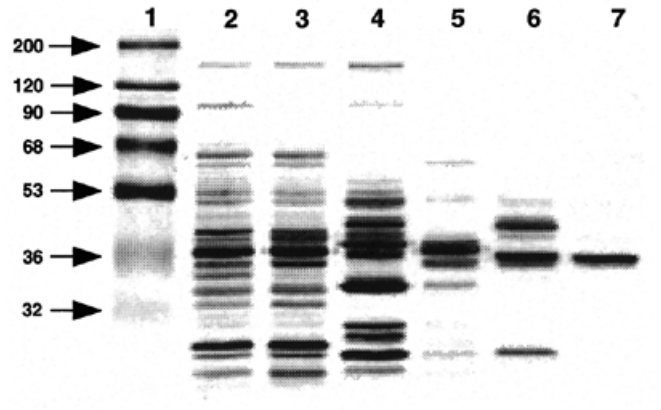

Fig. 2. SDS–PAGE of samples obtained during purification of Neu5Ac-9-phosphate synthase. Lane 1, molecular mass markers; lane 2, cytosol; lane 3, ammonium sulfate precipitation; lane 4, hydroxlapatite chromatography; lane 5, phenyl-Sepharose chromatography; lane 6, MonoQ chromatography; lane 7, gel filtration chromatography. Lanes 2–6, each lane contained 100 ng of protein; lanes 6 and 7, each lane contained 10 μU Neu5Ac-9-phosphate synthase.

Substrate specificity

It has been reported that human Neu5Ac-9-phosphate synthase can use Man-6-phosphate as a substrate, as well as ManNAc-6-phosphate (Lawrence *et al.*, 2000). Condensation of Man-6-phosphate with PEP results in the formation of deaminoneuraminic acid (KDN)-9-phosphate. Rat Neu5Ac-9-phosphate synthase was therefore also investigated for KDN-9-phosphate synthase activity. Assays were performed using 3.3 mM Man-6-phosphate (this concentration is used in the standard assay) and using five times this concentration of Man-6-phosphate. Under both conditions no formation of KDN-9-phosphate, which could be detected by the thiobarbituric acid assay as well as Neu5Ac-9-phosphate (Aminoff, 1961), was found.

The product of the reaction of Neu5Ac-9-phosphate synthase was characterized with the aid of radiolabeled compounds. Purified enzyme was assayed under standard conditions with addition of [^{14}C]-ManNAc 6-phosphate. The compounds were separated by paper chromatography. The product of the enzymatic reaction was Neu5Ac-9-phosphate and no formation of Neu5Ac was observed (data not shown). Therefore it can be concluded that Neu5Ac-9-phosphate synthase does not have an additional Neu5Ac-9-phosphatase activity.

Next we substituted ManNAc-6-phosphate with other sugar phosphates (GlcN-6-phosphate, Frc-6-phosphate, GlcNAc-1-P, GlcNAc-6-phosphate) and with ManNAc. Additionally, PEP was replaced by pyruvate. No enzyme activity was observed with any of these substrates, indicating a high specificity of Neu5Ac-9-phosphate synthase for ManNAc-6-phosphate and PEP. The enzyme showed K_m values of 35 μM for ManNAc-6-phosphate and 105 μM for PEP. In contrast to the findings of Watson *et al.* (1966) for hog submaxillary gland Neu5Ac-9-phosphate synthase, which displayed a nonlinear progression for PEP in a [S]/v versus [S] plot, the Lineweaver-Burk plot for the rat enzyme showed a linear progression. This may indicate that exclusively the hog enzyme is regulated by cooperativity.

Neu5Ac-9-phosphate synthase activity depended on the concentration of Mg^{2+}, the physiological divalent cation in the cytosol. Maximum activity was observed as a Mg^{2+} concentration of 5 mM (data not shown), in agreement with the findings of Watson *et al.* (1966) for the hog submaxillary gland enzyme. Mg^{2+} ions could be replaced by other divalent cations, such as Mn^{2+}, Fe^{2+}, or Ni^{2+}. At 5 mM, Mn^{2+}, Fe^{2+}, and Ni^{2+} showed 188%, 122%, and 62%, respectively, of the activity of Mg^{2+}. Enzyme activity in the presence of 5 mM Cu^{2+} and Ca^{2+}, respectively, was less than 5%, therefore these cations were not able to replace Mg^{2+}.

