Structure-function relations and application of the neisserial polysialyltransferase of serogroup B

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Die Menschen sind verschieden,

doch die Wahrheit ist Eine,

und alle, die sie suchen, auf welchem Gebiet es sei,

helfen einander.

Gottfried Wilhelm Freiherr von Leibniz

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Zusammenfassung

Die Polysialyltransferase (polyST) aus *Neisseria meningitidis* der Serogruppe B (*Nm*B) synthetisiert das Kapselpolysaccharid, das aus α2,8-verknüpfter Polysialinsäure (polySia) besteht. Da diese Kapsel einen wichtigen Virulenzfaktor darstellt ist sie ein geeigneter Ansatzpunkt für die Entwicklung neuer Medikamente. Darüber hinaus beschrieben jüngste Studien verschiedene biotechnologische Anwendungen von polySia, was dazu führte, dass dieses Enzym in den Fokus industrieller Herstellung dieses Zuckers gerückt ist.

In dieser Arbeit konnte die erste erfolgreiche Expression aktiven und löslichen Proteins in einer ausreichenden Menge erzielt werden. Das ermöglichte die erste detaillierte biochemische Beschreibung einer gereinigten polyST. Im Gegensatz zu bereits publizierten Daten, die mit polyST haltigen Fraktionen gewonnen wurden, konnte gezeigt werden, dass eine Membranauflagerung nicht essentiell für die enzymatische Aktivität ist. Nach der Etablierung eines kontinuierlichen spektrophotometrischen Assays konnte eine kinetische Charakterisierung der polyST durchgeführt werden. Untersuchungen zum Reaktions-mechanismus zeigten, dass das synthetisierte Polymer Längen mit mehr als 100 Einheiten betragen kann und in einer nicht-prozessiven Weise erstellt wird. Um die kleinste katalytische Einheit der polyST zu definieren wurden Studien mit sowohl N- als auch C-terminalen Verkürzungen durchgeführt. Diese zeigten, dass die gesamte C-terminale Domäne unverzichtbar ist, aber bis zu 33 Aminosäuren vom N-terminalen Ende entfernt werden können, ohne einen Aktivitäts- oder Löslichkeitsverlust nach sich zu ziehen. Durch Sequenzanalysen prokaryontischer Sialyl- und Polysialyltransferasen konnten zwei hoch konservierte Sialylmotive, nämlich ein D/E-D/E-G- und ein HP-Motiv identifiziert werden. Nachfolgende Charakterisierungen der dazugehörigen Mutanten belegten den Einfluss in Substratbindung und Katalyse.

Saturated-transfer-differene-NMR Experimente ermöglichten erste Einblicke in die Substratbindung zum Enzym. Interessanterweise wird die Bindung des Donorzuckers CMP-Neu5Ac hauptsächlich durch den Nukleotidteil vermittelt. Die relativ lockere Bindung der Sialinsäure ermöglicht wahrscheinlich einen effizienten Übertrag. Im Gegensatz dazu wird der Akzeptor, trimere Sialinsäure, komplett gebunden, was auf eine ausgedehnte Bindungsstelle hindeutet.

Um die polyST auch biotechnologisch einzusetzen wurde ein System zur enzymatischen Synthese und Abbau von polySia entwickelt. Eine Reaktion mit vier Enzymen und *in situ* Regeneration des CTP's ergab die größten Ausbeuten von 5 mg pro 10 ml Reaktionsvolumen an polySia mit ähnlicher Kettenlänge. Eine Reinigung des Polymers bis zur Homogenität

konnte mit einem einfachen 3-Schritt Protokoll geschehen. Schließlich wurde die Prozessivität des Abbaus der polySia durch Endosilalidasen aus Phagen untersucht. Dabei konnte gezeigt werden, dass eine negative Beeinflussung einer spezifischen Bindungsstelle eine zufälligere Längenverteilung der Abbauprodukte nach sich zieht. Aus biotechnologischer Sicht sind diese Endosialidasen, der Wildtyp, wie auch die verwendeten Mutanten wertvolle Werkzeuge in der Formgebung enzymatisch hergestellter polySia.

Abstract

The polysialyltransferase (polyST) of *Neisseria meningitidis* serogroup B is responsible for synthesis of capsular polysaccharide, namely α2,8-polysialic acid (polySia). As the capsular polysaccharide is a major virulence factor, this enzyme represents an interesting target for therapeutic strategies. Further, several recent studies demonstrating the potential of polySia in a range of biotechnological applications has brought this enzyme into the spotlight for its potential use in industrial scale synthesis of polySia.

In this study the first successful expression of active and soluble recombinant polyST of *Neisseria meningitidis* serogroup B in a sufficient amount was achieved and allowed the first comprehensive biochemical characterisation of purified polyST. In contrast to already published data with polyST containing fractions, the dispensability of membrane attachment for enzymatic activity could be shown. A kinetic characterisation of the protein was enabled by establishment of a continuous spectrophotometric assay. Further mechanistic investigation of polySia synthesis showed formation of polymers far exceeding 100 units and proved a non-processive manner of chain elongation. To narrow down the minimal catalytic domain of the polyST, N- and C-terminal truncations of the polyST were carried out, revealing the importance of a complete C-terminal domain. However, 33 amino acids could be removed from the N-terminus without the loss of activity or solubility. Sequence alignments of prokaryotic sialyltransferases and polySTs exposed the first two highly conserved sialylmotifs named D/E-D/E-G-motif and HP-motif. Subsequent biochemical characterisation of corresponding mutants confirmed crucial involvement in substrate binding and catalysis.

Saturated-transfer-difference-NMR experiments gave first insight into substrate binding to the enzyme. Interestingly, binding of donor-sugar CMP-Neu5Ac is predominantly mediated through the ribose and cytosine moieties. The relatively loose binding of the sialic acid moiety presumably allows for effective transfer. In contrast, the acceptor substrate, the trimeric sialic, acid was shown to be bound in total, revealing the presence of an extended binding site.

To address the biotechnological potential of the polyST, a system for enzymatic synthesis of α2,8-linked polySia has been established and methods of biodegradation investigated. A one-pot, 4-enzyme, cyclic reaction with *in situ* regeneration of the high energy phosphate donor, CTP, gave the greatest yields of polySia. 5 mg of small range disperse polySia could be synthesised in a 10 ml reaction and purified to homogeneity with a straightforward 3-step purification protocol. Finally, the processivity of biodegradation of polySia by phage-derived endosialidase EndoNF was investigated. As a consequence of interfering with the integrity of a specific polySia binding site, brake-down products are more random-sized. In terms of

biotechnological applications these endosialidases, the wild type as well as the mutants, are useful tools for trimming of enzymatically synthesised polySia.

Chapter 1 - General Introduction

1.1 - *Neisseria meningitidis*

Neisseria meningitidis is one of 24 bacterial strains that account for the genus of *Neisseria*, which are immobile, aerobic, gram-negative diplococcoi. Major causes of human diseases are *N. gonorrhoeae* and *N. meningitidis* (Feder and Garibaldi, 1984; Snyder and Saunders, 2006). The latter is restricted to the nasopharynx of humans which is the only known habitat of the pathogen (Johansson *et al*., 2003; Stephens *et al*., 1983). Transmission from human to human takes place via airborne droplets entering the respiratory system. Generally, a few days after resettlement of the new host, severe invasive infections occur sporadically (Ala'Aldeen *et al*., 2000; Stephens, 1999; van Deuren *et al*., 2000). But mostly *N. meningitidis* lives in probiotic relation with its host, and is found in 10-20% of healthy individuals (Cartwright *et al*., 1987; Claus *et al*., 2005; Stephens, 1999; van Deuren *et al*., 2000). Exceptionally in isolated groups, prevalences of 100% have been reported (Andersen *et al*., 1998).

1.1.1 - Serogroup classification

Meningococci are divided into distinct serogroups based primarily on the different capsular polysaccharides, present on the surface of the bacteria. Since the beginning of last century, 13 serogroups are known of which the most prevalent serotypes are A, B, C, W-135, X, and Y (see also Tab. 1-1). Serogroup A and X capsular polysaccharides are composed of N-acetyl mannosamine-1-phosphate and N-acetyl glucosamine-1-phosphate repeating units, with an α1,6-glycosidic linkage (Bundle *et al*., 1973, 1974a, 1974b; Jennings *et al*., 1977). Instead serogroup B and C are homopolymers of N-acetyl neuraminic acid (Neu5Ac, shown in Figure 1-3) in α2,8-Neu5Ac (serogroup B), or α2,9-Neu5Ac (serogroup C) linked repeating units (Bhattacharjee *et al*., 1975).

Tab. 1-1: *Neisserial* serogroups and capsular polysaccharides.

serogroup	capsular polysaccharide
A	[→6)-α-D- Man*p*NAc-(1→OPO3→]n
B	[→8)-α-Neu*p*5Ac-(→]n
C	[→9)-α-Neu*p*5Ac-(2→]n
W-135	[→6)-α-D-Gal*p*-(1-4)-α-Neu5Ac-(2→]n
X	[→6)-α-D- Glc*p*NAc-(1→OPO3→]n
Y	[→6)-α-D-Glc*p*-(1-4)-α-Neu*p*5Ac-(2→]n

Serogroups W-135 and Y are characterised by a hetero-polymer of galactose / Neu5Ac (W-135) or glucose / Neu5Ac (Y) repeating units, respectively (Bhattacharjee *et al*., 1976). Additional markers, taken into account for classification include outer membrane proteins e.g. PorA and PorB, and Lipo-oligosaccharides (LOS) (Jolley *et al*., 2007).

1.1.2 - Epidemiology

Meningococcal meningitis arises sporadically throughout the world. The highest burden of meningococcal disease occurs in Africa in an area that stretches from Senegal to Ethiopia, known as the "Meningitis Belt". In this region major epidemics are due to *N. meningitidis* serogroups A and C. Serogroup A is also the major cause of meningococcal disease in Asia. In contrast, in the temperate zones, several local outbreaks of *N. meningitidis* serogroup C (*Nm*C) have been reported. In particular, in the US and Canada in 1992 and 1993 and later in Spain in 1995 to 97, nevertheless serogroups B and C together account for the majority of cases in Europe and the Americas (see Fig. 1.1). In the last decade, the meningococcal meningitis disease has predominantly increased in New Zealand where in average 500 cases occur each year. Most of these cases are caused by serogroup B.

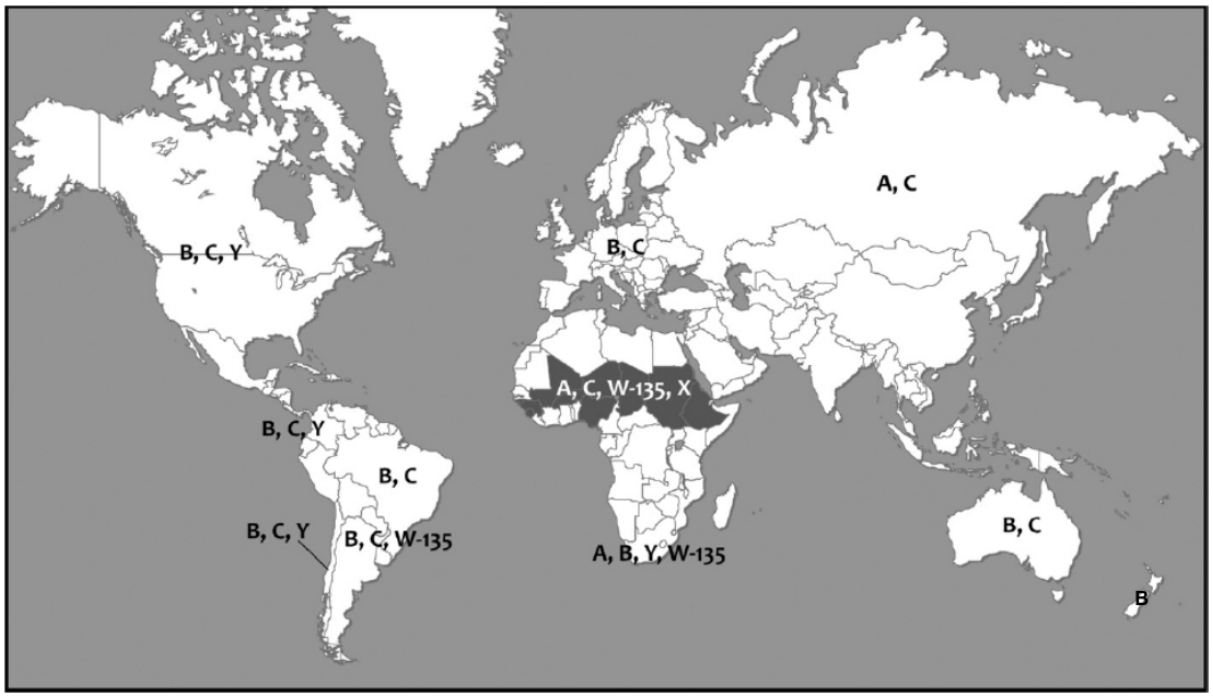


Fig. 1-1: Worldwide distribution of major meningococcal serogroups. The "Meningitis Belt" is highlighted in grey (modified from Harrison *et al.*, 2009)

1.1.3 - Pathogenesis and treatment of neisserial meningitis

After airborne contagion with *Neisseria meningitidis*, the bacteria adhere at the epithelium of the human nasopharynx and start to colonise. Proliferation of the meningococci causes morphological changes in the epithelial cells (Stephens and Farley, 1991) followed by induced, receptor mediated transcytosis through the epithelial barrier (Stephens *et al*., 1983; Stephens and Farley, 1991) and into the bloodstream (Yazdankhah and Caugant, 2004; Stephens, 2007). In the circulation, meningococci are able to evade the immune system and proliferate, which causes severe septicaemia. Moreover, crossing of the blood brain barrier allows the bacteria to gain access to the cerebrospinal fluid (Cartwright and Ala'Aldeen, 1997). The mechanism of the crossing is not fully understood, but a mechanism similar to crossing the epithelial barrier is discussed (Nassif *et al*., 2002; Unkmeir *et al*., 2002a, b). Finally, proliferation in the subarachnoid space and release of LOS causes the symptoms of meningitis (Halstensen *et al*., 1993; Spanaus *et al*., 1997; Waage *et al*., 1989). Despite proper treatment, meningitis caused by invasive meningococci remains a prevalent disease with 10% case-fatality. Patients might die in few hours after having the first symptoms and survivors often suffer from sequelae such as deafness, limb loss or neurological damage (Connolly and Noah, 1999; Kirsch *et al*., 1996; Tzeng and Stephens, 2000; Brandtzaeg and van Deuren, 2002).

Vaccination against neisserial meningitis is possible in the cases of serogroups A, C, W-135, and Y, but vaccines against serogroup B and X are not yet developed. Treatment of acute neisserial meningitis is based on application of broad-spectrum antibiotics, which in general has good results, but increasing resistance to penicillin, sulpha antibiotics, and chloramphenicol (Jorgensen *et al*., 2005) provide impetus for the development of alternative therapeutic strategies.

1.1.4 - Virulence factors of *Neisseria meningitidis*

The first contact made by *Neisseria meningitidis* to the epithelium of the nasopharynx is mediated by Type IV pili. Essential for this contact is the protein PilC1 which is either associated with the pili or attached to the outer-membrane of the bacterium (Rahman *et al*., 1997; Rudel *et al*., 1995). After retraction of the pili, adhesins like Opa and Opc procure direct attachment and uptake into the cells (Virji *et al*., 1995). Subsequently, intracellular proliferation and transcytosis depend on the ability of the *Neisseria* to recruit iron (Bonnah *et*

al., 2004). Leupeptin sensitive proteases of the meningococci are shown to cause rapid degradation of ferritin which liberates remarkable amounts of iron (Larson *et al*., 2004). Crossing the basal lamina and entering the circulation is not fully understood, but again the involvement of the adhesins Opa and Opc and others are discussed to bind to components of the extracellular matrix and the basal lamina like laminin, fibonectin and collagen. (Virji *et al*., 1995). Once in the bloodstream, survival of the *Neisseria meningitidis* strictly depends on the expression of LOS and capsular polysaccharide. Manipulation that prevented the expression of these factors, generated bacteria that were rapidly lysed by the host complement system (Jarvis and Vedros, 1987; Kahler *et al*., 1998; Vogel *et al*., 1997; 1999).

The extended serum resistance observed for capsulated bacteria is reasoned by molecular mimicry of the bacteria. First, the four terminal sugar moieties of the LOS α-chain of the Immunotype L3, 7, and 9 (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc) are matching the hosts lacto-N-neotetraose (Meyer *et al*., 1991; Ram *et al*., 1999; Tsai, 2001). Secondly the capsular polysaccharide of the serogroup B meningococcus (see Tab. 1-1) is chemically and immunologically identical to the polysialic acid found on the human neuronal cell adhesion molecule NCAM and is composed of α2,8-linked Neu5Ac units (see Chapter 1.2.4) (Mackinnon *et al*., 1993; Hammerschmidt *et al*., 1994; Vogel *et al*., 1997; Vogel and Frosch, 1999; Meyer *et al*., 1991). This capsule prevents the bacteria from phagocytosis by macrophages and is, therefore, the primary virulence factor of *Neisseria meningitidis* serogroup B (de Vries *et al*., 1996; Estabrook *et al*., 1998; Unkmeir *et al*., 2002). Additionally, because of its negative charge, the capsule is highly hydrated (Costerton *et al*., 1981) which protects the bacteria from desiccation (Musher, 2003). Interference with capsule biosynthesis renders bacteria more vulnerable in- and outside the host. Consequently, strategies to combat meningococcal spread are largely concentrated at blocking capsule biosynthesis.

