The \textit{siaA} gene involved in capsule polysaccharide biosynthesis of \textit{Neisseria meningitidis} B codes for N-acylglucosamine-6-phosphate 2-epimerase activity

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Abstract

The capsule polysaccharide of \textit{Neisseria meningitidis} serogroup B is composed of a homopolymer of \(\alpha-2\rightarrow8\) linked N-acetyl-neuraminic acid (sialic acid). The enzymes required for sialic acid biosynthesis and polymerization are encoded in region A of the capsule gene complex. We here describe the enzymatic activity of the \textit{siaA} gene product as determined by biochemical analysis. \textit{siaA} was overexpressed in \textit{Escherichia coli} and the SiaA protein was purified to homogeneity. Enzymatic assays revealed that SiaA did not accept N-acetyl-glucosamine as substrate, but only N-acetyl-glucosamine-6-phosphate (EC 5.1.3.9). SiaA catalyzes the isomerization of N-acetyl-glucosamine-6-phosphate to form N-acetyl-mannosamine-6-phosphate. This reaction represents the first step in capsule biosynthesis of \textit{N. meningitidis} B. ß 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

\textit{Neisseria meningitidis} is a major cause of bacterial meningitis in man. In particular, serogroup B strains are predominantly isolated from patients suffering from meningococcal sepsis and meningitis in Central Europe and North America. The capsular polysaccharide of \textit{N. meningitidis} B is considered the major virulence factor of the bacteria. The capsule is composed of a homopolymer of \(\alpha-2\rightarrow8\) linked N-acetylneuraminic acid (NeuNAc, sialic acid) and mediates resistance to phagocytosis and lysis by serum complement [1,2]. Four enzymatic steps are needed for sialic acid biosynthesis. The genes encoding the required enzymes are located in region A of the capsule gene complex in the order \textit{siaA}, \textit{siaB}, \textit{siaC} and \textit{siaD} (Fig. 1) [3,4]. The corresponding SiaB, SiaC and SiaD proteins have been previously characterized as CMP-NeuNAc synthetase, NeuNAc condensing enzyme and polysialyl transferase, respectively [4,5].

Based on sequence homologies (24.7\% identity to the \textit{Escherichia coli} neuC gene product) [6] and investigation of a \textit{siaA} knock-out mutant [4], the protein encoded by \textit{siaA} was presumed to be an N-acylglucosamine 2-epimerase (EC 5.1.3.8) which catalyzes the isomerization of N-acetyl-d-glucosamine (GlcNAc) and N-acetyl-d-mannosamine (ManNAc) as the first step in sialic acid biosynthesis. Functional characterization of the enzyme had not been carried out previously, however, and therefore its identity remained unclear. In fact, in some sialic acid containing bacterial species, GlcNAc-6P-2-epimerase (EC 5.1.3.9) activity has been determined in crude cell extracts [7]. On the other hand, it is known that in \textit{Neisseria} as well as in other bacteria, sialic acid is synthesized by condensation of phosphoenolpyruvate (PEP) and ManNAc, but not from ManNAc-6P [8,9]. The isomerization of GlcNAc-6P and ManNAc-6P consequently would require a specific phosphatase to remove the phosphate group from ManNAc-6P before the condensation reaction can occur. In contrast, in mammalian cells NeuNAc biosynthesis is initiated by a bifunctional UDP-GlcNAc-2-epimerase/ManNAc kinase [10], then PEP and ManNAc-6P are condensed to form NeuNAc-9P which is then dephosphorylated by a specific phosphatase to yield free NeuNAc [2,8,11].
To clarify the function of SiaA, which may be useful for synthetic applications [12], and to elucidate the sialic acid biosynthesis pathway in *N. meningitidis*, we purified and analyzed the recombinant protein by different biochemical assays in this study.