Inactivation of Neu5Ac-9-phosphate synthase by cysteine-specific chemical modifiers

Putative essential amino acid residues in the active site of Neu5Ac-9-phosphate synthase were identified with the aid of specific chemical modifiers. Modification of lysines by 2,4,6-trinitrobenzenesulfonic acid and arginines by phenylglyoxal and histidines by diethylpyrocarbonate had no influence on enzyme activity (data not shown). On the other hand, the cysteine modifiers *N*-ethylmaleimide and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were very effective inhibitors of Neu5Ac-9-phosphate synthase activity. *N*-Ethylmaleimide (1 μM) inactivated the enzyme completely after 30 min. DTNB was even more effective. A 10-min incubation with 0.3 μM DTNB had no effect, presumably due to the reaction of DTNB with traces of redox reagents in the buffer, but in the range of 0.3 μM to 0.8 μM a clear concentration dependency was observed (data not shown). DTNB at 0.8 μM was sufficient for complete enzyme inhibition. These data show that at least one highly reactive cysteine is located in or near the active site of Neu5Ac-9-phosphate synthase.

Interestingly, the inhibitory effect of DTNB was positively influenced by Mg^{2+} in a concentration-dependent manner. An incubation of Neu5Ac-9-phosphate synthase with 0.6 μM DTNB for 30 min in the absence of Mg^{2+} resulted in a residual activity of 35%. The identical assay in the presence of 5 mM

Table II. MS analysis of peptides derived from tryptic digest of Neu5Ac-9-phosphate synthase

Peptide	$(M+H^+)_{experimental}$ (Da)	$(M+H^+)_{theoretical}$ (Da)	Sequence
1	1065.2	1064.6	ALERPYTSK[a]
2	1303.8	1303.6	HLEFSHDQYK
3	1315.3	1314.6	MPLELELCPGR
4	1541.0	1540.7	VGSGDTNNFPYLEK

[a]Sequence confirmed by post–source decay sequencing.

Mg^{2+} showed a residual enzyme activity of only 20%. The effect was amplified by increasing concentrations of Mg^{2+} (data not shown). Assuming that Mg^{2+} is not required for the modification reaction, this indicates that Mg^{2+} has an allosteric effect on the structure of the active site of the enzyme and exposes the cysteine(s) more effectively to DTNB.

To localize the active site cysteine(s) in more detail, the enzyme was preincubated with its substrates before DTNB treatment. Whereas ManNAc-6-phosphate did not protect the enzyme against inhibition, PEP partially protected the cysteine(s) against modification (Figure 3). Addition of Mg^{2+} to the assay increased the protection effect of PEP, but no further effect was observed by addition of ManNAc 6-phosphate. Therefore we conclude that the active site cysteine(s) play an important role in binding PEP or a PEP/Mg^{2+} complex.

Discussion

Neu5Ac-9-phosphate synthase is a key enzyme in mammalian Neu5Ac biosynthesis. It catalyzes the condensation of a 6-carbon sugar with a 3-carbon α-keto acid to form a unique 9-carbon sugar. This sugar, Neu5Ac, is the precursor of nearly all sialic acids. In this study, Neu5Ac-9-phosphate synthase was purified to homogeneity. The enzyme had a specific activity of about 500 mU/mg protein. Watson *et al.* (1966) reported the purification of Neu5Ac-9-phosphate synthase from hog submaxillary glands, which resulted in an enzyme with a specific activity in the same range. Although they have not checked the preparation for homogenity, our results point out that they already may have prepared homogenous enzyme. Neu5Ac-9-phosphate synthase was characterized as a dimer of 37-kDa subunits. This is in good agreement with the findings of Lawrence *et al.* (2000) and Nakata *et al.* (2000), who showed that human as well as murine Neu5Ac-9-phosphate synthase consist of 40-kDa subunits and postulated that at least the murine enzyme has a dimeric structure.

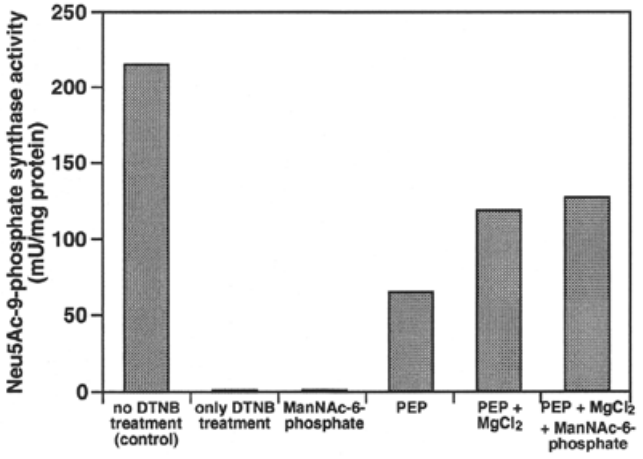

Fig. 3. Protection of Neu5Ac-9-phosphate synthase activity against inhibition by DTNB. Purified enzyme (0.5 µg) in a dithiotreitol-free buffer was pretreated 15 min at 37°C with 8.3 mM PEP, 3.3 mM ManNAc-6-phosphate, and 5 mM $MgCl_2$, respectively, or with the indicated combinations, and then treated 30 min at 37°C with 0.8 µM DTNB. Enzyme activity was determined as described in *Materials and methods*.