1.1.5 - Capsule biosynthesis of *Neisseria meningitidis*

The capsule of *Neisseria meningitidis* serogroup B, C, W-135, and Y is synthesised and transported to the outer membrane by gene-products of the cps-locus (Frosch *et al*., 1989). The AT-content of this locus represents 70% and is therefore significantly higher than the rest of the meningococcal genome. For that reason it is hypothesised that these genes are acquired during evolution (Claus *et al*., 1997, 2004; Frosch *et al*., 1991). The cps gene-cluster comprises 24 kb and is segmented into three regions A, B, and C (Frosch *et al*., 1989). Region A harbours the genes *siaA*, *siaB*, *siaC*, and *siaD* for synthesis of the polysaccharide. Region B, with genes *lipA* and *lipB*, codes for proteins that modify the capsular polysaccharide in the

cytoplasm, which is then trans-located to the outer membrane by the gene-products of region C, *ctrA – ctrD* (Boulnois *et al*., 1987; Boulnois and Roberts, 1990). All cps-loci are highly conserved within the serogroups B, C, W-135, and Y with the exception of the *siaD*-gene encoding the polysialyltransferase (polyST) (Claus *et al*., 1997; Roberts, 1996), that catalyses the synthesis of the serogroup specific polymer (see Tab. 1-1).

All enzymes involved in capsular biosynthesis and translocation are proposed to be arranged in a functional complex spanning both, the inner and outer membrane (see Fig. 1-2). The presence of a capsule biosynthesis complex implicates a tight connection of polysialic acid synthesis and translocation (Steenbergen and Vimr, 2003; Bliss and Silver, 1996; Steenbergen and Vimr 2008).

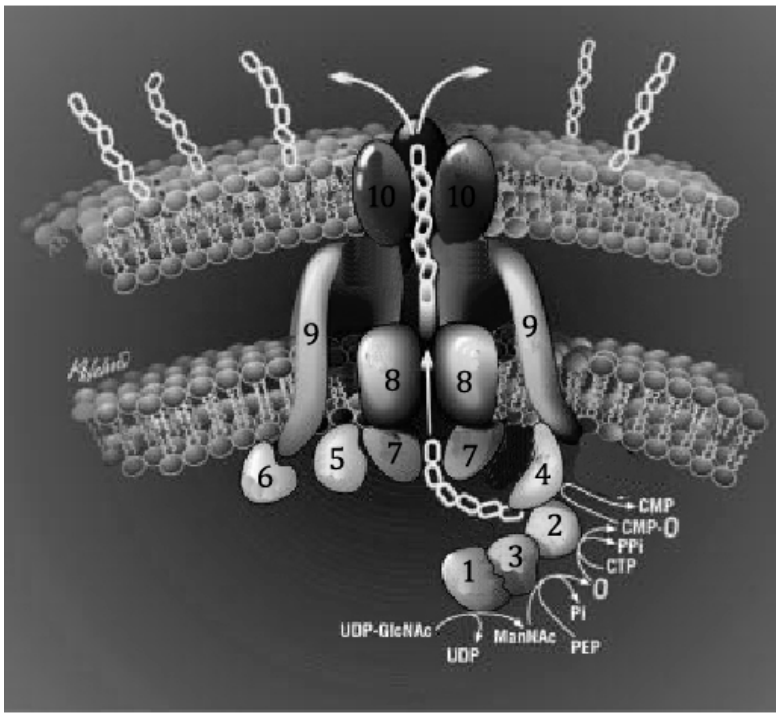
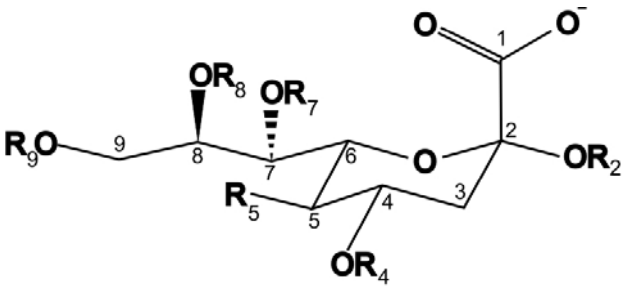


Fig. 1-2: Capsule biosynthesis complex as proposed for *Nm*B and *Nm*C. Inner and outer membrane of *Neisseria meningitidis*. Polysialic acid is shown as yellow chain. Enzymes involved in capsular synthesis: 1, siaA, epimerase; 2, siaB, CMP-sialic acid synthetase; 3, siaC, sialic acid syntase; 4, siaD, polyST; polymer modifying: 5, lipA; 6, lipB; and translocation: 7, ctrA; 8, ctrB; 9, ctrC; 10, ctrD (Modified from Steenbergen and Vimr 2003).

1.2 – Polysialic acid

Polysialic acid is a homopolymer of sialic acid units (see also Fig. 1-4). Sialic acids are derivatives of the neuraminic acid that is a nine carbon sugar containing a carboxylic function at C1 position (Fig. 1-3). To date, more than 50 natural sialic acids that occur in a wide range of organisms reaching from mammals to bacteria are known. The most prevalent sialic acid is the C5 N-acetylated sialic acid (Neu5Ac). Polymers of N-acetyl neuraminic acid (polySia) with α2,8-linkages, α2,9-linkages or alternating α2,8- α2,9- linkages have been described (Bundle *et al*., 1974). Based on NMR-evaluations, polySia is believed to exhibit a helical conformation in solution, with approximately nine residues per turn (Brisson *et al*., 1992). PolySia chains with a degree of polymerisation (DP) above eleven have been shown to form filamentous networks (Toikka *et al*., 1998). The enzymatic assembly of polySia is mediated by so-called polysialyltransferases.



R_2 = -H; -glycosyl
R_4 = -H; -acetyl
R_5 = $-NH_3$, -N-acetyl, -N-glycolyl, -OH, $-OCH_3$
R_7 = -H, -acetyl
R_8 = -H, -acetyl, $-CH_3$, $-SO_3^{2-}$, -glycosyl
R_9 = -H, -acetyl, $-PO_3^{3-}$, $-SO_3^{2-}$, -glycosyl

Fig. 1-3: Neuraminic acid and derivatives. Sialic acids are nine carbon sugars harbouring a carboxylic function at C1 position. Substituents have been described to occur on positions C2, C4, C5, C7, C8, and C9.

1.2.1 Biosynthesis of polysialic acid

The biosynthesis of polySia in prokaryotes starts from N-acetyl glucosamin-6-phosphate which is converted to N-acetyl mannosamine-6-phosphate by action of the N-acetyl glucaosamin-6-phosphate-2-epimerase (Gosh and Chance, 1964). The following dephosphorylation generates N-acetyl mannosamine, which is subsequently transferred to phosphoenolpyruvate to form Neu5Ac by action of the sialic acid synthase (Blacklow and Warren, 1962; Roseman, 1962). The CMP-Neu5Ac synthetase activates the sugar by transferring CMP from CTP in C2 position and releasing pyrophosphate. Finally, serogroup specific polySTs use CMP-Neu5Ac as a donor substrate to transfer Neu5Ac onto an appropriate acceptor. In the case of *Neisseria meningitidis* serogroup B the Neu5Ac is transferred onto the growing chain forming α2,8-linked polySia (see also Tab. 1-1; Fig. 1-4).

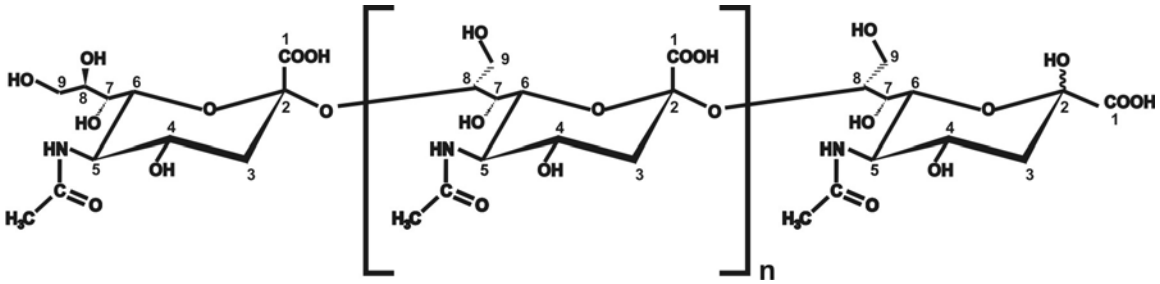


Fig. 1-4: α2,8-linked polySia

1.2.2 - Polysialyltransferases

Up to now, only few studies focused on bacterial polySTs, which are grouped into one carbohydrate active enzyme (CAZy) family, GT-38. The existing studies show the polyST of *E. coli* K1 not to be able to start *de novo* synthesis of polySia, but to act on exogenously added acceptors including oligo- and polysialic acids, sialogangliosides and synthetic acceptors (Steenbergen and Vimr, 1990; Cho and Troy, 1994; McGowen *et al*., 2001). Indeed, the nature of the priming endogenous acceptor is still unknown. In the case of *E. coli* K92 polyST the functional complex was found to be larger than a monomer (Vionnet *et al*., 2006). Sequence alignments of two *E. coli* polySTs (K1 and K96) and two neisserial polySTs (serogroup B and C) show highest identity between the *E. coli* enzymes (82% identity) and meningococcal enzymes (65% identity), but only 33% identity between the two genera. Moreover, the neisserial enzymes are elongated by a C-terminal domain not present in the *E. coli* polySTs. At the beginning of this study, neither data on isolated bacterial polySTs have been available, nor have any conserved motifs been described for bacterial sialyltransferases.
However, eukaryotic polySTs, which share no homology with the bacterial enzymes, are described in more detail, as they share three sialyl sequence motifs. The L-motif (large motif) for CMP-Neu5Ac binding, the S-motif (short motif) binds to both CMP-Neu5Ac and acceptor, and the VS-motif (very short motif) is important for activity (Datta and Paulson, 1995; Datta *et al*., 1998, Kitazume *et al*., 2001). Also highly conserved cystein residues are described by Angata *et al*. (2001).

1.2.3 - Degradation of polysialic acid by endosialidases

Enzymatic degradation of polySia is carried out by either exo- (EC 3.2.1.18) or endo-sialidases (EC 3.2.1.129). Exosialidases cleave sialic acid residues from the non-reducing end of sialylated glycoconjugates. In contrast, endosialidases exclusively cleave within the polySia chain and are, therefore, the only enzymes known to specifically degrade polySia. For

this reason, endosialidases are widely used tools in polySia research (Rutishauser, 2008). Endosialidases derive from tailspike proteins of bacteriophages were they facilitate infection of encapsulated bacteria by digesting the α2,8-linked polySia capsule. Bacteriophage-borne endosialidases (endoN) exhibit minimum substrate lengths ranging from a trimer to an octamer (Hallenbeck *et al*., 1987; Pelkonen *et al*., 1989; Miyake *et al*., 1997). Interestingly, despite the apparent importance of dynamic regulation of polysialylation in vertebrates, no endosialidases are known in vertebrate systems.

1.2.4 - Polysialic acid in vertebrates

PolySia is found in vertebrates as a posttranslational modification mainly of the neural cell adhesion molecule NCAM (Finne *et al*., 1983; Moebius *et al*., 2007). It was also found to be the major glycan structure of the CD36 scavenger receptor in human milk (Yabe *et al*., 2003). Additionally, Zuber *et al*. (1992) found polySia on the α-subunit of the voltage-gated sodium channel in rat brain. Most recently, neuropilin-2 was found to be posttranslationally modified by polysialylation, on maturating human dendritic cells (Curreli *et al*., 2007).
PolySia is broadly expressed during ontogenetic growth and remains important in neuronal cell plasticity in the adult brain (Conchonaud *et al*., 2007; Kleene and Schachner, 2004; Roth *et al*., 1988; Rutishauser and Landmesser, 1996; Weinhold *et al*., 2005; Mühlenhoff *et al*., 2009).

1.2.5 - Polysialic acid in bacteria

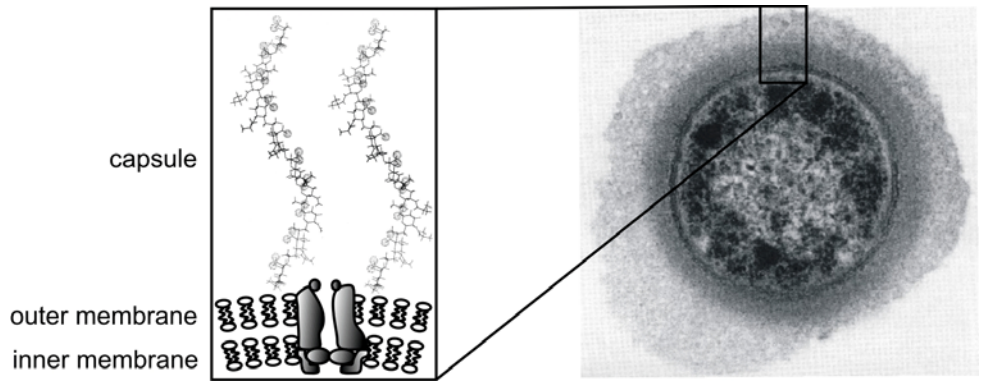


Fig. 1-5: Encapsulated bacterium. Cross-section of gram negative *Escherichia coli* K1 (right), schematic of inner-, and outer membrane and capsule of α2,8-linked polySia (left).

PolySia has been found as a capsular polysaccharide on the cell surface of several gram-negative bacteria. The most prominent representatives are the *Neisseria meningitidis*

serogroup B and *E. coli* K1. But also the capsules of *Moraxella nonliquefaciens* and *Pasteurella haemolytica* A2 are composed of the same polymer (Devi *et al*., 1991; Barrallo *et al*., 1999). These pathogens are surrounded by a thick layer of capsular polysaccharide of up to 400 nm depth, containing up to 200 sialic acid residues per chain. This capsule is negatively charged and hydrophilic, which confers a high water binding capacity. This prevents the organism from desiccation (Musher, 2003). The structural similarity of the human and bacterial polySia (Finne *et al*., 1983) known as antigen mimicry, allows these neuro-invasive pathogens to evade the human immune system. In contrast capsules of α2,9-linked polysialic acid are high inflammatory agents, highlighting the low immunogenicity of α2,8-linked polySia as a key function of such capsules.

1.2.6 - Polysialic acid as a new biomaterial in medical applications

Regenerative medicine and tissue engineering demand for materials that possess several key characteristics. Biocompatibility and the possibility of producing tailor shaped 2- and 3-dimensional scaffolds are as important as a balance between stability and biodegradability. In recent years polysaccharides have been recognised as promising new biomaterials. Imitation of naturally occurring polySia by a bio-identical polymer that can be synthesised *in vitro* holds promise for the production of a highly biocompatible polymer with very low immunogenicity (Finne *et al*., 1983). Additionally, the presence of numerous functional groups allows multiple modifications for cross linking or decoration with desired factors (Berski *et al*., 2008).

A sufficient stability of polySia in the human system is demonstrated by the long circulation half-life. The absence of known endosialidase activity in humans opens the possibility that the degradation of polySia based materials can be precisely controlled by the application of exogenous endosialidases. The suitability of polySia as a basis material to produce 3-dimensional matrices that can be degraded in a controlled manner has been demonstrated (Berski *et al*. 2008), in association with our laboratory. Further, evaluation of those materials in cell culture experiments revealed polySia to be a promising alternative in reconstructive therapeutic strategies (Bruns *et al*., 2007; Haile *et al*., 2007, 2008; Stark *et al*., 2008).

The polysialylation of protein therapeutics has also been shown to hold great potential for improving critical pharmacodynamic properties in a growing number of protein therapeutics. After decoration with polySia, a prolonged half live and reduced drug clearance and immunogenicity has been demonstrated for protein drugs. Amongst them, asparaginase, a

cytostatic drug in cancer treatment, insulin and Factor VIII of the blood clotting cascade. Modification of the proteins took place at the ∈-amino groups of their lysine residues (Fernandez *et al*., 2001; Jain *et al*., 2003).

Together, these findings show the relevance of polySia as a biomaterial and demand therefore for the supply of sufficient high-grade material. To date, industrial production of this polymer is based on fermentation (Rode *et al*., 2008) and available form several companies, but there is no chemical or enzymatic approach for the synthesis of polySia. A promising opportunity is therefore the use of polysialyltransferases as they naturally occur, or as engineered recombinant protein.

1.3 - Objectives

Biomedical applications of polySia as described above have led to a demand for the supply of sufficient high-grade material, which until today is isolated from fermented bacteria. Aiming at establishing a chemo-enzymatic reaction cascade for the production of polySia my diploma work was concentrated at the isolation and functional expression of a bacterial α2,8-polyST. In a comparative study, polyST of *Nm*B was identified as a promising candidate. In continuation of this work goals defined for my doctorial thesis were (i) the establishment of the expression and homogenous purification of recombinant *Nm*B polyST. In the following, (ii) the determination of the minimal catalytic domain of the enzyme and (iii) delineation of structure-function relations were scheduled. Finally (iv) up-scaled production of the enzyme and (v) use of the same for the in vitro production of polySia were set to achieve.

Chapter 2

Biochemical characterisation of a *Neisseria meningitidis* polysialyltransferase reveals novel functional motifs in bacterial sialyltransferases

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Preface – About the Manuscript

Neisseria meningitidis is the major cause of bacterial meningitis which occurs sporadically in epidemic waves throughout the world. Current treatment is exclusively based on antibiotics, but recent occurrence of multi-resistant meningococci demand for the development of alternative healing strategies. To date very few studies focused on the polysialyltransferase of *Neisseria meningitidis*, that is the key-enzyme of capsule formation, and therefore a potential drug target.

In the present study a comprehensive biochemical characterisation of the polysialyltransferase (polyST) of *Neisseria meningitidis* Serogroup B (*Nm*B) is presented. My contribution to this study comprised the recombinant expression and purification of a variety of *Nm*B-polyST constructs and activity as well as solubility testing, the definition of the minimal active domain and processivity determinations. Further, I established and performed the continuous spectrophotometric assay for kinetic analysis of wild-type and mutant polySTs that revealed two highly conserved functional motifs to be crucial for activity. Finally, Dr. Katharina Stummeyer, Prof. Dr. Rita Gerardy-Schahn and I wrote the paper.