### 2. Materials and methods

#### 2.1. Purification of the recombinant meningococcal SiaA protein

For overexpression of the *N. meningitidis* SiaA protein, *E. coli* SURE cells harboring plasmid pUE15 (siaA gene is cloned in expression vector pKK223-3) [4] were grown in LB medium supplemented with ampicillin (100 mg l$^{-1}$) in a 10 l fermentor. In mid log phase, IPTG (1 mM final concentration) was added to induce synthesis of the SiaA protein. Cells were grown to a density of OD$_{578}$nm 1.9 and subsequently harvested by centrifugation in a flow-through rotor (57 g wet weight) and stored at $-20^\circ$C. Crude cell extracts as well as highly purified SiaA protein were employed for enzymatic assays. Bacterial cells were suspended in 5 mM Tris- HCl pH 7.7, disrupted with glass beads in a homogenator, and insoluble cell debris was separated by centrifugation. Supernatant was assayed either as crude cell extract or was further purified by FPLC in three steps using anion exchange and gel permeation chromatography. For purification, the supernatant was first applied to a DEAE Sepharose column. Fractions obtained from the column by continuous gradient elution (0–500 mM NaCl) were monitored for the desired protein by SDS–PAGE. Fractions containing the 40 kDa protein were pooled and applied to a Q-Sepharose column. Appropriate fractions obtained by continuous gradient elution (100–350 mM NaCl) were again pooled and concentrated by ultrafiltration before being applied to a Superdex 200 column. After elution with 10 mM Tris–HCl pH 7.7, 100 mM NaCl, the purified SiaA protein was used for enzyme assays after concentration by ultrafiltration.

#### 2.2. Assay for GlcNAc-2-epimerase activity

In order to analyze the isomerization of GlcNAc by the enzymatic activity of SiaA, the following reaction mixture was employed: 500 µl of protein solution (crude extract or purified SiaA enzyme), 50 µl of 300 mM pyruvate solution, 50 µl of 240 mM GlcNAc solution and 0.22 U of neuraminic acid aldolase. ManNAc resulting from the isomerization of GlcNAc was to be consumed by the addition of pyruvate and commercial neuraminic acid aldolase which catalyzes the reversible aldol formation of NeuNAc [12]. The reaction mixture was incubated at 37°C, and at time intervals of 30, 90 and 150 min, respectively, 100 µl aliquots were withdrawn to analyze the presence of NeuNAc. Quantification of the produced NeuNAc was carried out by the colorimetric thiobarbituric acid assay which is specific for 3-deoxy-2-keto-aldonic acids (Fig. 3) [13,14].

#### 2.3. Assay for GlcNAc-6P-2-epimerase activity

For analysis of GlcNAc-6P-2-epimerase activity of the *N. meningitidis* SiaA, the putative substrate GlcNAc-6P was synthesized from GlcNH$_2$ according to the procedure of Whitesides [15]. The above described assay for GlcNAc-2-epimerase activity was modified to include an additional dephosphorylation step in order to cleave phosphate esters from the equilibrated sugar substrates (see Fig. 3). In detail, 50 µl of 240 mM GlcNAc-6P solution were added to 500 µl of crude cell extract and incubated for 135 min at 37°C. Aliquots (100 µl) of the reaction mixtures were adjusted to pH 6.2–6.5 by addition of 20 µl of 20 mM HCl, and were then incubated with 10 U of acid phosphatase for 45 min at room temperature. After neutralization using 20 µl of 20 mM NaOH, 20 µl of 250 mM pyruvate solution and 0.4 U of neuraminic acid aldolase were added, and incubation was allowed for 90 min at 37°C. Finally, the synthesized NeuNAc was determined by the standard thiobarbituric acid assay [13].

### 3. Results and discussion

#### 3.1. Overexpression and purification of the recombinant SiaA protein from *N. meningitidis*

In order to elucidate the enzymatic function of *N. meningitidis* SiaA, the siaA gene was overexpressed in *E. coli*. For this purpose, siaA was amplified by PCR and cloned into expression vector pKK223-3. The resulting plasmid pUE16 [4] was employed to transform *E. coli*.
SURE cells. A prominent band at 40 kDa was visible when cell lysates of transformants were separated by SDS-PAGE (Fig. 2). The predicted molecular mass of the meningococcal SiaA protein is 41.4 kDa. For enzyme purification, cells were grown in LB medium supplemented with ampicillin in a 10 l fermentor. For induction of SiaA synthesis, IPTG was added to the culture. Protein purification was carried out by anion exchange and gel permeation chromatography. By this means, highly purified SiaA was obtained (Fig. 2). Crude cell extracts (supernatant from disrupted cells) as well as purified SiaA protein were employed for enzymatic assays.