To confirm that the 37-kDa polypeptide represents rat Neu5Ac-9-phosphate synthase, it was tryptically digested and the resulting peptides were analyzed by MALDI-TOF MS. Four peptides corresponded exactly to theoretical tryptic peptides of the murine enzyme, indicating a high sequence similarity of the two proteins. Additionally, the human and mouse enzymes show 93% sequence similarity (Lawrence *et al.*, 2000; Nakata *et al.*, 2000), suggesting that mammalian enzymes are generally closely related. On the other hand, human Neu5Ac-9-phosphate synthase differs from the murine enzyme in that it can use Man-6-phosphate as a substrate to synthesize KDN-9-phosphate (Lawrence *et al.*, 2000). KDN is a sialic acid sharing many features with Neu5Ac, occurring in glycoconjugates and displaying variations in the α-ketosidic linkage to the penultimate sugar residue (Inoue *et al.*, 1996). We found no KDN-9-phosphate synthase activity for the rat Neu5Ac-9-phosphate synthase. Admittedly, we used a thiobarbituric acid assay for detection of sialic acids, which was about 10-fold less sensitive than the high-performance liquid chromatography (HPLC) analysis used by Lawrence *et al.* (2000), but Nakata *et al.* (2000) used HPLC also for the analysis of KDN-9-phosphate synthase activity of the murine enzyme and ruled it out completely. This indicates that a separate KDN-9-phosphate synthase, which has already been found in trout testis (Angata *et al.*, 1999), has to be postulated at least for rodents. Site-directed mutagenesis of amino acids not conserved between rodent and human Neu5Ac-9-phosphate synthase may be used to engineer the rodent enzymes to a human enzyme or vice versa.

Synthesis of Neu5Ac is strongly regulated by the initial enzyme of the pathway, UDP-GlcNAc 2-epimerase/ManNAc kinase (Hinderlich *et al.*, 1997), by a CMP-Neu5Ac-mediated feed back inhibition of the epimerase activity (Kornfeld *et al.*, 1964). We analyzed the Neu5Ac-9-phosphate synthase for regulatory mechanisms. Dependency of the enzymatic reaction on substrate concentrations fitted perfectly with Michaelis-Menten kinetics, indicating no regulation by cooperativity effects. Potential allosteric effectors like CMP-Neu5Ac or UDP-GlcNAc had also no effect on enzymatic activity (data not shown). Nevertheless, Neu5Ac-9-phosphate synthase activity may be coupled directly to the regulation of UDP-GlcNAc 2-epimerase/ManNAc kinase, by the availability of the substrate ManNAc-6-phosphate. Expression of two separate synthases for KDN-9-phosphate and Neu5Ac-9-phosphate favors a more coordinated and separately regulated biosynthesis of the two sialic acids.

Divalent cations are essential for the activity of Neu5Ac-9-phosphate synthase. Stephens and Bauerle (1992) showed, for the related *E. coli* 3-deoxy-arabino-heptulosonate 7-phosphate synthase, that an active site cysteine is directly involved in binding of the cation and the substrate PEP. We showed that the cysteine-specific chemical modifier, DTNB, completely inhibited Neu5Ac-9-phosphate synthase activity, and that optimal protection against inactivation was provided by a PEP/Mg^{2+} complex. Furthermore, a cysteine serves as a nucleophile in C-O bond cleavage at PEP during the catalytic process of *E. coli* 3-deoxy-manno-octulonate 8-phosphate synthase (Hedstrom and Abeles, 1988). A general role of cysteines in the mechanism of the aldol addition of the sugar

and PEP is therefore suggested. Both mouse and human Neu5Ac-9-phosphate synthase contain eight cysteine residues. Due to the very high sequence homology of the mammalian enzymes, this should also count for rat Neu5Ac-9-phosphate synthase. An aligment with several related bacterial Neu5Ac synthases (Figure 4) showed only one highly conserved cysteine residue among the species. This strongly favours mammalian Cysteine-184 as the catalytically important one. A site-directed mutagenesis study may clarify this in the future.