Biochemical characterisation of a *Neisseria meningitidis* polysialyltransferase reveals novel functional motifs in bacterial sialyltransferases

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Summary

The extracellular polysaccharide capsule is an essential virulence factor of *Neisseria meningitidis*, a leading cause of severe bacterial meningitis and sepsis. Serogroup B strains, the primary disease causing isolates in Europe and America, are encapsulated in α-2,8 polysialic acid (polySia). The capsular polymer is synthesized from activated sialic acid by action of a membrane associated polysialyltransferase (*Nm*B-polyST). Here we present a comprehensive characterisation of *Nm*B-polyST. Different from earlier studies we show that membrane association is not essential for enzyme functionality. Recombinant *Nm*B-polyST was expressed, purified and shown to synthesize long polySia chains in a non-processive manner *in vitro*. Subsequent structure-function-analyses of *Nm*B-polyST based on refined sequence alignments allowed the identification of two novel functional motifs in bacterial sialyltransferases. Both motifs (D/E-D/E-G and HP) are highly conserved among different sialyltransferase families with otherwise little or no sequence identity. Their functional importance for enzyme catalysis and CMP-Neu5Ac binding was demonstrated by mutational analysis of *Nm*B-polyST and emphasised by structural data available for the *Pasteurella multocida* sialyltransferase *Pm*ST1. Together our data are the first description of conserved functional elements in the highly diverse families of bacterial (poly)sialyltransferases and thus provide an advanced basis for understanding structure-function-relations and for phylogenetic sorting of these important enzymes.

Introduction

Neisseria meningitidis (*Nm*) is a leading cause of bacterial meningitis and sepsis in children and adolescents. Sporadic cases as well as outbreaks and epidemic waves are observed. Despite the availability of potent antimicrobial agents, case-fatality rates are high and survivors frequently suffer from sequelae such as limb loss and deafness (van Deuren *et al.*, 2000). Essential virulence factors of disease causing meningococci are their extracellular polysaccharide capsules. Serogroup B strains (*Nm*B), the primary disease causing isolates in Europe and America, are encapsulated in α-2,8 polysialic acid (polySia). The *Nm*B capsule was shown to mediate resistance to phagocytosis and complement-mediated bacteriolysis and it is chemically and immunologically identical to polySia found in the human host (Mackinnon *et al.*, 1993; Hammerschmidt *et al.*, 1994; Vogel *et al.*, 1997; Vogel and Frosch, 1999). This mimicry also prevented the generation of effective polysaccharide based vaccines against *Nm*B-strains. Bypass of host defence mechanisms by polySia capsules has not only been described for *Nm*B, but is also an important virulence determinant of other disease causing pathogens such as *Escherichia coli* K1 and K92, *Moraxella nonliquefaciens*, *Pasteurella haemolytica* and *Neisseria meningitidis* serogroup C (Troy, 1992). These polySia capsules of *Nm*C and *E. coli* K92 are connected by α-2,9 (Bhattacharjee *et al.*, 1975) or alternating α-2,8/α-2,9 (Egan *et al.*, 1977) glycosidically linked sialic acids, respectively. Because of the critical function in bacterial pathogenesis enzymes involved in polySia biosynthesis are interesting targets for therapeutic intervention. Biosynthesis of polySia is catalysed by membrane associated polysialyltransferases (polySTs) at the cytoplasmic side of the inner membrane of gram negative bacteria (Masson and Holbein, 1983; Troy, 1992). The polymerisation

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reaction proceeds by transfer of sialic acid from the donor substrate CMP-Neu5Ac to the nonreducing end of a growing polySia chain and was proposed to be processive, since no reaction intermediates could be detected (Steenbergen and Vimr, 2003). Polysialyltransferases have been cloned from *E. coli* K1 and K92 as well as from *N. meningitidis* serogroup B and C (Weisgerber *et al.*, 1991; Vimr *et al.*, 1992; Edwards *et al.*, 1994; Claus *et al.*, 1997). Homology is highest within the pair of *E. coli* (82% identity) and meningococcal enzymes (65% identity) and is lower between the two genera (33% identity). Interestingly, the neisserial enzymes are elongated by a C-terminal domain not present in the *E. coli* polySTs.

While only few studies included the neisserial polySTs (Masson and Holbein, 1983; Swartley *et al.*, 1997; Steenbergen and Vimr, 2003), the *E. coli* enzymes have been studied more extensively (Whitfield, 2006; Troy, 1992). They are unable to start *de novo* biosynthesis of polySia, but elongate exogenously added acceptors including oligo- and polysialic acids, sialylgangliosides and synthetic acceptors (Steenbergen and Vimr, 1990; Cho and Troy, 1994; McGowen *et al.*, 2001). The nature of the priming endogenous acceptor is still unknown, but recently gene products NeuE and KpsC were shown to be required for *de novo* synthesis of polySia in *E. coli* (Andreishcheva and Vann, 2006) and the functional complex of the *E. coli* K92 polysialyltransferase was found to be larger than a monomer (Vionnet *et al.*, 2006). Construction of chimeric proteins between the closely related *E. coli* K1 and K92 enzymes, synthesizing α-2,8- and alternating α-2,8/α-2,9-linked polySia, respectively, mapped the region responsible for linkage specificity to primary sequence elements located between amino acids 53 and 85 in both enzymes (Steenbergen and Vimr, 2003). However, so far no data on isolated proteins are available. Attempts to purify or solubilise polySTs failed and resulted in inactivation of the enzymes and it was therefore proposed, that membrane association is required for polyST activity (Steenbergen and Vimr, 2003; Vionnet *et al.*, 2006). The lack of purified native or recombinant protein furthermore prevented detailed structure function analyses of these important enzymes.

No conserved motifs have been described for bacterial sialyltransferases. This is different from the eukaryotic sialyltransferases, where four conserved motifs have been shown to be involved in binding of donor and acceptor substrates and in enzyme catalysis (Drickamer, 1993; Livingston and Paulson, 1993; Geremia *et al.*, 1997; Jeanneau *et al.*, 2004). Moreover, based on primary sequence similarities all eukaryotic sialyl- and polysialyltransferases were grouped into a single family GT-29 of the CAZy (http://www.cazy.org) while the bacterial enzymes are distributed into four families (Coutinho *et al.*, 2003). Bacterial polysialyltransferases are found in CAZy-family GT-38, while families GT-42, GT-52 and GT-80 contain sialyltransferases that sialylate bacterial lipooligosaccharide (LOS). Structural information is available for the *Campylobacter jejuni* sialyltransferase cst-II (member of CAZy-family GT-42) (Chiu *et al.*, 2004) and the sialyltransferase *Pm*ST1 of *Pasteurella multocida* (member of CAZy-family GT-80) (Ni *et al.*, 2006). While cst-II belongs to the glycosyl-transferase-A-like (GT-A) structural group, *Pm*ST1 has a GT-B like fold.

In the current study we focused on the characterisation of the polyST from *Neisseria meningitidis* serogroup B. For the first time, we succeeded with soluble expression and purification of recombinant *Nm*B-polyST and, thereby, demonstrate that membrane association is not a prerequisite for the formation of functional enzyme. We also show that removal of the C-terminal extension present in *Nm*B but not in the homologous *E. coli* enzymes, completely abolished enzymatic activity, proving it as an essential functional domain. Using site directed mutagenesis and refined protein alignment strategies, we identified two functionally important motifs, which are highly conserved in a number of bacterial (poly)sialyltransferases of otherwise unrelated sequences. With these data we provide first evidence for the conservation of catalytic features among bacterial sialyl- and polysialyltransferases and thereby improve the basis for design of sialyltransferases specific drugs.

Results

Recombinant expression of NmB-polyST.

Detailed structure-function-analyses of bacterial polysialyltransferases were hitherto prevented by insufficient supply of the enzymes. Expression levels were described to be low and polyST activity was found associated with bacterial membranes (Shen *et al.*, 1999; Steenbergen and Vimr, 2003; Vionnet *et al.*, 2006). Since attempts to solubilize polyST were either unsuccessful or accompanied by inactivation of the enzyme, all analyses have been performed with crude membrane fractions as enzyme source. With the aim to obtain purified enzyme in yields sufficient to perform structure-function studies, we began the current work with a systematic search for conditions that would allow production of recombinant *Nm*B-polyST. To test the influence of N-terminal fusion parts on *Nm*B-polyST expression and activity, constructs were generated with either short N-terminal epitope tags (T7, Strep II) or with additional large fusion parts like NusA and maltose binding protein (MBP) (Fig. 1A). The constructs were expressed in *E. coli* BL21 (DE3) and soluble and insoluble fractions of the bacterial lysates were analysed for expression and activity of *Nm*B-polyST. Though soluble protein could be detected for all constructs (Fig. 1B) the addition of large N-terminal fusion parts, considerably increased the amount of active protein in the soluble fractions (Fig. 1C). Compared to polySTs carrying only short N-terminal epitope tags, additional fusion of NusA or MBP increased the soluble activity of NusA- and MBP-polyST two and three fold, respectively. The specific activity of both fusion proteins was also increased two fold compared to enzymes with short tags (Fig. 1D). Subsequent experiments were, therefore, carried out with either NusA- or MBP-fusion proteins.

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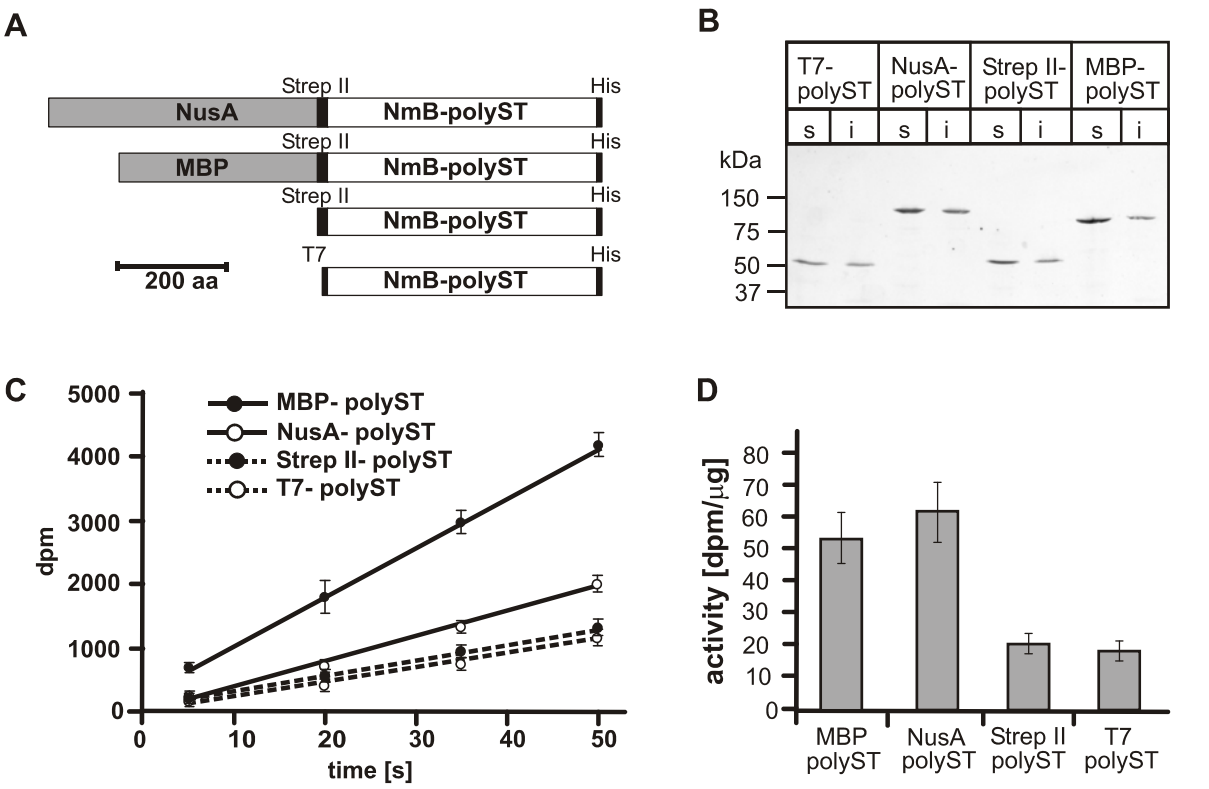

Fig. 1. Influence of N-terminal fusion tags on *NmB*-polyST expression and activity. (A) Schematic representation of *Nm*B-polyST fusion proteins. *Nm*B-polyST is shown as white box while short epitope tags (His, T7, Strep II) and large fusion partners (MBP, NusA) are given as black and grey boxes, respectively. The length of the black ruler represents 200 amino acids. (B) Western blot analysis of *Nm*B-polyST fusion proteins. Proteins were expressed in *E. coli* BL21(DE3) and equal amounts of soluble (s) and insoluble (i) fractions were separated by SDS-PAGE. C-terminally epitope-tagged fusion proteins were detected by Western blot analysis with anti-His-tag antibody. (C) Enzymatic activity of *Nm*B-polyST fusion proteins in the soluble fractions. PolyST activity was analysed using the radiochemical activity assay. Reactions were incubated at room temperature and aliquots were assayed for radiolabelled polyST at the indicated time points. Each value represents the average of three independent determinations with the standard deviation indicated. (D) Specific activities of *Nm*B-polyST fusion proteins were standardised by *Nm*B-polyST expression levels, which were determined by immunoblotting and infrared fluorescence detection.

Definition of the minimal active domain of* Nm*B-polyST.

It has been reported for other bacterial sialyltransferases that N- or C-terminal truncations significantly increased protein solubility by eliminating membrane interaction domains (Chiu *et al.*, 2004; Ni *et al.*, 2006). Consequently, the second experimental series was designed to define the minimal catalytic domain of *Nm*B-polyST. N- and C-terminal truncations of the enzyme were generated as NusA-fusion proteins carrying a C-terminal His-tag for detection. Full length and truncated proteins were expressed in *E. coli* and soluble and insoluble cell fractions were tested for expression and enzymatic activity of *Nm*B-polyST. As depicted in Figure 2, approximately 50 % of the wild-type polyST were soluble (Fig. 2B) and enzymatically active (Fig. 2D), whereby the detected activity was 3-fold higher in the soluble than in the insoluble fraction. Removal of 23 (Δ23*Nm*B-polyST) and 33 (Δ33*Nm*B-polyST) amino acids from the N-terminus had only slight effects on solubility and activity of *Nm*B-polyST. However, deletion of the first 64 amino acids (Δ64*Nm*B-polyST) shifted the majority of the expressed protein to the insoluble fraction and no enzymatic activity was detected in the soluble or the insoluble fraction.

Primary sequence analysis revealed that *Nm*B-polyST carries a C-terminal extension of 95 amino acids that is not present in the polySTs of *E. coli* K1 and *E. coli* K92 (Fig. 2 A). To investigate the role of this additional protein part, we generated a set of truncated *Nm*B-polySTs lacking the C-terminal domain either partially (*Nm*B-polySTΔ22, *Nm*B-polySTΔ60) or completely (*Nm*B-polySTΔ94, *Nm*B-polySTΔ95, *Nm*B-polySTΔ97). As displayed in Fig. 2C, all variants could be expressed as soluble proteins at similar or, in the case of constructs with entirely deleted C-terminal domain, even higher levels than the full-length enzyme. However, each C-terminal truncation completely abolished enzymatic activity (Fig. 2D) , indicating that the C-terminal domain is indispensable for *Nm*B-polyST activity.

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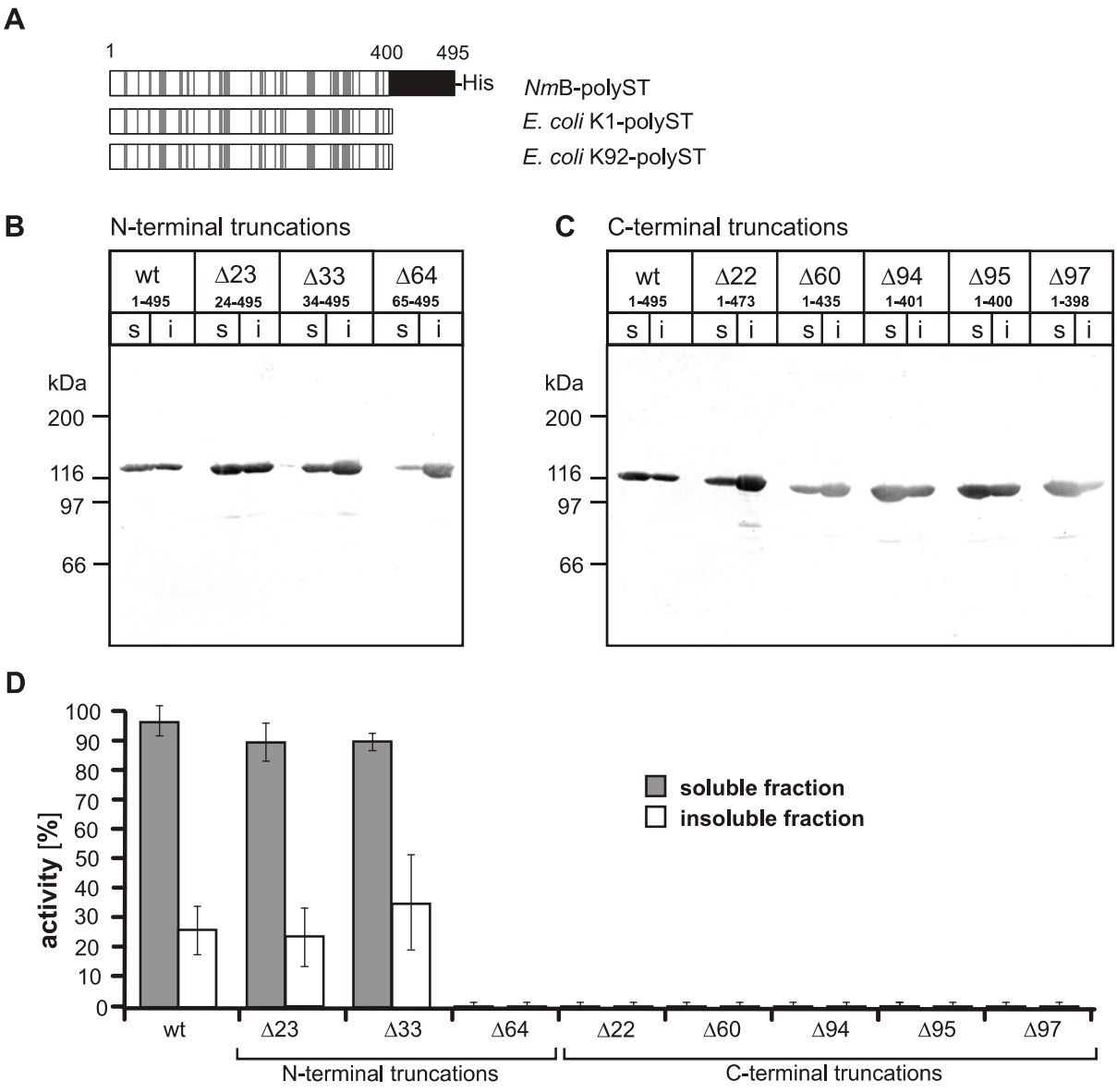