3.2. Analysis of GlcNAc-2-epimerase activity

The principle of the assay was to isomerize GlcNAc by the enzymatic activity of SiaA. The resulting ManNAc was then to be consumed by addition of pyruvate and commercial neuraminic acid aldolase which catalyzes the reversible aldol formation of NeuNAc [12]. Subsequently, the produced NeuNAc was quantified by the colorimetric thioarbituric acid assay which is specific for 3-deoxy-2-keto-aldonic acids (Fig. 3) [13,14]. By this method, specific detection of ManNAc was verified, despite of high GlcNAc and pyruvate concentrations in the reaction mixture. Control reactions included substitution of the enzymatic reaction mixture by water, GlcNAc solution by water, GlcNAc by glucosamine (GlcNH₂) (negative controls), and GlcNAc by ManNAc (positive control), respectively.

By this procedure, we were unable to detect any GlcNAc-2-epimerase activity of the SiaA protein, neither in the crude extract nor in the purified enzyme preparation (data not shown). Also, incubation of the reaction mixture for up to 14 days did not give a positive result for the catalyzed formation of NeuNAc via ManNAc. However, the assay conditions are suitable for detecting ManNAc formation as could be seen from the positive control (data not shown). We therefore conclude that the siaA gene product from N. meningitidis B does not exhibit GlcNAc-2-epimerase activity.

3.3. Determination of GlcNAc-6P-2-epimerase activity

Next, we analyzed the SiaA protein from N. meningitidis B for GlcNAc-6P-2-epimerase activity. The putative substrate GlcNAc-6P was synthesized from GlcNH₂ according to the procedure of Whitesides et al. [15]. Initially, we tested whether GlcNAc-6P would be a substrate of yeast glucose-6-phosphate-dehydrogenase (G6P-DH) which oxidizes Glc-6P to gluconolactone-6P in a NAD⁺-coupled reaction and can thus be monitored photometrically. Since G6P-DH did not accept GlcNAc-6P, the above described assay for GlcNAc-2-epimerase activity was thus modified to include an additional dephosphorylation step in order to cleave phosphate esters from the equilibrated sugar substrates (see Fig. 3). As before, the synthesized NeuNAc was determined by the standard thioarbituric acid assay [13]. In control reactions, both the aldolase and GlcNAc-6P were independently replaced by water, respectively.

By this assay, only the sample reactions containing crude or purified SiaA preparations gave a positive signal, indicating that the N. meningitidis SiaA protein catalyzes...
the isomerization of GlcNAc-6P to form ManNAc-6P (Fig. 4). From the synthesized amount of NeuNAc a corresponding GlcNAc-6P-2-epimerase activity of approximately 0.1 U g$^{-1}$ of wet cells can be deduced as a minimum estimate (actual activity is likely higher owing to the equilibrium nature of the assay and/or possibly insufficient conversions). Specific activity of the purified SiaA protein diminished during the course of purification (2 mU g$^{-1}$) which may indicate an inherent protein structural instability or the loss of an as yet unidentified cofactor upon chromatography. This is in accordance with the observations of Ghosh and Roseman who reported an instability or the loss of an as yet unidentiﬁed cofactor of the puriﬁed enzyme from Enterobacter cloacae (formerly Aerobacter cloacae) [7].

The enzymatic function of the siaA gene product from N. meningitidis B has been identiﬁed by biochemical assays to be that of a GlcNAc-6-phosphate 2-epimerase which catalyzes the equilibration of GlcNAc-6-phosphate with ManNAc-6-phosphate. This reaction represents the ﬁrst step in polylsialic acid capsule biosynthesis of B meningococci, as ManNAc is a prerequisite for the condensation with PEP to form NeuNAc. It is likely that corresponding enzymes from homologous genes of NeuNAc biosynthetic pathways of other bacteria, e.g. the neuC gene product of E. coli K1 [6], may utilize GlcNAc-6P as a substrate. Since the NeuNAc condensing enzyme (encoded by siaC in N. meningitidis B and by neuB in E. coli) utilizes ManNAc but not ManNAc-6P, a speciﬁc phosphatase may be required to remove the phosphate moiety at position C-6 before NeuNAc condensation can proceed. It is also possible that dephosphorylation may be catalyzed by one of the ubiquitous phosphatases present in these cells, since in the case of N. meningitidis B no speciﬁc phosphatase is encoded within the capsule gene complex.

References