Materials and methods

Materials

ManNAc-6-phosphate was prepared as described previously (Jourdian and Roseman, 1962). [^{14}C]-ManNAc-6-phosphate was prepared identically, using [^{14}C]-mannosamine (ICN; Eschwege, Germany) as educt. All other chemicals were from Sigma (Deisenhofen, Germany) and Boehringer Mannheim (Mannheim, Germany). All chromatography media and supplies were from Pharmacia (Freiburg, Germany).

Neu5Ac-9-phosphate synthase assay

Neu5Ac-9-phosphate synthase activity was assayed by a modification of the method of Warren (1959). In brief, incubation mixtures contained 50 mM Na_2HPO_4, pH 7.5, 12.5 mM $MgCl_2$, 8.3 mM PEP, 3.3 mM ManNAc-6-phosphate, and various amounts of enzyme solution in a final volume of 125 µl. Incubations were carried out at 37°C for 30 min and stopped by heating at 100°C for 3 min. After centrifugation for 3 min at 13,000 × *g*, 137 µl of periodic acid solution (2.5 mg/ml in 57 mM H_2SO_4) were added and incubated for 15 min at 37°C. Then 50 µl sodium arsenite solution (25 mg/ml in 0.5 M HCl) were added, and the tubes were shaken vigorously to ensure complete elimination of the yellow-brown color. After this step 100 µl of 2-thiobarbituric acid solution (71 mg/ml adjusted to pH 9.0 with NaOH) were added, and the samples were heated to 100°C for 7.5 min. The solution was extracted with 1 ml of butanol/5% 12 M HCl, and the phases were separated by centrifugation. The absorbance of the organic phase was measured at 549 nm.

The radiolabeled assay was performed as described in the previous paragraph with addition of 50 nCi [^{14}C]-ManNAc-6-phosphate. The assay was stopped by addition of 200 µl ethanol. Radiolabeled compounds were separated by descending paper chromatography as described by Zeitler *et al.* (1992). The respective R_f values were 0.08 for Neu5Ac-9-phosphate, 0.17 for ManNAc-6-phosphate, and 0.24 for Neu5Ac.

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. One unit of enzyme activity was defined as the formation of 1 µmol of Neu5Ac-9-phosphate per min at 37°C. Specific activity was expressed as mU per mg of protein.

Purification of Neu5Ac-9-phosphate synthase

Step 1: Preparation of rat liver cytosol. Male Wistar rat livers were removed under light ether anesthesia and transferred to 2 volumes of homogenizing buffer containing 10 mM NaH_2PO_4, pH 7.5, 1 mM ethylenediamine tetra-acetic acid, 1 mM dithiothreitol (buffer A), and 1 mM phenylmethylsulfonyl fluoride. The tissue was homogenized with an Ultraturrax for 1.5 min at 10,000 rpm. The homogenate was ultracentrifuged at 100,000 × *g* for 60 min.

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Mouse   MPLELELCPGRWVGGKHPCF IIAEIGQNHQGDIDVAKRMI RTAKECGADCAKFQKSELEF KFNRKALERPY_TSKHSWGK TYGEHKRHLEFSHDQYKELQ  99
Human   MPLELELCPGRWVGGQHPCF IIAEIGQNHQGDLDVAKRMI RMAKECGADCAKFQKSELEF KFNRKALERPY_TSKHSWGK TYGEHKRHLEFSHDQYRELQ  99
E.coli                MSNIY IVAEIGCNHNGSVDIAREMI LKAKEAGVNAVKFQTFKADK LISAIAPKAEYQIKNTGELE SQLEMTKKLEMKYDDYLHLM  85
Strept                  MVY IIAEIGCNHNGDINLAKKMV DVAVSCGVDAVKFQTFKAEK LISKFAPKAEYQKETTGTAD SQLEMTKRLELSFEEYLEMR  83
Campyl                MKKAL IIAEAGVNHNGDLNLAKKLI EVAAKSGADFVKFQSFKAEL CVSKNAKKAAYQLKTTAKDE SQLEMIKKLELDFNAHQLLI  85
Legion                 MTCF IIAEAGVNHNGDLQLAKELV YAAKESGADAVKFQTFKADT LVNKTVEKAEYQKNNAPESS TQYEMLKALELSEEDHYLLS  84
B.subt    MAAFQIANKTVGKDAPVF IIAEAGINHDGKLDQAFALI DAAAEAGADAVKFQMFQADR MYQK__DPGLY_KTAAGKDV SIFSLVQSMEMPAEWILPLL  95