Fig. 2. Analysis of expression and activity of wild-type and truncated *NmB*-polyST. (A) Schematic representation of bacterial polySTs The N-terminal domain homologous in polysialyltransferases of *E. coli* K1, K92 and *N. meningitidis* serogroup B is shown as white box, with conserved amino acid stretches indicated in grey. The 95 amino acid comprising C-terminal domain of *Nm*B-polyST, which is not conserved in the *E. coli* enzymes, is depicted in black. (B-C) Western blot analysis of wild-type and truncated *Nm*B-polySTs. NusA-fusion proteins of full-length and N- and C-terminally truncated *Nm*B-polyST were expressed in *E. coli* BL21(DE3). Soluble (s) and insoluble (i) fractions (resuspended in the same volume and buffer as the soluble fractions) were prepared and equal amounts were analysed by SDS-PAGE and Western blotting using anti-His-tag antibody. (D) Enzymatic activity of soluble und insoluble fractions was analysed using the radiochemical polyST assay. The data were standardised by *Nm*B-polyST expression levels that were determined by immunoblotting and infrared fluorescence detection, and are given relative to the enzymatic activity of the soluble wild-type fraction. Each value represents the average of three independent determinations with the standard deviation indicated

***The fusion protein MBP-NmB-polyST produces capsular polySia* in vivo.**

Our trials to express recombinant *Nm*B-polyST in the *E. coli* expression strain BL21(DE3) clearly revealed beneficial effects of large N-terminal fusion parts on the expression of active, soluble enzyme. To analyse, however, if polyST fusion proteins maintain enzymatic activity also *in vivo*, wild-type and MBP-*Nm*B-polyST were subcloned into a neisserial expression vector and transformed into the polyST deficient neisserial strain 2517. Parental strain and transformants were analysed for capsular polySia in a quantitative whole-cell ELISA. Equal loading of the microplates with bacteria was confirmed in a parallel ELISA directed against the meningococcal major outer membrane protein PorA. Interestingly, no difference in capsule expression was observed between strains complemented with wild-type or the MBP-fusion construct (Fig. 3A). Moreover, Western blot analysis of the neisserial lysates revealed a

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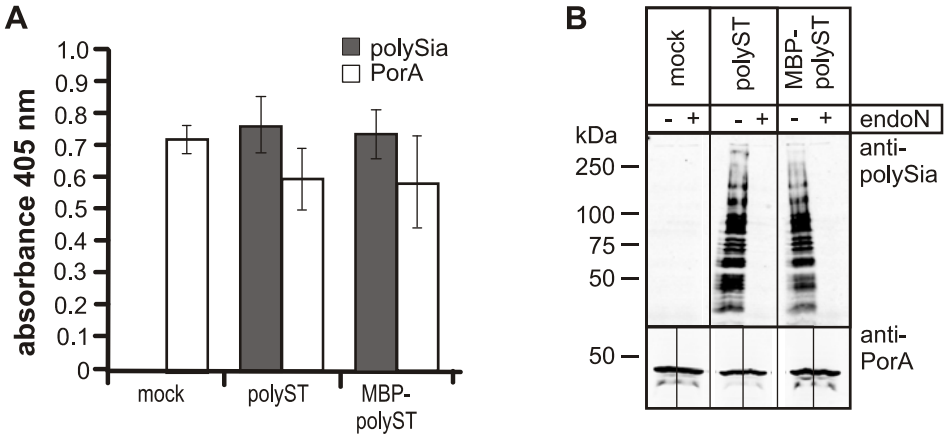

Fig. 3. *In vivo* activity of *Nm*B-polyST fused to maltose binding protein. (A) MBP-*Nm*B-polyST and native *Nm*B-polyST carrying no additional tags were cloned into an neisserial expression vector and transformed into the polyST deficient Neisseria strain 2517. Capsular polySia of parental strain (mock) and transformants was quantified by whole-cell ELISA using mab 735. Equal loading of the wells with *Neisseria* was controlled using mab P1.2 for detection of the meningococcal major outer membrane protein PorA. Each value represents the average of three independent determinations with the standard deviation indicated. (B) Lysates of parental strain (mock) and transformants were additionally analysed by SDS-PAGE and Western blotting using mab 735 before and after treatment with polySia degrading endoN (top). Equal sample loading was confirmed in a parallel Western blot immunostained with anti-PorA antibody P1.2 (bottom).

similar polySia staining of wild-type and fusion protein that was not detectable in samples treated with endoN (Fig. 3B), which is a bacteriophage derived enzyme that degrades polySia with high substrate specificity (Mühlenhoff *et al.*, 2003; Stummeyer *et al.*, 2005). This clearly demonstrates that MBP-*Nm*B-polyST is enzymatically active *in vivo*.

Purification of recombinant NmB-polyST.

Since MBP-*Nm*B-polyST was shown to be active *in vitro* and *in vivo* and because MPB could be directly utilised for affinity chromatography, purification was optimized for the MBP-*Nm*B-polyST construct schematically depicted in Fig. 1A. The fusion construct additionally carries two short epitope tags (N-terminal Strep II-, C-terminal His-tag), that were used for detection of the protein throughout the purification. After over expression in *E. coli* BL21(DE3) at 15 °C, the recombinant MBP-*Nm*B-polyST was purified in two consecutive steps by MBP-affinity and size exclusion chromatography. Trials to also utilise the C-terminal His-tag for purification failed, indicating that the epitope is not accessible in the native enzyme. As shown in Fig. 4A, the applied purification procedure yielded a highly enriched MBP-*Nm*B-polyST (protein of 100 kDa). Major bands of smaller molecular weight still visible in the Coomassie stained SDS-PAGE of the purified pool, were also detected in a Western blot directed against the StrepII-tag, but not in a blot stained with an anti-His-tag antibody. This indicates that these bands represent breakdown products of MBP-*Nm*B-polyST caused by C-terminal degradation.

Throughout the purification, polyST activity was monitored by the radiochemical polyST assay using colominic acid as acceptor. Enzyme activity was detected in all MBP-*Nm*B-polyST containing fractions and specific activity increased about 20-fold from cell lysate to the elute obtained after gel filtration (see Fig. 4A, bottom). As depicted in Figure 4B, the fusion protein eluted in a single peak at the upper limit of the applied Superdex 200 gel filtration column, which suggests association of MBP-*Nm*B-polyST to hexameric or higher-order oligomers. However, the purified protein was soluble and completely remained in the supernatant after high speed centrifugation (100 000 xg, Fig. 4C). One litre of expression culture yielded 1.3 mg of the highly enriched MBP-*Nm*B-polyST. The protein was concentrated to 2 mg/ml and stored at 4 °C for 3-4 weeks without detectable loss of activity or further degradation.

*Soluble recombinant MBP-*Nm*B-polyST produces polySia chains in a non-processive manner.*

Capsular polysaccharides purified from serogroup B *Neisseria meningitidis* were shown to exhibit a high degree of polymerisation (DP) (Gotschlich *et al.*, 1969; Frosch and Müller, 1993). This implies that *Nm*B-polyST is able to produce large polymers when the enzyme is part of the native capsule biosynthesis complex associated with the inner bacterial membrane. To analyze products synthesised by purified *Nm*B-polyST in more detail, pentameric (DP5) α-2,8-linked oligosialic acid was used as primer for the recombinant enzyme and the polymerisation reaction was started by addition of radiolabelled sugar donor substrate CMP-[^{14}C]-

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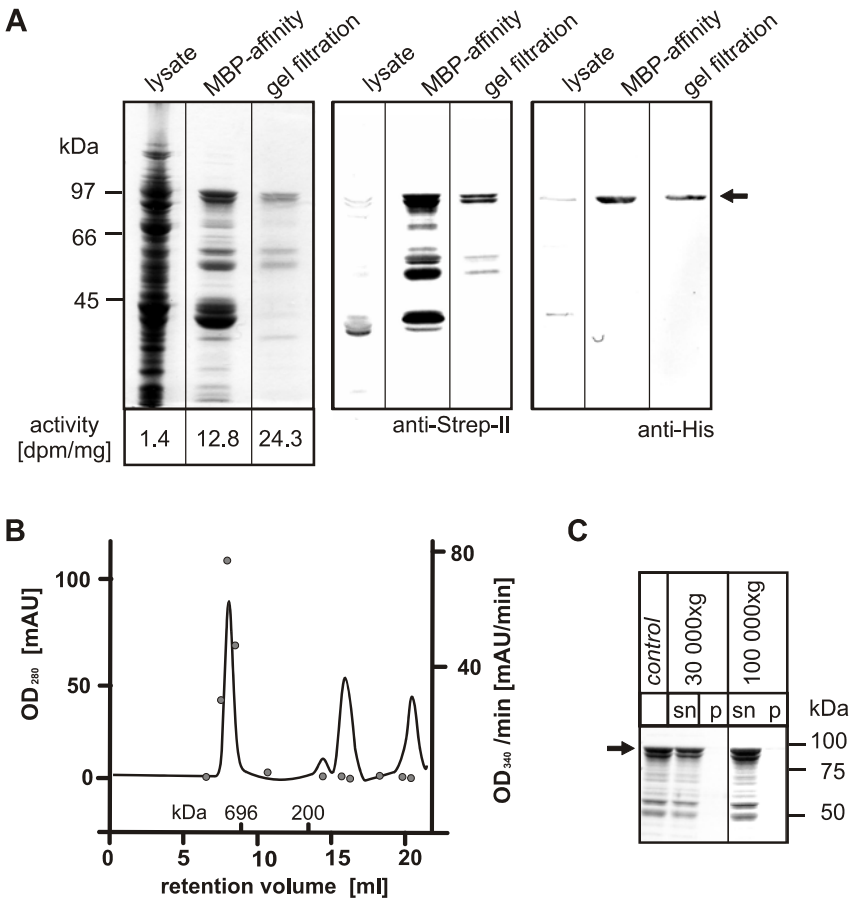

Fig. 4. Purification of *NmB*-polyST. (A) The MBP-fusion protein of *Nm*B-polyST was expressed in *E. coli* BL21 (DE3) and purified in a sequence of MBP-affinity and size exclusion chromatography. Protein containing fractions were analysed by Coomassie stained SDS-PAGE (left panel) and Western blot directed against the N-terminal StrepII-tag (middle panel) and the C-terminal His-tag (right panel). Protein bands corresponding to MBP-*Nm*B-polyST are indicated by an arrow. Enzyme activity was monitored by the radiochemical polyST assay and specific activities were calculated as indicated. (B) Size exclusion chromatography of MBP-*Nm*B-polyST. Elution positions of standard proteins (Thyroglobuline (669 kDa), β-Amylase (200 kDa)) are indicated. Enzymatic activity of the collected fractions (secondary y-axis, grey circles) was determined using the enzyme linked polyST assay. (C) High speed centrifugation of purified *Nm*B-polyST. Samples were centrifuged as indicated and equal amounts of supernatant (sn) and pellet (p) fraction, which was resuspended in the same volume as the supernatant, were analyse by Coomassie stained SDS-PAGE.

Neu5Ac. Products were separated by high percentage polyacrylamide gelelectrophoresis and visualised by autoradiography. Because polySia can be specifically degraded with bacteriophage endosialidases (endoN) (Mühlenhoff *et al.*, 2003; Stummeyer *et al.*, 2005) the nature of the synthesised products was controlled by treating one of two parallel samples with endoN prior to electrophoresis. As shown on the gel in Figure 5A, which separates polymerisation products from DP10 to >100, a large fraction of the synthesised polySia was longer than 100 residues per chain. All reaction products were specifically degraded by endoN. This demonstrates that the recombinant MBP-*Nm*B-polyST is able to synthesize long polySia chains starting with short oligomeric acceptors *in vitro*. However, in addition to the high molecular weight polySia also shorter oligosialic acid reaction products were detectable (Figure 5A). To validate the presence of short reaction products and analyze the mode of chain elongation in more detail, we studied the influence of acceptor concentrations on chain length distribution. Therefore, DP5 concentrations were altered over three orders of magnitude starting with 1 µM, while the concentration of enzyme and donor substrate CMP-Neu5Ac was kept constant. As shown in Figure 5B, increasing acceptor concentrations augment the concentration of short and medium sized reaction products until - at the point of equimolar concentrations of DP5 and CMP-Neu5Ac (lanes 4 and 5) - virtually all radiolabelled products remain below DP11 (Fig. 5B). An identical correlation of chain length distribution and acceptor concentration was obtained for trimeric (DP3) α-2,8-linked oligo sialic acid (Fig. S1). These data argue against processivity of *Nm*B-PolyST *in vitro*, since in case of a highly processive mechanism synthesis of few long chains should be favoured over synthesis of many short chains. To evaluate these data in a second assay system, we used our recently developed fluorescence based polyST assay (Vionnet and Vann, 2007) that utilises the trisialylganglioside analogue GT3-FCHASE as artificial acceptor substrate. In a first experiment GT3-FCHASE was incubated with purified MBP-*Nm*B-polyST and increasing concentrations of the donor substrate CMP-Neu5Ac (0, 5, 50, 500 µM). After an incubation time of 30 min the synthesized reaction products were analysed by ion exchange HPLC. As shown in Figure 6, the chain length of synthesized polySia increased with increasing concentrations of donor substrate and resulted in a complete conversion of the acceptor substrate to high molecular weight polymer at a CMP-Neu5Ac concentration of 500 µM. This clearly demonstrates synthesis of long polySia chains. To verify the suggested non-processive elongation mode of *Nm*B-polyST (Fig. 3B and Fig. S1), we performed a time course experiment under reaction conditions that, as shown above, result in synthesis of long polySia chains (Fig. 7). If the reaction was stopped after

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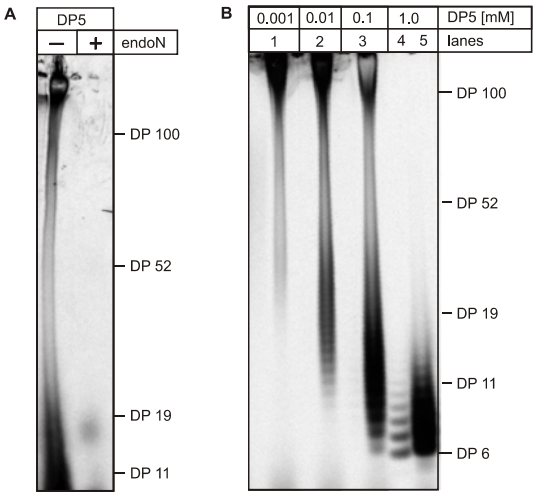

Fig. 5. PolySia biosynthesis of purified *Nm*B-polyST. (A) Purified *Nm*B-polyST was assayed for 30 min in the presence of 0.1 mM pentameric α2,8-linked sialic acid (DP5) and 1 mM CMP-[C^{14}]Neu5Ac. Subsequently, half of the sample was digested with polySia-specific endoN. Radiolabelled reaction products were separated by acrylamide-gelelectrophoresis (10%) and detected by autoradiography. The following dyes were used as standards and correspond to polySia chain length given in brackets: trypan blue (DP100), xylene cyanol (DP52) bromo-phenol blue (DP19), bromcresol purple (DP11). (B) Dependence of chain length distribution on acceptor concentration. The assay was performed as described in (A) but DP5 concentrations were varied as indicated. Samples were separated on a 25% polyacrylamide gel. To display single oligomeric reaction products at the highest acceptor concentration more clearly, less sample volume was applied in lane 4.

2 min predominantly short oligosialic acid containing products were found (Fig. 7A) that were further elongated as the polymerisation progressed (Fig. 7B,C) to finally yield high molecular weight polymer (Fig. 7D). This is reflected by a shift of the detected product peaks to longer retention times (Fig. 7). The occurrence of intermediates in the polymerisation reaction argues, in agreement with the data obtained from the radioactive assay system, for non-processive chain elongation by MBP-*Nm*B-polyST and indicates that the enzyme dissociates from the product after each addition of sialic acid.

Identification of two conserved motifs in bacterial sialyl- and polysialyltransferases.