Mouse   SYAQEIGIFFTASGMDEMAV EFLHELNVPFFKVGSGDTNN FPYLEKTAKKGRP___MVIS SGM___QSMDTMKQVYQIVK P_____LNPNFCFLQCTSAY 188
Human   RYAEEVGIFFTASGMDEMAV EFLHELNVPFFKVGSGDTNN FPYLEKTAKKGRP___MVIS SGM___QSMDTMKQVYQIVK P_____LNPNFCFLQCTSAY 188
E.coli  EYAVSLNLDVFSTPFDEDSI DFLASLKQKIWKIPSGELLN LPYLEKIAKLPIPDKKIIIS TGM___ATIDEIKQSVSIFI NNKV__PVGNITILHCNTEY 180
Strept  DYAISKGVETFSTPFDEESL EFLISTDMPIYKIPSGEITN LPYLEKIGKQQKK___VILS TGM___AVMEEIHQAVNILR QN_____GTTDISILHCTTEY 173
Campyl  SHAKQCGIAFLSTAFDLESI ELLDDLGLEVFKIPSGEITN LPYLKKIAKLNKK___IILS TGMSNLGEIEAALEVLCKEG T_____QRANITLLHCTTEY 177
Legion  ELANSLGIEFMSTGFDEQSI DFLISLGVKRLKIPSGEITN VPYLQHCASKKLP___LIIS TGM___CDLQEVRVAIDTVK PYYGNSLSDYLVLLHCTSNY 178
B.subt  DYCREKQVIFLSTVCDEGSA DLLQSTSPSAFKIASYEINH LPLLKYVARLNRP___MIFS TAGA__EISDVHEAWRTIRA E_____GNNQIAIMHCVAKY 185

Mouse   PLQPEDANLRVISEYQKLFP DIPIGYSGHE_TGIAISVAA VALGAKVLERHITLDKTWKG SDHSASLEPGELAELVRSVR LVERALGSPTKQLLPCEMAC 287
Human   PLQPEDVNLRVISEYQKLFP DIPIGYSGHE_TGIAISVAA VALGAKVLERHITLDKTWKG SDHSASLEPGELAELVRSVR LVERALGSPTKQLLPCEMAC 287
E.coli  PTPFEDVNLNAINDLKKHFP KNNIGFSDHS_SGFYAAIAA VPYGITFIEKHFTLDKSMSG PDHLASIEPDELKHLCIGVR CVEKSLGSNSKVVTASERKN 279
Strept  PTPYPSLNLNVIHTLKDEFK DLTIGYSDHS_IGSEVPIAA AAMGAEVIEKHFTLDTNMEG PDHKASATPDILAALVKGVR IVEQALGRFEKIPDPVEEKN 272
Campyl  PAPFNEVNLKAMQTLKNAFN LDV_GYSDHT_KGIHISLTA IALGASVIEKHFTLDKNMSG PDHKASLEPDELQELCTKIR EIESALGDGIKQASKSERKN 275
Legion  PASYQDVNLKAMQTLADEF_ QLPVGYSDHT_LGILVPTLA VGMGACVIEKHFTMDKSLPG PDHLASMDPEEMKNLVQSIR DAETVLGSGEKKPSDNELPI 276
B.subt  PAPPEYSNLSVIPMLAAAFP EAVIGFSDHSEHPTEAPCAA VRLGAKLIEKHFTIDKNLPG ADHSFALNPDELKEMVDGIR KTEAELKQGITKPVSEKLLG 285

Mouse   NEKLGKSVVAKVKIPAGTTL TLDTLTVKVGEPKGYPPEDI FNLAGKKVLVTIEEDDTVME ESVESHSKKIKA 359
Human   NEKLGKSVVAKVKIPEGTIL TMDMLTVKVGEPKAYPPEDI FNLVGKKVLVTVEEDDTIME ELVDNHGKKIKS 359
E.coli  KIVARKSIIAKTEIKKGEVF SEKNITTKRPGNGISPMEWY NLLGKIA_EQDFIPDELIIH SEFKNQGE 346
Strept  KIVARKSVVALKPIKKGDIY SIENITVKRPGNGISPMNWY DILGQEA_QDDFEEDEVIRD SRFENQLPEL 341
Campyl  IEIARKSLVAKKKIKKGEIF SEENLTTKRPASGISAMRYD EYLGKKA_SKDYEEDELIHE 334
Legion  RALVRRSITLRRDLVKGAQI SKEDLILLRPGTGIAPSEIS NIVGSRL_SMNLSAGTNFVM GTY 338
B.subt  SSYKTTTAIEGE_IRNFAYR GIFTTAPIQKGEAFSEDNIA VLRPGQK_PQGLHPRFFELL TSGVRAVRDIPADTGIVWDDILLKDSPFHE 373
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Fig. 4. Alignment of amino acid sequences of mammalian Neu5Ac-9-phosphate synthases and bacterial Neu5Ac synthases. Mouse, mouse Neu5Ac-9-phosphate synthase (accession no. AB041263); Human, human Neu5Ac-9-phosphate synthase (accession no. AF257466); *E. coli*, *E. coli* Neu5Ac synthase (accession no. U05248); Strept, *Streptococcus agalactiae* Neu5Ac synthase (accession no. AB017355); Campyl, *Campylobacter coli* Neu5Ac synthase (accession no. AF195053); Legion, *Legionella pneumophila* Neu5Ac synthase (accession no. AJ007311); B.subt, *Bacillus subtilis* Neu5Ac synthase (accession no. P39625). Black boxes indicate cysteine residues homologous to mammalian Neu5Ac-9-phosphate synthases. The alignment was performed using MacMolly software (SoftGene, Germany).

Step 2: Ammonium sulfate precipitation. The supernatant from step 1 was fractionated with 1.5 M and 3.5 M ammonium sulfate, respectively. The precipitates were collected by centrifugation at 20,000 × *g* for 20 min. The pellet from the second centrifugation was dissolved in buffer A, pH 7.0, and centrifuged at 30,000 × *g* for 10 min.

Step 3: Desalting on Sephadex G-25. The supernatant from step 2 was applied to a column (26 mm ID × 60 cm) of Sephadex G-25. The column was eluted with buffer A, pH 7.0, and the protein fraction with absorbance at 280 nm was collected.

Step 4: Hydroxylapatite chromatography. The fraction from step 3 was applied to a column (26 mm ID × 20 cm) of hydroxylapatite, equilibrated with buffer A, pH 7.0. The column was eluted with 130 ml of a linear gradient starting with buffer A, pH 7.0, and finishing with 200 mM NaH_2PO_4, pH 7.0, 1 mM ethylenediamine tetra-acetic acid, 1 mM dithiothreitol. Fractions were collected at a flow rate of 1 ml/min.

Step 5: Hydrophobic interaction chromatography on phenyl-Sepharose. Fractions containing enzyme activity from step 4 were adjusted to 3 M NaCl and then applied to a column (26 mm ID × 20 cm) of phenyl-Sepharose. The column was washed with 100 ml of buffer A containing 3.0 M NaCl. The enzyme was eluted with 140 ml of a linear gradient of 3 M to 0 M NaCl in buffer A at a flow rate of 1 ml/min.

Step 6: Anion exchange chromatography on MonoQ. Fractions with enzyme activity from step 5 were desalted (see step 3) and then applied to a MonoQ HR 10/10 column. The column was washed with 25 ml buffer A and eluted with 50 ml of a linear gradient of 0 M to 0.25 M NaCl. Fractions were collected at a flow rate of 0.5 ml/min.

Step 7: Gel filtration chromatography on Superdex 200. Fractions with the highest enzyme activities from step 6 were concentrated to 0.3 ml on UH 100/25 Ultra Thimbles (Schleicher & Schuell, Germany), then applied to a Superdex 200 HR 10/30 column. The column was equilibrated with buffer A and eluted with the same buffer. The fractions were collected at a flow rate of 0.25 ml/min.

All procedures were carried out at 0–4°C. Fractions obtained during purification were assayed for Neu5Ac-9-phosphate synthase activity as described in *Neu5Ac-9-phosphate synthase assay,* and analyzed for purity by SDS–PAGE according to the method of Laemmli (1970). Gels were silver stained for protein as described elsewhere (Heukeshoven and Dernick, 1985).

Peptide MS

Prior to digestion, 10 µg of purified Neu5Ac-9-phosphate synthase were treated with 55 mM iodacetamide for 20 min in the dark at room temperature. The protein was then digested with 0.2 µg of trypsin in 50 mM ammonium bicarbonate and 5 mM $CaCl_2$ at 37°C for 16 h. The MS analysis of proteolytically derived peptides was performed using a Bruker Reflex MALDI-TOF mass spectrometer with α-cyano-4-hydroxycinnimanic acid as a matrix. For the analysis of fragment ions generated by post–source decay (Kaufmann *et al.*, 1993) the FAST™ method developed by Bruker was used.