To identify amino acid residues critical for (poly)sialyltransferase activity, we searched for common sequence motifs in the available bacterial sialyltransferase sequences. By iterative steps of pairwise and mutiple alignments combined with visual inspection of the sequences we identified two short motifs (D/E-D/E-G and HP). Both motifs are present in a range of enzymes with otherwise little homology (Fig. 8) that, based on functional and sequence properties, had been allocated to different

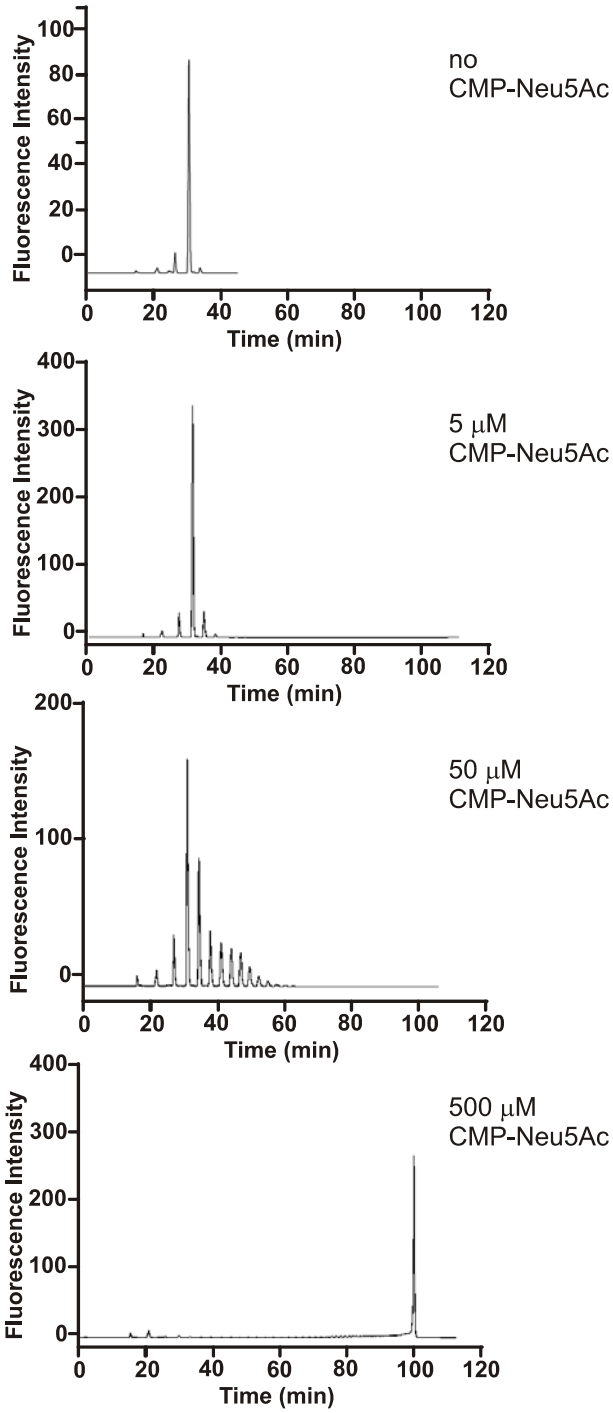

Fig. 6. Dependence of GT3-FCHASE extension by purified *Nm*B-PST on CMP-Neu5Ac concentration. Purified *Nm*B-polyST was incubated with GT3-FCHASE and CMP-Neu5Ac for 5 min as described in experimental procedures. The respective CMP-Neu5Ac concentrations are indicated. The reaction mixtures were then adjusted to 25% ethanol. The supernatants were applied to a DNA Pac PA-100 column and chromatographed with a gradient of $NaNO_3$ according to Inoue *et al.* (Inoue *et al.*, 2001; Inoue and Inoue, 2003).

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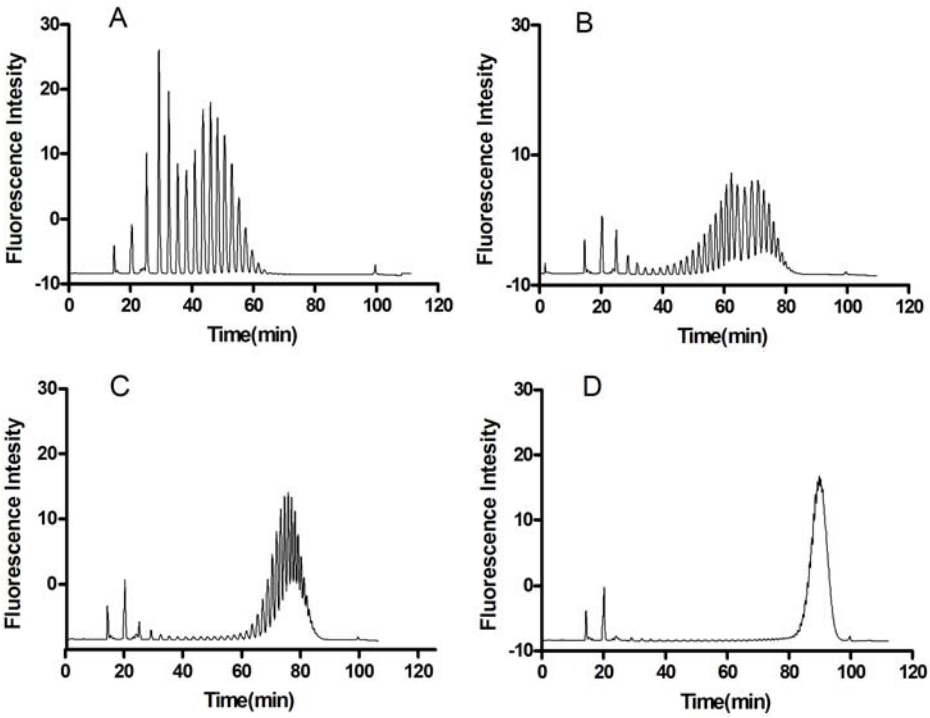

Fig. 7. Time course of elongation of GT3-FCHASE by *Nm*B-polyST. Purified *Nm*B-polyST (37 µg/ml) was incubated with GT3-FCHASE and 0.5 mM CMP-Neu5Ac for (A) 2 min., (B) 5 min., (C) 10 min., and (D) 30 min prior to adjusting the reaction mixtures to 25% ethanol. The supernatants were applied to a DNA Pac PA-100 column and chromatographed with a gradient of $NaNO_3$ according to Inoue *et al.* (Inoue *et al.*, 2001; Inoue and Inoue, 2003).

CAZy families (GT-38 and GT-52) and to pfam family 05855. While GT-38 contains bacterial polysialyltransferases, GT-52 includes bacterial LOS sialyltransferases and pfam 05855 groups sialyltransferases similar to the sialyltransferase Lst of *Haemophilus ducreyi* (Bozue *et al.*, 1999). The D/E-D/E-G- and the HP-motif identified in this study are conserved in all members of the three families and, interestingly, are also found in the bacterial LOS sialyltransferases grouped in GT-80. However, the relation to GT-80 family members is not easily seen in an alignment, since the stretch of sequence between the D/E-D/E-G- and HP-motif is approximately 50 amino acids longer than in the other families.

D/E-D/E-G- and HP-motifs are crucial for* Nm*B-polyST activity.

To investigate the functional relevance of the identified motifs, point mutations were introduced into *Nm*B-polyST by site directed mutagenesis. Histidine and proline of the HP-motif as well as the glycine and its adjacent glutamate residue of the D/E-D/E-G-motif were individually changed to alanine, resulting in the mutants H278A, P279A, E153A and G154A. Mutants were expressed in *E. coli* BL21(DE3) and lysates were analysed for protein expression and activity by Western blot and the radiochemical polyST activity assay respectively. As shown in Figure 9A, all mutants were expressed at the level of the wild-type protein and the ratio between soluble and insoluble protein was in all cases similar to wild-type. In contrast, enzyme activity was dramatically impaired in all mutants (Fig. 9B). While mutations in the D/E-D/E-G-motif (E153A and G154A) fully inactivated the protein, the H278A and P279A mutants of the HP-motif maintained residual activity (below 10% of wild-type). However, when both residues of the HP-motif were changed to alanine simultaneously (H278A/P279A) enzyme activity was abolished.

To confirm the functional relevance of the D/E-D/E-G- and HP-motif *in vivo*, mutant meningococcal strains were generated by homologous recombination and capsular phenotypes of the mutant strains were analysed by slide agglutination using the polySia specific antibody 735. In agreement with the *in vitro* studies, *Neisseria* strains exhibiting the E153A, G154A or the double mutation H278A/P279A did not synthesize polySia capsules while capsules were still produced in strains carrying the single mutations H278A and P279A (Fig 9C). However, a whole cell ELISA used to quantitatively compare polySia synthesis in wild-type bacteria and mutants clearly demonstrated impairment of capsule biosynthesis in mutants with residual *in vivo* activity. While the PorA control was similar for wild-type and mutants, the polySia signal was significantly reduced to 30% and 60% for the mutants carrying the amino acid exchanges H278A and P279A, respectively. This demonstrates that mutations within the HP-motif not only decrease enzymatic activity *in vitro*, but also reduce capsule production *in vivo*.

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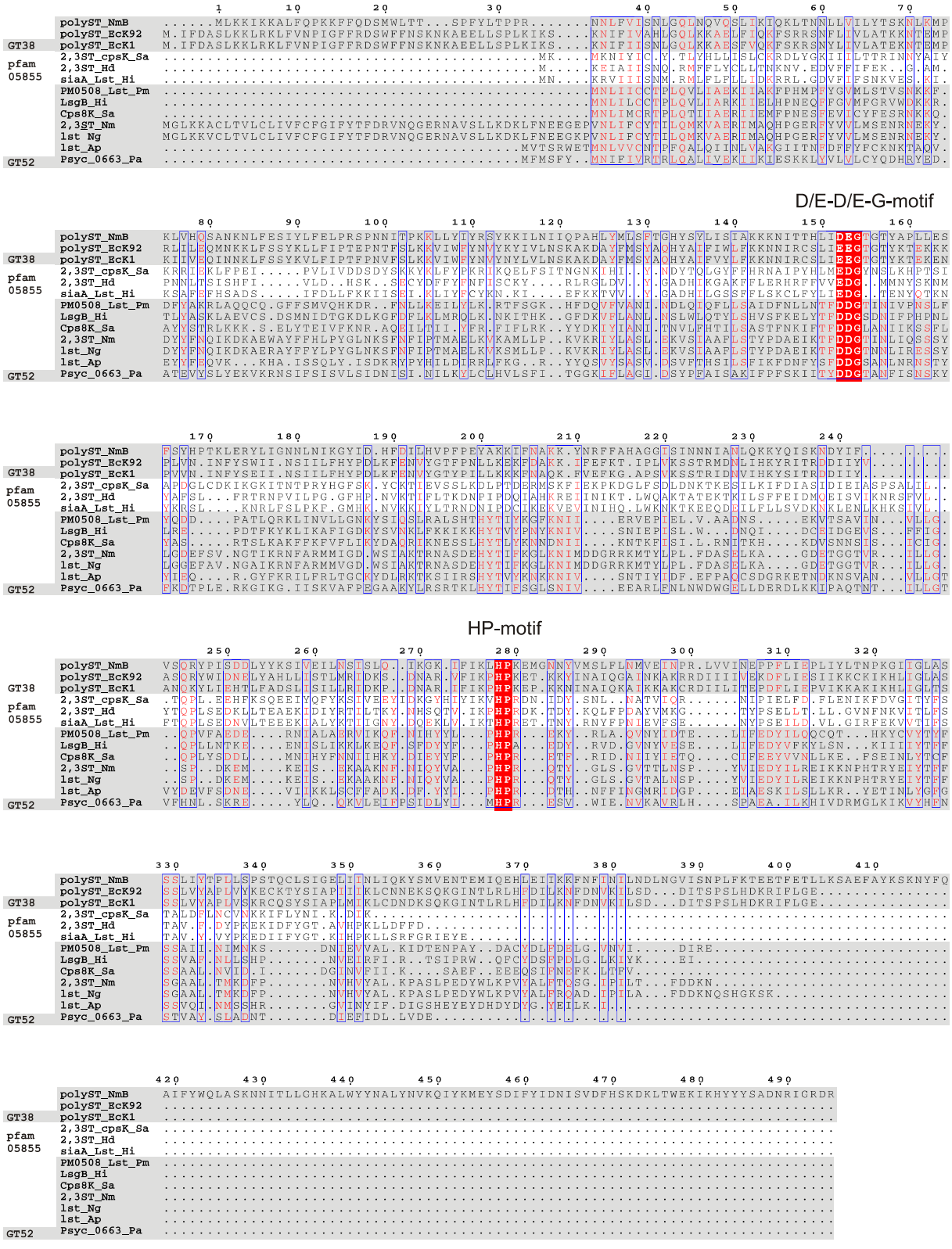

Fig 8. Conserved motifs in bacterial sialyl- and polysialyltransferases. Sequence alignment of bacterial sialyltransferases identifying two short motifs conserved in CAZy families GT-38 (bacterial polysialyltransferases), GT-52 (bacterial LOS-sialyltransferases) and in pfam family 05855 (similar to *H. ducreyi* sialyltransferase Lst). To improve clarity of the illustration only three representatives of pfam 05855 are shown. PolyST_*Nm*B (AAA20478), polyST_EcK92 (AAA24215), polyST_EcK1 (CAA43053), 2,3ST_cpsK_Sa (EAO062164), 2,3-ST_Hd (AAD28703), SiaA_Lst_Hi (AAL38659), PM0508_Lst_Pm (AAK02592), LsgB_Hi (AAX88755), Cps8K_Sa (AAR29926), 2,3_ST_*Nm* (AAC44544), lst_Ng (AAY41933), lst_Ap (AAS66624), Psyc_0663_Pa (AAZ18522). Alignments were made using Multalign (Corpet, 1988).

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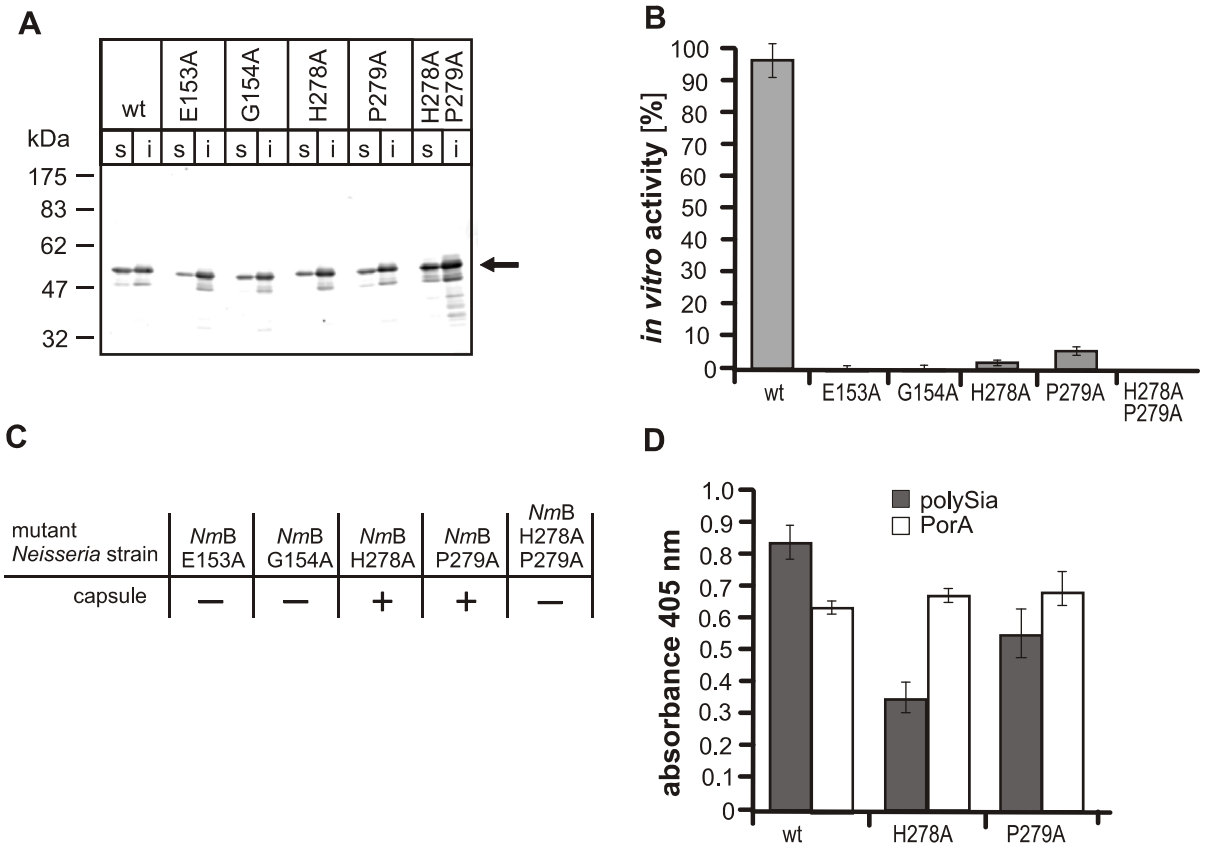

Fig. 9. Analysis of *Nm*B-polyST mutants *in vivo* and *in vitro.*
(A) Single point mutations were introduced into the D/E-D/E-G and HP-motif of *Nm*B-polyST. Wild-type and mutant enzymes were expressed in *E. coli* and soluble (s) and insoluble (i) fractions of the bacterial lysates were analysed for expression by SDS-PAGE and Western blotting using anti-T7-tag antibody. (B) PolyST activity was determined in protein lysates with similar expression levels by the radiochemical assay. Relative activities were calculated compared to the wild-type enzyme (100%). (C) Mutant strains of *N. meningitidis* were generated by replacing the native polyST gene with mutant polySTs carrying the respective point mutations. Capsule expression was analysed with the polySia specific antibody 735 by slide agglutination. (D) Capsular polySia of mutant strains with residual capsule expression was quantified by ELISA using mab 735. Equal loading of the wells with *Nm*B was controlled using mab P1.7 for detection of the meningococcal major outer membrane protein PorA. Each value represents the average of three independent determinations with the standard deviation indicated.

An enzyme linked assay system for the functional characterisation of purified poly-sialyltransferases.

With the aim to compare wild-type and mutant polySTs with partial activity (particularly mutants H278A and P279A) we decided to perform kinetic studies. As a first step towards this goal the glycosyltransferase assay developed by Gosselin and co-workers (Gosselin *et al.*, 1994) was adapted to assay polyST activity. This spectrophotometric assay links the release of CMP by polyST during polySia synthesis to NADH oxidation and thus enables continuous monitoring of the enzyme reaction. PolyST activity towards short oligosialic acid acceptors (DP2 to DP5) was measured at constant CMP-Neu5Ac and enzyme concentrations. As shown in Fig. 10A, efficient chain elongation required oligomers of at least DP3. These findings are in agreement with the acceptor dependence obtained when polyST containing membrane preparations of *E. coli* K1 or K92 were assayed in radiochemical or HPLC-based test systems (Steenbergen and Vimr, 1990; Ferrero *et al.*, 1991; Chao *et al.*, 1999).
Subsequently, kinetic parameters of MBP-*Nm*B-polyST were determined for the donor substrate CMP-Neu5Ac (Figure 10B, Table 1). The calculated K_m value of 0.42 mM is five fold higher than that obtained for the membrane bound enzyme from *E. coli* K1 polyST (Kundig *et al.*, 1971; Vijay and Troy, 1975). However, because apart from enzyme source and preparation but also assay- and buffer conditions differ between earlier and current studies, further interpretation of the observed variations in K_m appears unreasonable. In summary, the enzyme linked assay provided a useful tool to carry out kinetic analysis of polySTs and greatly facilitated the acquisition of data which, until now, have required single point measurements and elaborate detection methods.

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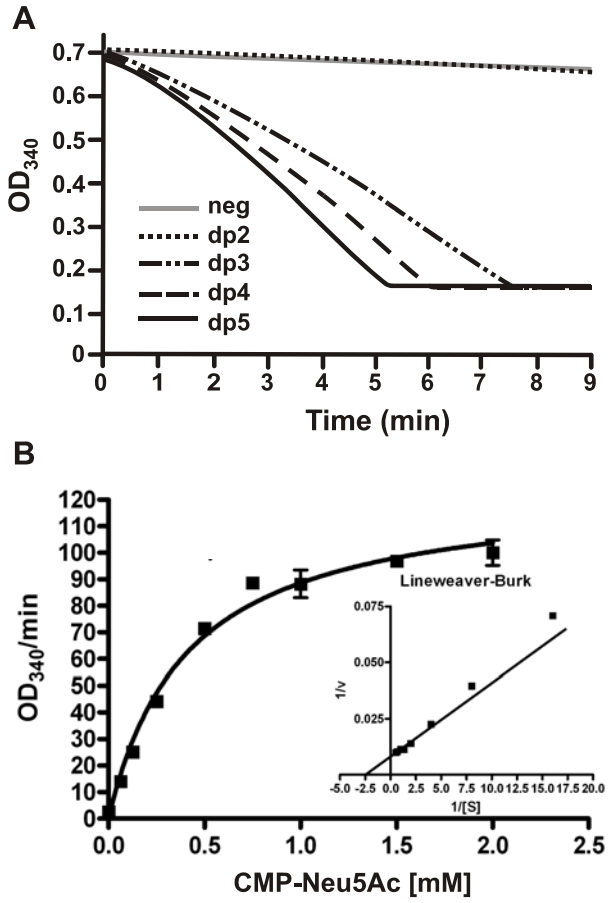

Fig. 10. Enzymatic characterisation of *Nm*B-polyST. (A) To analyze acceptor specificity, oligomeric α2,8-linked sialic acids with a degree of polymerisation (DP) ranging from DP2 to DP6 (0.5 mM) were assayed at constant enzyme (4 µM) and CMP-Neu5Ac concentration (1 mM) using the continuous spectrophotometric assay. PolyST activity is detected as decreasing absorption at 340 nm (NADH oxidation). (B) To determine K_m and V_{max} of *Nm*B-polyST for the donor substrate CMP-Neu5Ac, measurements were performed at 30°C and 0.28 mg/ml colominic acid. The resulting substrate velocity curve and Lineweaver-Burk plot are depicted. Kinetic parameters were obtained by nonlinear regression in Prism (GraphPad Software).

The HP-motif is involved in CMP-Neu5Ac binding.

Because *Nm*B-polySTs carrying the point mutations H278A and P279A retained residual activity *in vitro*, kinetic properties of these mutants could be determined. Both mutants were expressed as MBP-fusion proteins, purified and tested in the enzyme-linked polyST assay. As listed in Table 1, V_{max} values for CMP-Neu5Ac were decreased by factor 4 (P279A) and 6 (H278A) with respect to the wild-type enzyme and, in both proteins, K_m values for CMP-Neu5Ac were 3- to 5-fold increased. These data suggest that the HP-motif of *Nm*B-polyST is involved in binding of the donor substrate CMP-Neu5Ac. To further analyse the effect of both mutations on acceptor binding, K_m values for colominic acid were determined. Interestingly, Michaelis constants were not significantly influenced by either mutation indicating that (i) the HP-motif does not vitally participate in acceptor binding and that (ii) the introduced point mutations did not cause major structural changes since the acceptor binding site appeared to be largely unaffected. Remarkably, the recently solved crystal structure of *Pasteurella multocida* sialyltransferase *Pm*ST1 (Ni *et al.*, 2006) revealed a similar CMP-Neu5Ac binding function of the HP-motif in the GT-80 sialyltransferase family. The enzyme consists of two Rossmann domains that form a deep cleft in which the active site is located and has been crystallised in the presence and absence of donor and acceptor substrates. The active site of *Pm*ST1 with bound donor analogue CMP-3F(α)Neu5Ac and acceptor lactose (Ni *et al.*, 2007) is shown in Figure 11 and illustrates that the histidine residue of the HP-motif (H311 in *Pm*ST1) is directly involved in CMP-Neu5Ac binding. It forms hydrogen-bonds to the phosphate group of CMP and to the carboxylate-function of the sialic acid moiety. Moreover, this structure shows that also the D/E-D/E-G-motif is located directly at the active site cleft of *Pm*ST1 (DDG) and is involved in binding of donor and acceptor substrates. The second aspartic acid residue of the DDG-sequence forms hydrogen bonds to the acceptor lactose and to the hydroxyl-group in position C4 of the sialic acid moiety. In combination the structural and biochemical data

Table 1. Kinetic parameters of wild-type and mutant *Nm*B-polyST.

	CMP-Neu5Ac [1]		Colominic acid [2]
*Nm*B-polyST	V_{max} (µmol min^{-1} mg^{-1})	K_m (mM)	K_m [3] (mM)
WT	25.8 ± 3.6	0.42 ± 0.03	0.63 ± 0.10
H278A	4.0 ± 1.1	1.37 ± 0.12	0.40 ± 0.14
P279A	6.0 ± 1.0	2.15 ± 0.51	0.51 ± 0.13

[1] Determined at constant acceptor concentration of 0.28 mg/ml colominic acid.
[2] Determined at constant donor concentration of 1 mM CMP-Neu5Ac
[3] Kinetic constants were calculated based upon an average chain length of 32 residues for colominic acid as estimated using the thiobarbituric acid assay procedure according to Skoza and Mohos (Skoza and Mohos, 1976)
Kinetic values were obtained using the continuous polysialyltransferase assay and are presented as the means ± s.e. of three independent determinations.

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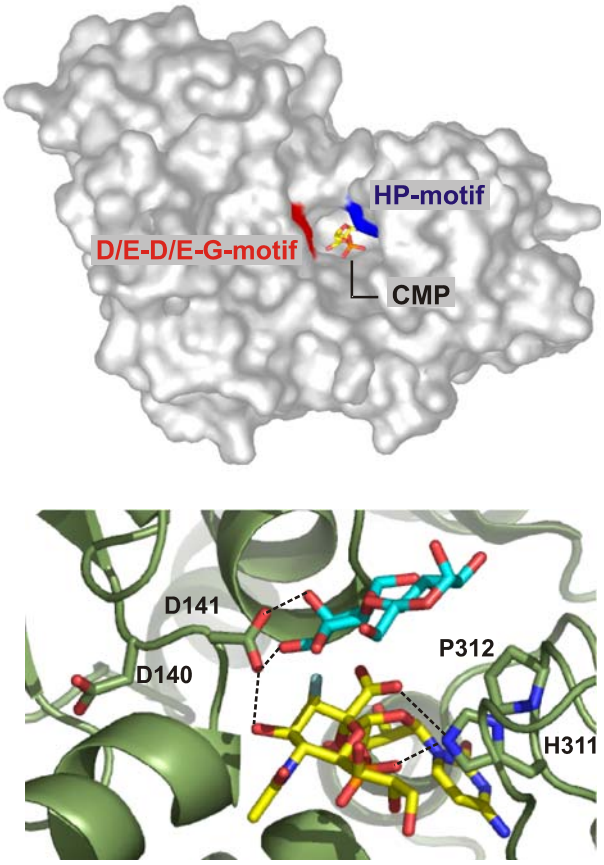

Fig. 11. Location of the conserved HP- and D/E-D/E-G-motifs in *P. multocida* sialyltransferase *Pm*ST1. (A) Surface representation of *Pm*ST1 (PDB entry 2EX1). The motifs are coloured in red (D/E-D/E-G) and blue (HP) while bound CMP is shown in yellow stick representation. (B) Active site view of *Pm*ST1. Amino acids that are part of the two motifs are labelled and depicted in green stick representation. Hydrogen bonds are shown as dotted line while the bound donor analogue CMP-3F(α)-Neu5Ac is depicted in yellow and the bound acceptor lactose is shown in cyan. Figures were generated with Pymol (http://www.pymol.org).

provide strong evidence that the D/E-D/E-G- and HP-sequences are crucial for CMP-Neu5Ac binding and enzyme catalysis, in bacterial sialyl- and polysialyltransferases that harbour these motifs.

Discussion

In this study, we functionally characterised the polysialyltransferase responsible for capsule biosynthesis in *Neisseria meningitidis* serogroup B. Though polySTs are interesting therapeutic targets to combat these pathogens, no data on isolated proteins and virtually no information on structure-function-relationships, which are crucial for the rational design of inhibitors, have been reported. Attempts to purify or solubilise membrane associated polySTs failed or resulted in inactivation of the enzymes, so that membrane association was proposed to be essential for polyST activity (Steenbergen and Vimr, 2003; Vionnet *et al.*, 2006).

With the aim to overcome the lack in structure-functional information we started this study by screening for production systems that enable expression and purification of active *Nm*B-polyST. Soluble expression of active protein was significantly improved after addition of large N-terminal fusion parts and allowed purification of *Nm*B-polyST fused to maltose binding protein. Purified MBP-*Nm*B-polyST was enzymatically active and, as described for membrane bound bacterial polySTs of *E. coli* K1 and K92 (Steenbergen and Vimr, 1990; Ferrero *et al.*, 1991; Chao *et al.*, 1999), able to synthesize long polySia chains from oligomeric primers of at least DP3. Consequently, our data provide clear evidence that membrane association is not a prerequisite for *Nm*B-polyST activity. Furthermore, we demonstrate that the purified enzyme elongates polySia chains in a non-processive manner. This is in contrast to *in vivo* studies carried out in *E. coli* K1 that suggest a processive mode of polySia biosynthesis (Steenbergen and Vimr, 2003). PolySTs were proposed to be part of a large capsule biosynthesis complexs, in which biosynthesis and translocation of polySia across the inner and outer bacterial membranes are tightly linked (Steenbergen and Vimr, 2003) and the functional *E. coli* K92 polysialyltransferase complex was found to be larger than a monomer (Vionnet *et al.*, 2006). Thus, potential interaction partners of polyST could increase the efficiency of polySia biosynthesis and thereby increase processivity. We (W.V. and J.V.) recently demonstrated that also membrane bound *E. coli* K92 polyST is a non-processive enzyme *in vitro* (Vionnet and Vann, 2007). But the utilised membranes also lacked other gene products of the K92 capsule biosynthesis cluster. However, even polySia biosynthesis assayed with intact membrane preparations of *E. coli* K1, which most likely include all relevant interaction partners of polyST, was found to be less efficient with exogenously added sialyloligomers than with endogenous or lipid bound acceptors (Cho and Troy, 1994; Chao *et al.*, 1999; McGowen *et al.*, 2001). This may argue for a more effective binding of the endogenous acceptor and hence result in increased processivity. In conclusion, the finding that recombinant soluble *Nm*B-polyST is non-processive does not exclude processivity of the enzyme in the living system. The investigation of functional properties of recombinant *Nm*B-polyST in complex with other factors of the capsular biosynthetic machinery is, therefore, an important aim in future studies. Interestingly, in this regard is the finding that MBP-*Nm*B-polyST expressed in a polyST deficient *Neisseria* strain complemented the defect (see Fig. 3). The fusion protein may thus provide an interesting tool for studies aimed at identifying polyST mediated protein-protein-interactions *in vivo.*

Mapping of the minimal catalytic domain of *Nm*B-polyST has shown that the C-terminal domain, though not conserved in the homologous *E. coli* enzymes, is essential for catalytic activity. All C-terminally truncated *Nm*B-polySTs were completely inactive. Studies are underway to analyse if potential

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variations in folding, in the oligomerisation status or in substrate binding properties are responsible for inactivation of the truncated polySTs (Fig. 2).

To characterize the catalytic domain of *Nm*B-polyST in more detail, we aimed at identifying key residues for polyST function. So far no functional residues have been described for bacterial polysialyltransferases and no sequence relationships to other bacterial sialyltransferases have been reported. In contrast to the eukaryotic sialyltransferases, which are grouped into a single CAZy family (GT-29) and harbour four highly conserved and well described sialylmotifs (Drickamer, 1993; Livingston and Paulson, 1993; Geremia *et al.*, 1997; Jeanneau *et al.*, 2004) the less homologous bacterial sialyltransferases are found in four different CAZy families. GT-38 contains bacterial polysialyltransferases, while GT-42, GT-52 and GT-80 include bacterial LOS sialyltransferases. Heretofore no conserved sequence features relating members of different CAZy families were described. In the current study we combined sequence alignments and visual inspections and identified two short motifs in bacterial sialyltransferases, the D/E-D/E-G-motif and the HP-motif. Both motifs are conserved throughout the CAZy families GT-38 and GT-52 and GT-80, with the only exception of a *Haemophilus ducreyi* sialyltransferase (AAP9506, HA instead of the HP), and are in addition found in pfam family 05855. The latter contains bacterial sialyltransferases similar to the Lst of *Haemophilus ducreyi* that have not yet been included in the CAZy database. By mutagenesis studies of *Nm*B-polyST we demonstrate that the D/E-D/E-G-motif is essential for polyST activity. Single amino acid substitutions within this motif (E153A and G154A) completely abolished enzyme activity *in vitro* and *in vivo*. By contrast, the simultaneous mutation of H278 and P279 to alanine in the HP-motif is required to destroy polyST activity. Single mutations within this motif did not obstruct polyST activity but resulted in reduced capsule production and lowered catalytic efficiency of the enzyme as shown by *in vivo* studies and kinetic analysis of the purified mutants. Most interestingly, since the K_m of the donor substrate CMP-Neu5Ac was increased the residues forming the HP-motif are likely to be involved in CMP-Neu5Ac binding. The binding of the acceptor colominic acid was, in contrast, not influenced by single mutations of the HP-motif since the determined Michaelis constant were not significantly affected. Moreover, the unaltered binding properties of the acceptor argue against severe misfolding of these mutants.

Support for the functional relevance of the D/E-D/E-G- and the HP-motif in bacterial sialyltransferases is furthermore provided by the recently solved crystal structure of the *Pasteurella multocida* sialyltransferase *Pm*ST1 (Ni *et al.*, 2006). *Pm*ST1 is a member of CAZy family GT-80 and belongs to the glycosyltransferase-B structural superfamily. Interestingly, this fold was also predicted for the bacterial polysialyltransferases of GT-38 including *Nm*B-polyST (Breton *et al.*, 2006). *Pm*ST1 harbours both motifs (D/E-D/E-G- and HP) close to its active site cleft, whereby the histidine residue of the HP-motif (H311) forms a hydrogen-bond to the phosphate group of bound CMP. Mutation of this residue to alanine resulted in a 30-fold reduction of activity and a 2-fold increase in K_m for CMP-Neu5Ac and it was suggested that H311 stabilises the CMP leaving group in *Pm*ST1 catalysis (Ni *et al.*, 2007). Since the H278A mutation had similar effects on the kinetic properties of *Nm*B-polyST, this residue may have a related function in polyST catalysis. So far no data on mutagenesis of the equivalent proline residue (P312) of *Pm*ST1 is available. However, mutation of this residue to alanine is likely to influence the structure of the loop harbouring the HP-motif and may thereby displace the histidine residue. A similar effect may explain the reduced activity of the P279A mutation in *Nm*B-polyST. Strikingly, also residues of the D/E-D/E-G-motif were found to be essential for *Pm*ST1 function. Though the first aspartic acid residue (D140) of the motif points towards the protein core and is most likely not involved in substrate interactions, the second aspartic acid (D141) protrudes into the active site cleft of *Pm*ST1 (Fig. 11). Moreover, this residue was shown to interact with the *Pm*ST1 acceptor lactose and suggested to act as general base in *Pm*ST1 catalysis. Mutation of D141 to alanine virtually inactivated the enzyme (20000-fold reduction of activity) (Ni *et al.*, 2007). This is again in perfect agreement to the inactive E153A mutant of *Nm*B-polyST and may suggest an analogous function of this residue in polyST catalysis. Indirect evidence that the protein stretch containing the conserved D/E-D/E-G-motif is located close to the active site also in CAZy GT-52 family members was provided by Wakarchuk and coworkers (Wakarchuk *et al.*, 2001). They showed that the neisserial α2,3-sialyltransferase Lst switches to a bifunctional α2,3/6-sialyltransferase mode upon mutation of the single residue G168 and predicted this residue to be positioned in an acceptor binding cavity. Interestingly, G168 is found only two residues upstream of the D/E-D/E-G-motif (residues 164-166) in this enzyme. Finally it should be mentioned that structural information is available for a second sialyltransferase the cst-II from *Campylobacter jejuni* (Chiu *et al.*, 2004). However, this enzyme is a member of CAZy-family GT-42, which is the only bacterial sialyltransferase family so far that does not harbour HP- and D/E-D/E-G-motifs.

In conclusion, the establishment of efficient expression, purification and assay procedures for *Nm*B-polyST allowed us to identify and characterize key groups for polyST function. Alignments with bacterial sialyltransferases revealed two functional motifs, the D/E-D/E-G- and the HP-motif, that are conserved in otherwise unrelated bacterial sialyltransferases of CAZy families GT-38, GT-52 and GT-80 as well as in pfam 05855. The functional importance of both motifs for enzyme catalysis and/or CMP-Neu5Ac binding was demonstrated by mutational analysis of *Nm*B-polyST and is emphasised by structural and biochemical data available for the *Pasteurella multocida* sialyltransferase *Pm*ST1. Our data therefore allow to hypothesise that basic features of substrate binding and enzyme catalysis are conserved in a wide range of bacterial sialyltransferases and improve the basis for design of sialyltransferase specific drugs.

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Experimental procedures

Materials.

Colominic acid and CMP-Neu5Ac were purchased from Sigma. Oligomeric sialic acids were from Nacalai Tesque. The pMAL-c Vector was from New England Biolabs, pET vectors were purchased from Novagen.