Kinetic analysis

Kinetic data were determined for Neu5Ac-9-phosphate synthase purified as described in *Purification of Neu5Ac-9-phosphate synthase*. The enzyme activity assay contained 50 mM Na_2HPO_4, pH 7.5, 12.5 mM $MgCl_2$, a variable concentration of PEP in the presence of 3.3 mM ManNAc-6-phosphate, or a variable concentration of ManNAc-6-phosphate in the presence of 8.3 mM PEP. Michaelis constants (K_m) were determined by Lineweaver-Burk plots.

Effect of divalent cations

Neu5Ac-9-phosphate synthase from the final purification step was assayed in 50 mM NaH_2PO_4, pH 7.5, 1 mM dithiothreitol, 8.3 mM PEP, 3.3 mM ManNAc-6-phosphate after addition of the following salts (7 mM, if not otherwise indicated): $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeSO_4$, $NiCl_2$, and $CuCl_2$.

Inactivation of Neu5Ac-9-phosphate synthase by chemical modifiers

Dithiothreitol-free enzyme was freshly prepared from MonoQ fractions by gel filtration as described under purification step 7, with the exception that dithiothreitol was removed from the elution buffer. Modification was performed by incubating Neu5Ac-9-phosphate synthase with the indicated amounts of different modifiers. Extent of inactivation was monitored by measuring residual enzyme activities in aliquots withdrawn from the incubation mixture and from control experiments without modifier at the indicated times. All inactivation experiments were carried out at 37°C.

The enzyme was protected against inactivation by incubation with 8.3 mM of PEP, 3.3 mM ManNAc-6-phosphate, or 5 mM $MgCl_2$ before adding the modifier. Inactivation and corresponding control experiments were carried out and monitored as described.

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Abbreviations

DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); HPLC, high-performance liquid chromatography; KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneuraminic acid; PEP, phospho*enol*pyruvate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

References

Aminoff, D. (1961) Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucins. *Biochem. J.*, **81**, 384–392.

Angata, T., Nakata, D., Matauda, T., Kitajima, K., and Troy, F.A. II (1999) Biosynthesis of KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid). *J. Biol. Chem.*, **274**, 22949–22956.

Bradford, M.M. (1976) A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Ferrero, M.A., Reglero, A., Fernandez-Lopez, M., Ordas, R., and Rodriguez-Aparicio, L.B. (1995) *N*-Acetyl-D-neuraminic acid lyase generates the sialic acid for colominic acid biosynthesis in *Escherichia coli* K1. *Biochem. J.*, **317**, 157–165.

Fukuda, M. (1996) Possible roles of tumor-associated carbohydrate antigens. *Cancer Res.*, **56**, 2237–2244.

Hedstrom, L. and Abeles, R. (1988) 3-Deoxy-manno-octulonate 8-phosphate synthase catalyzes the C-O bond cleavage of phosphoenolpyruvate. *Biochem. Biophys. Res. Commun.*, **157**, 816–820.

Heukeshoven, J. and Dernick, R. (1985) Simplified method for silver staining of proteins in polyacylamide gels and the mechanism of silver staining. *Electrophoresis*, **6**, 103–112.

Hinderlich, S., Stäsche, R., Zeitler, R., and Reutter, W. (1997) A bifunctional enzyme catalyzes the first two steps of *N*-acetylneuraminic acid biosynthesis in rat liver. Purification and characterization of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. *J. Biol. Chem.*, **272**, 24313–24318.

Inoue, S., Kitajima, K., and Inoue, Y. (1996) Identification of 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN, deaminoneuraminic acid) residues in mammalian tissues and human lung carcinoma cells. Chemical evidence of the occurrence of KDN glycoconjugates in mammals. *J. Biol. Chem.*, **271**, 24341–24344.

Jourdian, G.W. and Roseman, S. (1962) D-Glucosamine 6-phosphoric acid and D-mannosamine 6-phosphoric acid. In Coon, M.J. (ed), *Biochemical preparations* vol. 9. New York: John Wiley & Sons, pp. 44–47.

Jourdian, G.W., Swanson, A.L., Watson, D., and Roseman, S. (1964) Isolation of sialic acid 9-phosphatase from human erythrocytes. *J. Biol. Chem.*, **239**, 2714–2716.

Kaufmann, R., Spengler, B., and Lützenkirchen, F. (1993) Mass spectrometric sequencing of linear peptides by product ion analysis in a reflectron time-of-flight mass spectrometer using matrix-assisted laser desorption ionisation. *Rapid Commun. Mass Spectrotrom.*, **7**, 902–910.