***Cloning of* Nm*B- polyST expressionvectors*.**

*Nm*B-polyST was amplified by PCR using plasmid pUE3 (Frosch *et al.*, 1991) as template and the primer pair KS23 (5'-G CAT GGA TCC CTA AAG AAA ATA AAA AAA GCT-3') and KS41 (5'-GCA GGC GGC CGC TCT ATC TCT ACC AAT TCT-3') containing BamHI and NotI sites (underlined), respectively. The PCR product was subcloned into the respective sites of pET23a to result in an N-terminally T7- and C-terminally His_6-tagged construct (pET23a-*Nm*B-polyST). To generate an expression construct including an N-terminal NusA-fusion part followed by a StrepII-tag, adapters Strepa (5'-CTA GTG CTA GCT GGA GCC ACC CGC AGT TCG AAA AAG GCG CCC TGG TTC CGC GTG-3') and Strepas (5'-GAT CCA CGC GGA ACC AGG GCG CCCT TTT TCG AAC TGC GGG TGG CTC CAG CTA GCA-3') were inserted by adapterligation into the BamHI and SpeI restriction sites of pET43a generating pET43a-Strep. The *Nm*B-polyST gene was subcloned from pET23a-*Nm*B-polyST using restriction sites BamHI and NotI to generate the expression vector pET43a-Strep-*Nm*B-polyST that encodes for the polyST fused with an N-terminal NusA/Strep-tag and a C-terminal His_6-tag. To obtain the vector pMBP-Strep, the maltose binding protein (MBP) was amplified by PCR with primers FF03 (5'-GAT ATT CAT ATG AAA ACT GAA GAA GGT AAA CT-3') and FF04 (5'-CAT ATA CTA GTC CTA CCC TCG ATG GAT CC-3') from the vector pMAL-c and subsequently subcloned into the NdeI and SpeI restriction sites of pET43a-Strep. Finally, *Nm*B-polyST was subcloned from pET43a-Strep-*Nm*B-polyST using the NheI/NotI sites resulting in pMBP-Strep-*Nm*B-polyST that encodes for the polyST fused with an N-terminal MBP/Strep-tag and a C-terminal His_6-tag.

Generation of Truncated Proteins.

N-terminally truncated *Nm*B-polySTs were generated by PCR using the forward primers IO07 (5'- GCA TGG ATC CAC ATC TCC ATT TTA TCT TAC-3') for Δ23 *Nm*B-polyST, IO08 (5'- GCA TGG ATC CAA CAA TTT ATT TGT CAT ATC TA-3') for Δ33 *Nm*B-polyST and IO09 (5'- GCA TGG ATC CTT ATA TAC TTC TAA AAA CTT AAA A-3') for Δ64 *Nm*B-polyST and the reverse primer KS41 (5'- GCA GGC GGC CGC TCT ATC TCT ACC AAT TCT-3'). C-terminally truncated *Nm*B-polySTs were generated by PCR using the reverse primers FF01 (5'- GCA TGC GGC CGC ATC TTT ACT ATG AAA GTC-3') for *Nm*B-polyST Δ 22, FF02 (5'- GCA TGC GGC CGC CCC TAA TAA GGT AAT ATT G -3') for *Nm*B-polyST Δ 60, KS335 (5'- G CAG GCG GCC GC TTC AAA TGT TTC TTC TGT TTT AAA-3') for *Nm*B-polyST Δ94, KS334 (5'-GC AG GCG GCC GC AAA TGT TTC TTC TGT TTT AAA GA-3') for *Nm*B-polyST Δ95 and FF02 (5'- GCA GGC GGC CGC TTC TTC TGT TTT AAA GAG AG -3') for *Nm*B-polyST Δ97and the forward primer KS333 (5'- G CAT GGA TCC CTA AAG AAA ATA AAA AAA GCT CTT-3'). BamHI and NotI sites (underlined) in forward and reverse primers, respectively, were used for subcloning of the PCR products into the BamHI/NotI sites of pET43a-Strep. The resulting constructs encode for proteins with an N-terminal NusA-/Strep-tag and a C-terminal His_6-tag. The identity of all constructs was confirmed by sequencing.

Site-directed mutagenesis.

Single point mutations of *Nm*B-polyST were obtained by QuickChange site directed mutagenesis (Stratagene) following the manufacturers instructions using the plasmid pET23a-*Nm*B-polyST as template. Mutated polySTs were subsequently subcloned into the BamHI and NotI sites of pET23a resulting in expression of N-terminally T7-tagged proteins. The identity of all constructs was confirmed by sequencing. Mutagenic primers are given below with the mutated base triplets underlined: pET23a-G154A: KS52 (5'-G ACT CAT TTA ATT GAT GAA GCG ACT GGA ACA TAT GCT CC-3') and KS53 (5'-GG AGC ATA TGT TCC AGT CGC TTC ATC AAT TAA ATG AGT C-3'); pET23a-E153A: KS54 (5'-T ACG ACT CAT TTA AT T GAT GCA GGG ACT GGA ACA TAT GC-3') and KS55 (5'-GC ATA TGT TCC AGT CCC TGC ATC AAT TAA ATG AGT CGT A-3'); pET23a-H278A: KS56 (5'-ATT AAA GGA AAG ATA TTT ATT AAA CTA GCC CCA AAA GAG ATG GGC AAC AAC-3') and KS57 (5'-GTT GTT GCC CAT CTC TTT TGG GGC TAG TTT AAT AAA TAT CTT TCC TTT AAT-3'); pET23a-P279A: KS58 (5'-GGA AAG ATA TTT ATT AAA CTA CAC GCA AAA GAG ATG GGC AAC AAC TA-3') and KS59 (5'-TA GTT GTT GCC CAT CTC TTT TGC GTG TAG TTT AAT AAA TAT CTT TCC-3'). Mutants H278A and P279A were additionally introduced into the pMBP-Strep vector. Therefore, the polyST inserts of pET23a-H278A and pET23a-P279A were ligated into pET43a-Strep using restriction sites BamHI and NotI und subsequently subcloned into pMBP-Strep using the NheI and NotI sites.

Expression of recombinant* Nm*B-PolyST.

To optimise protein production, bacteria were either cultivated at 30°C (only polySTs fused to NusA) or at 15°C. For production at 30°C, freshly transformed *E. coli* BL21(DE3) were grown in PowerBroth medium (Athena ES) containing 200 µg/l carbenicillin at 30 °C and 225 rpm. At an optical density OD_{600nm} = 1.8 expression was induced by adding 1 mM IPTG. Bacteria were harvested 3 h after induction by centrifugation (6000 xg for 15 min, 4°C). For 15°C production, bacteria were grown at 30°C to an optical density of OD_{600nm} = 0.9. Cultures were than rapidly cooled (ice bath) and further grown at 15°C

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until OD_{600nm} = 1.8 was reached. Transgene expression was induced by the addition of 1 mM IPTG and cells were harvested 24 h after induction. All pellets were washed once with PBS and stored at -20°C.

Separation of soluble and insoluble fractions of* Nm*B-PolyST.

To analyze *Nm*B-PolyST expression and enzymatic activity in bacterial lysates, cells were resuspended in 50 mM Tris-HCl pH 8.0, 40 mM $MgCl_2$ and lysed by sonication. Soluble and insoluble fractions were obtained following centrifugation (16000 xg, 15 min, 4°C). The insoluble fraction (pellet) was resuspendend in 50 mM Tris-HCl pH 8.0, 40 mM $MgCl_2$ in volume equal to that of the soluble fraction.

Purification of recombinant MBP-*Nm*B-PST fusion protein.

Bacterial pellets from 0.5 litre cultures were resuspended in binding buffer (20 mM Tris; pH 7.4; 1 mM EDTA; 1 mM DTT; 25 mM NaCl) including protease inhibitors (40 mg/ml Bestatin, 1 µg/ml Pepstatin and 1 mM PMSF) to give a final volume of 20 ml. Cells were disrupted by sonication and samples were centrifuged (30 min; 16000xg, 4 °C) and filtered (Sartorius Minisart 0.8 µm). For affinity absorption of MBP-*Nm*B-polyST, pre-swollen amylose resin (New England Biolabs) was added to the cleared supernatant and incubated at 4 °C for 1 h. Subsequently, the incubation mixture was transferred to a column and washed with 12 volumes of binding buffer at a flowrate of 0.5 ml/min. Bound protein was eluted with elution buffer (binding buffer containing 10 mM maltose). Fractions containing the fusion protein were pooled and passed through a desalting column (High Prep 26/10) equilibrated in 50 mM NaH_2PO_4 pH 8.0 and finally concentrated to 2 mg/ml using Amicon Ultra centrifugal devices (Millipore). To further enrich the MBP-*Nm*B-polyST fusion protein, a gel filtration chromatography step was applied. Samples were filtered (Millipore Ultrafree MC 0.2 µm) and loaded on a Superdex 200 10/300 GL column (GE Healthcare). Proteins were eluted at a flowrate of 0.5 ml/min with 50 mM NaH_2PO_4 pH 8.0 buffer and fractions of 0.5 ml were collected. Obtained protein samples were stable for more than three weeks if stored at 4 °C.

***SDS-PAGE and Immunoblotting*.**

SDS-PAGE was performed under reducing conditions using 2.5 % (v/v) β-mercaptoethanol and 1.5 % (w/v) SDS. For Western blot analysis, proteins were blotted onto nitrocellulose (Whatman). Proteins containing an N-terminal StrepII-tag were detected by StrepTactin-alkaline phosphatase-conjugate (StrepTactin-AP; IBA) according to the manufacturer's guidelines. His-tagged proteins were detected with 1 µg/ml penta-His antibody (Qiagen) followed by goat anti-mouse-IgG-AP (Dianova). For quantification by infrared fluorescence detection, samples and standard proteins were blotted onto PVDF membranes (Millipore). His-tagged proteins were detected with 1 µg/ml penta-His antibody (Qiagen) followed by 50 ng/ml of goat-anti-mouse IR680 antibody (LI-COR) and quantified according to the recommendations of the Odyssey infrared imaging system (LI-COR).

Radiochemical polyST assays.

PolyST activity in bacterial lysates was analysed as described previously (Weisgerber, 1990). Briefly, 10 µl lysate were mixed with 10 µl TMD-buffer (40 mM $MgCl_2$, 50 mM, Tris/HCl pH 8.0) and 2 µl colominic acid (100 mg/ml) as acceptor and reactions were started by adding 2 µl CMP-[^{14}C]Neu5Ac (13 mM, 1.55 mCi/mmol). Samples were incubated at 37°C and 5µl aliquots were spotted on Whatman 3MM CHR paper after the respective reaction time. Following descending paper chromatography, the chromatographically immobile ^{14}C-labelled polyST reaction products were quantified by scintillation counting. To analyse the products of purified *NmB*-polyST in more detail, reaction mixtures were analysed in the TBE-buffered (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) electrophoresis system described for analysis of acidic capsular polysaccharides (Pelkonen *et al.*, 1988). Enzyme reactions were carried out in TMD in a total volume of 24 µl as described above, using 10 µg enzyme and 0.001 to 1 mM α2,8-linked sialic acid (DP5) as acceptor. Control samples were subsequently treated with 1 µg polySia degrading endoN (Stummeyer *et al.*, 2005) for 20 min at 37 °C. Equal volumes of sample buffer (2 M sucrose in TBE) were added and samples were electrophoresed at 4 °C and 200 V over night. To visualize ^{14}C-labelled reaction products, gels were vacuum-dried immediately after electrophoresis and exposed to an imaging film (BioMax, Kodak)

Fluorescent polyST assays.

PolyST activity of purified *Nm*B-polyST was also monitored using the fluorescent acceptor GT3-FCHASE as acceptor (Vionnet and Vann, 2007). Briefly, GT3-FCHASE (0.23 µM), CMP-Neu5Ac (50-500 mM) and purified *Nm*B-polyST (30-180 µg/ml) were incubated in TMD-buffer (40 mM $MgCl_2$, 50 mM, Tris/HCl pH 8.0) at 37 °C. At the indicated time points (2-30 min) reactions were stopped by adjusting to 25% ethanol and samples were further analysed by HPLC as described (Vionnet and Vann, 2007).

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Continuous spectrophotometric Polysialyltransferase assay.

For rapid characterisation of purified polysialyltransferases, the glycosyltransferase testing system described by Gosselin and co-workers (Gosselin *et al.*, 1994) was adapted to polysialyltransferases. All measurements were carried out in 96-half area well plates (Greiner Bio-one) in a total volume of 106.5 µl. In detail, a master solution was prepared containing the linking enzymes pyruvate kinase (16.5 U/ml, Sigma), lactate dehydrogenase (23.5 U/ml) Sigma) and nucleotide monophosphate kinase (0.5 U/ml, Roche) in reaction buffer (1.5 mM ATP (Sigma), 1 mM PEP (Fluka), 0.13 mM NADH (Roche), 14 mM $MgSO_4$, 56 mM KCl in 100 mM Tris pH 7.5). As acceptor substrate either colominic acid or sialyloligomers were added in various concentrations. After addition of the donor substrate CMP-Neu5Ac (62 µM to 2 mM) samples were first monitored at OD 340 nm until a stable baseline was reached (3-5 min). This was essential to metabolize free CMP, which is always present in CMP-Neu5Ac preparations due to hydrolysis of the substrate. Finally, PST was added (10-20 µg/ml) and reactions were followed until the total amount of NADH was metabolised.

Generation and analysis of mutant* Neisseria *strains.

For mutagenesis of *Neisseria meningitidis* strains, the plasmids pET23a-G154A, pET23a-E153A, pET23a-H278A and pET23a-P279A needed to be modified to allow regular homologous recombination into the meningococcal chromosome and selection for the mutants. The *siaD* downstream region was amplified from serogroup B strain MC58 with primers GH149 (5'-GCG CGC CTC GAG AAT ACT ATG ACT TCT GA TCT CC-3') and GH150 (5'-GCG CGC CTC GAG CGA GTA ATT TGA CAA TAG AGC G-3') and integrated into each plasmid downstream of the mutated *siaD* gene using the respective XhoI sites (underlined). The resulting plasmids were linearised with NotI, blunt ended with T4 DNA polymerase and ligated with the kanamycin resistance cassette excised from pUC4K (GE Healthcare) by HincII. The final plasmids harbouring the kanamycin resistance cassette between the *siaD* gene and the *siaD* downstream region were used to transform serogroup B meningococcal strain MC58. Transformants were selected on GC agar supplemented with 100 µg/ml kanamycin. Recombination of the mutagenised motifs into the meningococcal siaD gene was verified by sequencing the PCR product obtained with primer pair GH157 (5'-CA GGC CAC TAC TCC TAT C TG-3')/Kana2 (5'-GAT TTT GAG ACA CAA CGT GG-3') with either primers GH157 and GH160 (5'-AGG TTC ATT AAT AAC TAC CAG C-3') (D/E-D/E-G-motif) or primers UE8a (5'-AA CGC TAC CCC ATT TCA-3') and GH160 (HP-motif). Furthermore, regular homologous recombination was confirmed by Southern blot hybridisations with the *siaD* gene and the kanamycin resistance gene used as a probe, respectively. The meningococcal capsule phenotype was analysed with mab735 by slide agglutination as described previously (Vogel *et al.*, 2001). Quantitative analysis of capsule expression was performed by whole-cell ELISA as described (Vogel *et al.*, 2001). Briefly, microtiter plates were precoated with poly-D-lysine (25 µg/ml in PBS) for 1 h at room temperature. After three washing steps with PBS, bacterial suspensions (20 µl/well, OD_{600}=0.10 in PBS) were applied for 2 h and cross-linked to poly-D-lysine by adding glutaraldehyde (0.05% in PBS) for 10 min. Plates were washed three times with PBS and nonspecific binding sites were saturated by incubation with 1% BSA in PBS for 1h. After three washing steps capsular polySia was detected by immunostaining using the polySia specific mab 735. The amount of bacteria bound to each well was controlled with a parallel set of microplates using mab P1.7 directed against the PorA antigen of the meningococcal strain MC58 for detection. Subsequently, plates were incubated with peroxidase coupled secondary antibody (Dianova) and analysed by colour reaction.

In vivo *analysis of MBP-*Nm*B-polyST.*

Neisserial expression constructs were generated for *in vivo* comparison of wild-type and MBP-*Nm*B-polyST. The MBP-polyST insert of the *E. coli* expression construct pMBP-Strep-*Nm*B-polyST was amplified with primers HC574 (5'-GCG CGC TCT AGA GAA GGA GAT ATA CAT ATG AAA AC-3') and HC572 (5'-GCG CGC GAT ATC TTA GTG GTG GTG GTG GTG G-3') and integrated between the SpeI and EcoRV sites of the neisserial expression vector pAP1 (Lappann *et al.*, 2006) resulting in pAP1-MBP-*Nm*B-polyST. For comparison also the polyST gene without further tags was amplified with primers HC573 (5'-GCG CGC GAT ATC AGA GAT ACA ATA ATG CTA AAG AAA ATA AAA AAA GC-3') and HC572 and integrated into the EcoRV site of pAP1 resulting in pAP1-*Nm*B-polyST. Further *in vivo* studies were performed using strain 2517, an unencapsulated polyST knock-out mutant of the meningococcal serogroup C strain 2120 (Ram *et al.*, 2003) that was transformed with the resulting plasmids. Wild-type and transformants were analysed for capsule expression by whole-cell ELISA as described above. Bacterial loading was controlled using anti-PorA antibody P1.2. Additionally, neisserial lysates were analsed by Western blot analysis. Bacteria were pelleted from 2 ml suspension cultures (OD_{600}=1.1), resuspended in 100 µl of Lämmli sample buffer (100 mM Tris pH 6.8, 1.7% SDS, 16.5% glycerol (v/v), 2.5% 2-mercaptoethanol, bromophenol blue) and lysed by sonication. The lysates were centrifuged (16.000 xg, 10 min, 4°C) and supernatants were divided in two aliquots. One aliquot was subsequently digested with 1 µg endoN (Stummeyer *et al.*, 2005) and incubated for 15 min at room temperatur followed by a 15 min incubation at 37°C. All samples were incubated for 10 min at 60°C prior to electrophoresis. SDS-PAGE and Western blot were performed as described above.