Kean, E.L. (1970) Nuclear cytidine 5′-monophosphosialic acid synthetase. *J. Biol. Chem.*, **245**, 2301–2308.

Kornfeld, S., Kornfeld, R., Neufeld, E.F., and O'Brien, P.J. (1964). The feedback control of sugar nucleotide biosynthesis in liver. *Proc. Natl Acad. Sci. USA*, **52**, 371–379.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lawrence, S.M., Huddleston, K.A., Pitts, L.R., Nguyen, N., Lee, Y.C., Vann, W.F., Coleman, T.A., and Betenbaugh, M.J. (2000) Cloning and expression of the human *N*-acetylneuraminic acid phosphate synthase gene with 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid biosynthetic ability. *J. Biol. Chem.*, **275**, 17869–17877.

Mortensen, B. and Huseby, N.E. (1997) Clearance of circulating gamma-glutamyltransferase by the asialoglycoprotein receptor. Enzyme forms with different sialic acid content are eliminated at different clearance rates and without apparent desialylation. *Clin. Chim. Acta*, **258**, 47–58.

Nakata, D., Close, B.E., Colley, K.J., Matsuda, T., and Kitajima, K. (2000) Molecular cloning and expression of the mouse *N*-acetylneuraminic acid 9-phosphate synthase which does not have deaminoneuraminic acid (KDN) 9-phosphate synthase activity. *Biochem. Biophys. Res. Commun.*, **273**, 642–648.

Rodriguez-Aparicio, L.B., Ferrero, M.A., and Reglero, A. (1995) *N*-Acetyl-D-neuraminic acid synthesis in *Escherichia coli* K1 occurs through condensation of *N*-acetyl-D-mannosamine and pyruvate. *Biochem. J.*, **308**, 501–505.

Roseman, S., Jourdian, G.W., Watson, D. and Rood, R. (1961) Enzymatic synthesis of sialic acid 9-phosphates. *Proc. Natl Acad. Sci. USA*, **47**, 958–961.

Schauer, R. (1985) Sialic acids and their role as biological masks. *Trends Biochem. Sci.*, **10**, 357–360.

Schauer, R., Kelm, S., Reuter, G., Roggentin, P., and Shaw, L. (1995). Biochemistry and role of sialic acids. In Rosenberg, A. (ed.) *Biology of the sialic acids*. New York: Plenum Press, pp. 7–67.

Stäsche, R., Hinderlich, S., Weise, C., Effertz, K., Lucka, L., Moormann, P., and Reutter, W. (1997) A bifunctional enzyme catalyzes the first two steps of *N*-acetylneuraminic acid biosynthesis in rat liver. Molecular cloning and functional expression of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. *J. Biol. Chem.*, **272**, 24319–24324.

Stephens, C.M. and Bauerle, R. (1992) Essential cysteines in 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. *J. Biol. Chem.*, **267**, 5762–5767.

Van Rinsum, J., van Dijk, W., Hooghwinkel, G.J.M., and Ferwerda, W. (1984) Subcellular localization and tissue distribution of sialic acid-forming enzymes. *N*-Acetylneuraminate-9-phosphate synthase and *N*-acetylneuraminate 9-phosphatase. *Biochem. J.*, **223**, 323–328.

Vann, W.F., Tavarez, J.J., Crowley, J., Vimr, E., and Silver, R.P. (1997) Purification and characterization of the *Escherichia coli* K1 neuB gene product *N*-acetylneuraminic acid synthetase. *Glycobiology*, **7**, 697–701.

Varki, A. (1992) Diversity in the sialic acids. *Glycobiology*, **2**, 24–40.

Warren, L. (1959) The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.*, **234**, 1971–1975.

Warren, L. and Felsenfeld, H. (1961) N-acetylmannosamine 6-phosphate and N-acetylneuraminic acid 9-phosphate as intermediates in sialic acid biosynthesis. *Biochem. Biophys. Res. Commun.*, **5**, 185–190.

Watson, D.R., Jourdian, G.W., and Roseman, S. (1966) The sialic acids VIII. Sialic acid 9-phosphate synthetase. *J. Biol. Chem.*, **241**, 5627–5636.

Yang, P., Major, D., and Rutishauser, U. (1994) Role of charge and hydration in effects of polysialic acid on molecular interactions on and between cell membranes. *J. Biol. Chem.*, **269**, 23039–23044.

Zeitler, R., Banzer, J.-P., Bauer, C., and Reutter, W. (1992) Inhibition of the biosynthesis of *N*-acetylneuraminic acid by metal ions and selenium *in vitro*. *Biometals*, **5**, 103–109.