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Supplementary material

The following supplementary material is available for this article:
Fig. S1. Elongation of trimeric a-2,8-linked sialic acid (DP3) by purified *Nm*B-polyST.
This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05862.x
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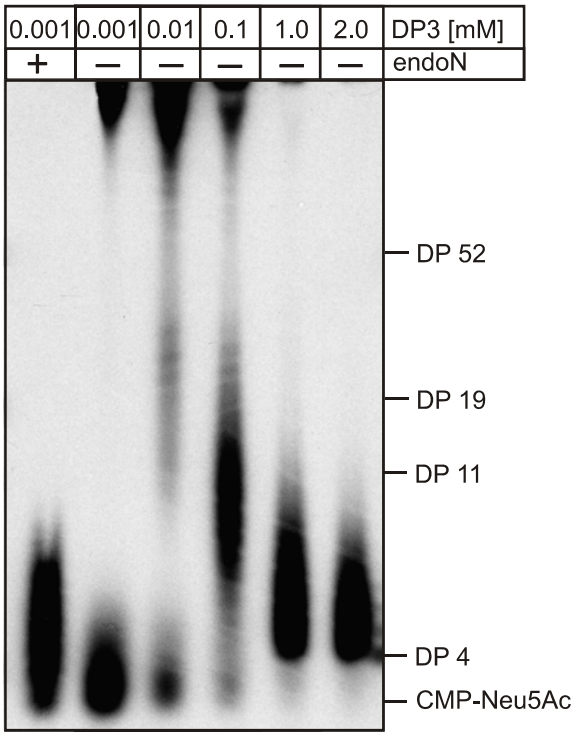

Figure S1. *Elongation of trimeric α-2,8-linked sialic acid (DP3) by purified NmB-polyST.* (A) Purified *Nm*B-polyST was assayed for 30 min in the presence of 1 mM CMP-[C14]Neu5Ac and increasing concentrations of DP3 as indicated. The sample including 0.001 mM DP3 was prepared as duplicate to subsequently digest the extra probe with polySia-specific endoN. Radiolabelled reaction products were separated by acrylamide-gelelectrophoresis (25 %) and detected by autoradiography. The following dyes were used as standards and correspond to polySia chain length given in brackets: trypan blue (DP100), xylene cyanol (DP52) bromophenol blue (DP19), bromcresol purple (DP11), phenole red (DP4).

Chapter 3

Neisseria meningitidis Serogroup B Polysialyltransferase: Insights into Substrate Binding

Friedrich Freiberger[b][+]; Raphael Böhm[a][+]; Katharina Stummeyer[b]; Rita Gerardy-Schahn[b]; Mark von Itzstein[a] and Thomas Haselhorst[a]

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Neisseria meningitidis Serogroup B Polysialyltransferase: Insights into Substrate Binding

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Preface – About the Manuscript

In this study for the first time binding properties of a polysialyltransferase and epitope mapping to its substrates are described. Nuclear magnetic resonance (NMR) as well as Saturation Transfer Difference-NMR (STD-NMR) studies on the polysialyltransferase of *Neisseria meningitidis* Serogroup B was done at the Griffith University, Gold Coast Campus, Australia.

I could shown that the CMP moiety of the donor sugar CMP-Neu5Ac is bound more tightly to the enzyme than the sialic acid residue to ensure an efficient glycosyl transfer. Furthermore I demonstrated the complete binding of DP3 by the polysialyltransferase by means of STD-NMR analysis. Finally an NMR based assay for activity without the need of labelled substrates or additional enzymes is presented. My contribution to this work comprises the purification of the polyST, establishment of the used protocols and all measurements, which were repeated and confirmed by Raphael Böhm who is the equal contributing author. Dr. Thomas Haselhorst, Raphael Böhm, and I wrote the paper.

Neisseria meningitidis Serogroup B Polysialyltransferase: Insights into Substrate Binding

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The polysialyltransferase from *Neisseria meningitidis* serogroup B (*Nm*B-polyST) catalyses the transfer of sialic acid (Sia) from activated sialic acid donor cytidine-5`monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) onto sialic acid containing acceptors, to synthesise polysialic acid (polySia). To better understand how this enzyme interacts with its substrates, we determined the binding epitope of CMP-Neu5Ac and trimeric α(2,8)-linked polySia (DP3) in complex with *Nm*B-polyST by means of Saturation Transfer Difference (STD) NMR experiments. We have discovered that the sialic acid residue of the donor substrate CMP-Neu5Ac has fewer contacts with the protein than the cytosine and ribose moieties. This loose binding to the enzyme ensures a fast and efficient transfer of the sialic acid residue to the acceptor. Interestingly, the enzyme accommodates all three sialic acid residues of the polySia DP3 acceptor, but a slightly higher saturation transfer could be observed for the *N*-acetyl group of the reducing terminal sialic acid moiety. We hypothesize that a more open binding of the internal and non-reducing terminal sialic acid residues of the acceptor substrate may facilitate an efficient release of the synthesized polysialic acid product. A 1D ^{1}H NMR-based investigation to monitor the biosynthesis of polySia without the need of fluorescent or radiolabelled material is also reported. Our study presented here reveals for the first time structural information about how this enzyme interacts with both polySia and CMP-Neu5Ac, facilitating the design of novel anti-*Nm*B-polyST inhibitors.

The human pathogen *Neisseria meningitidis* serogroup B (*Nm*B) is a major cause of bacterial meningitis in developed countries. This bacterium expresses capsular polysaccharides that are chemically and immunologically identical to the polysialic acid (polySia) expressed in the host organism.[1] This so-called antigen mimicry and the hydrated, negatively charged capsule of *Neisseria* circumvent opsonisation and activation of the humoral immune system including the production of antibodies. Two enzymes are mainly responsible for the Synthesis of polySia: Cytidine-5'monophosphate-N-sialic acid synthetase (CMP-Sia synthetase, CSS) and polysialyltransferase (polyST). While a number of biochemical and structural studies have been published for the NmB-CSS,[2-5] the lack of purified native or recombinant NmB-polyST in suitable amounts has prevented detailed structure function analyses of this important enzyme. We recently reported [6] the successful production and biochemical characterization of NmB-polyST.[7-8] The polysialyltransferase was expressed as a fusion protein with a soluble maltose-binding protein tag (MBP-NmB-polyST), and was shown to be capable of producing Sia large polymers. Here, we report for the first time Saturation Transfer Difference (STD) NMR experiments [9-14] to map the binding epitope of both the sialic acid donor CMP-Neu5Ac and the minimal NmB-polyST acceptor [6] trimeric α(2,8)-linked sialic acid (DP3). Using a 1D 1H NMR-based approach we have also investigated the transfer of CMP-Neu5Ac onto DP3.

Interactions of donor and acceptor substrates with *Nm*B-polyST were investigated with STD NMR experiments. The lack of purified native or recombinant enzyme has prevented detailed structure-function relationship analysis of *Nm*B-polyST so far. We recently reported the first successful expression, purification and biochemical characterization of this enzyme [6] but no structural data have been reported. To close this gap and to better understand how this enzyme interacts with its substrates, we present here for the first time structural insights of *Nm*B-polyST and the binding epitope of sialic acid donor and acceptor substrates from STD NMR spectroscopy. Figure 1a shows a 1D 1H NMR spectrum of CMP-Neu5Ac in complex with 200 µg MBP-*Nm*B-polyST. The corresponding STD NMR spectrum shown in Figure 1c reveals strong

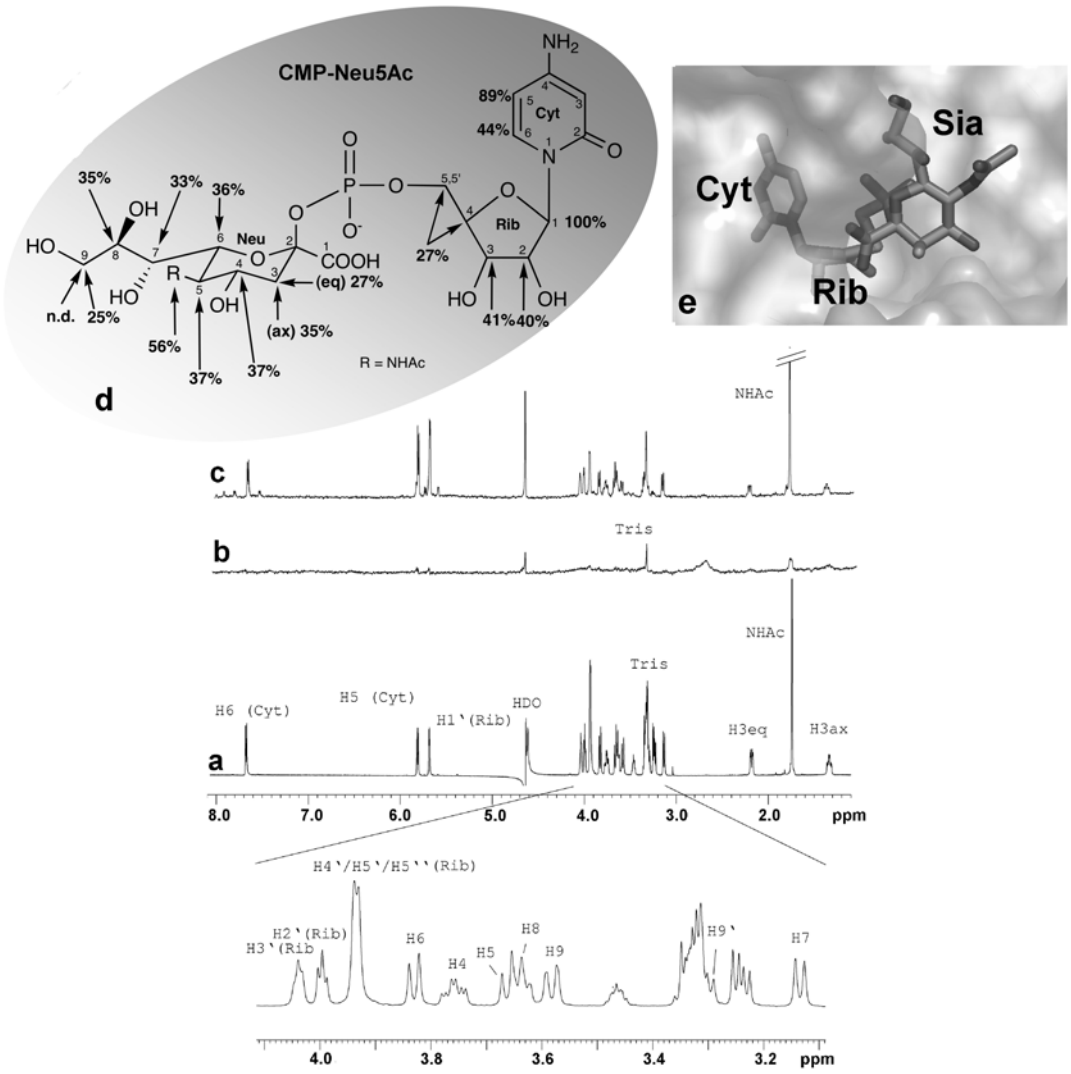

Figure 1. ^{1}H (a) and ^{1}H STD NMR experiments 0.8 mM of CMP-Neu5Ac in complex with maltose binding protein (MBP) (b) and MBP-*Nm*B-polyST fusion protein (c). The on-resonance frequency was set to -1.00 ppm and the off-resonance frequency to 33.3 ppm. The total saturation time was 2 sec. The residual water signal was removed using a WATERGATE sequence. All experiments were performed at 600 MHz and 283K in 20 mM deuterated TRIS containing 4 mM $MgCl_2$, pH 8.0. Epitope mapping of CMP-Neu5Ac when bound to *Nm*B-polyST (d). X-Ray crystal structure of the *Pasteurella multocida* sialyltransferase (*Pm*ST1) with bound CMP-3F(α)Neu5Ac (pdb code: 2IY7.pdb) [18] demonstrating a complete burial of the cytosine (Cyt) and ribose (Rib) moiety compared to the sialic acid portion (e).

STD NMR signals of activated sialic acid molecule. A control STD NMR spectrum of CMP-Neu5Ac when incubated with the maltose binding protein (MBP) is shown in Figure 1b. Only a few residual STD NMR signals, if any, can be detected leading to the assumption that CMP-Neu5Ac does not bind to protein MBP-tag and that the observed STD NMR signals shown in Figure 1c are genuine binding to NmB-polyST.

The binding epitope was determined by calculating relative STD effects according to the equation ASTD = (I0 - Isat) / I0 = ISTD / I0.[12] Intensities of the signals in the STD NMR spectrum (ISTD) were compared with intensities of signals in a reference spectrum (I0). The STD signal with the highest intensity was set to 100% and other STD signals were calculated accordingly (Figure 1d). It is obvious that protons associated with the ribose and cytosine moieties of CMP-Neu5Ac received the largest amount of saturation with the strongest STD NMR signals observed for H1 of the ribose unit (H1 Rib, 100%). The protons of the cytosine moiety received 89% (H5 Cyt) and 44% (H6 Cyt) saturation, whereas the sialic acid moiety received an average saturation of 36% demonstrating a less tight association with the protein. Since the sialic acid moiety has to be transferred to the acceptor molecule DP3 during the enzymatic reaction, it is reasonable that its contact with the protein's active site is weaker to ensure an efficient transfer.

The recently resolved x-ray crystal structure of the related Pasteurella multocida sialyltransferase

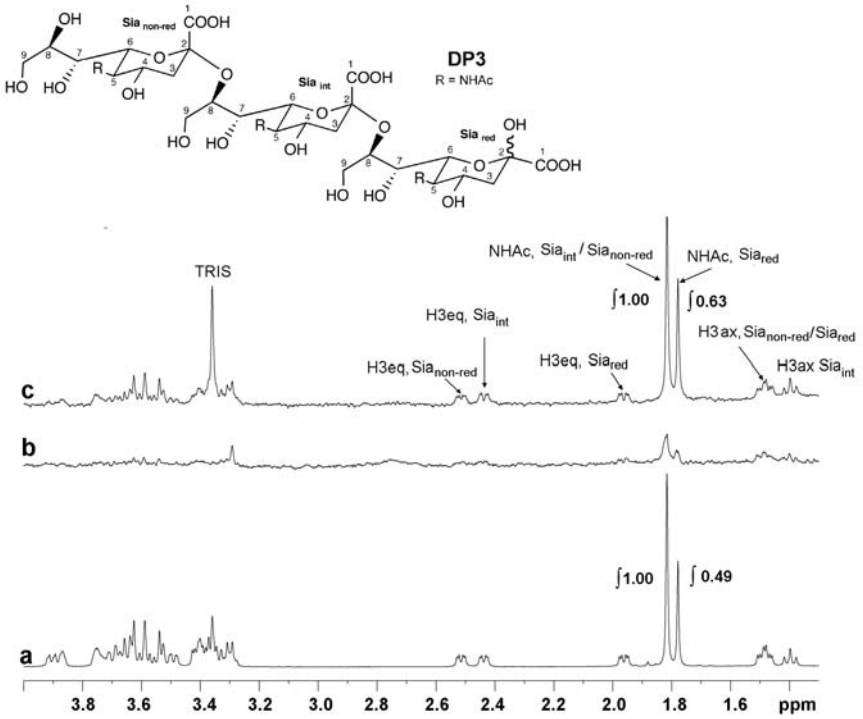

Figure 2. ^{1}H (a) and ^{1}H STD NMR experiments of trimeric α(2,8)-linked polySia (DP3) in complex with maltose binding protein (MBP) (b) and MBP-*Nm*B-polyST fusion protein (c). The on-resonance frequency was set to -1.00 ppm and the off-resonance frequency to 33.3 ppm. The total saturation time was 2 sec. The residual water signal was removed using a WATERGATE sequence. All experiments were performed at 600 MHz and 283K in 20 mM deuterated TRIS containing 4 mM MgCl2, pH 8.0.

(*Pm*ST1) with bound CMP-3F(α)Neu5Ac (pdb code: 2IY7.pdb) supports our hypothesis. The structure clearly shows the complete burial of the nucleotide moiety while the sialic acid moiety is more solvent exposed [15] as shown in Figure 1e. Interestingly, a similar effect was observed in an STD NMR spectroscopic analysis describing the binding epitope of UDP-Gal when bound to β(1,4)-galactosyltransferase T1.[16] In this previous study it was revealed that the galactose moiety of UDP-Gal, which is transferred by the enzyme to β-D-N-acetylglucosamine residues, receives less saturation from the protein than protons associated with the uracil and ribose moieties. This suggests a loose binding of the galactose residue thus ensuring an efficient glycosyl transfer.

The binding of polySia DP3, the minimal sialic acid acceptor molecule[6], was also evaluated with STD NMR spectroscopy. Figure 2a shows the ^{1}H NMR spectrum of a DP3:MBP-*Nm*B-polyST complex, whereas the corresponding STD NMR spectrum is shown in Figure 2c. Again, strong STD NMR signals reveal binding of the DP3 polySia acceptor molecule to the protein. To investigate whether or not the observed STD NMR signals were genuine binding to the *Nm*B-polyST portion and not to the MBP-fusion tag, an STD NMR experiment of polySia DP3 in the presence of MBP was conducted (Figure 2b). Only little, if any, signal intensity was observed, which clearly suggests that the observed STD NMR signals in Figure 2c are a genuine contribution of a polySia DP3:*Nm*B-polyST interaction. From the STD NMR signal intensity analysis (Figure 2c) it is obvious that the H3eq and H3ax protons of the Sia$_{non-red}$, Sia$_{int}$ and Sia$_{red}$ residues received a similar STD effect.

While the above data suggest that all three sialic acid residues of the DP3 acceptor molecule are in contact with the protein, the analysis of the STD NMR effects of the N-acetyl groups of DP3 revealed a slightly predominant interaction of the reducing terminal sialic acid residue (Sia$_{red}$). The integral value in the ^{1}H NMR spectrum (Figure 2a) of the N-acetyl signal from the non-reducing terminal (Sia$_{non\ red}$) and internal (Sia$_{int}$) sialic acid unit was set to 1.0 (equivalent to 6 protons). The corresponding integral of the signal of the reducing terminal sialic acid moiety (Sia$_{red}$) was 0.49 (equivalent to 3 protons). The analysis of the STD NMR spectrum (Figure 2c) revealed that the integral value of the reducing terminal sialic acid moiety (Sia$_{red}$) was 0.63 (equivalent to 3 protons) compared to the 6 protons of the non-reducing terminal (Sia$_{non-red}$) and internal (Sia$_{int}$) sialic acid unit (integral value of 1.0). This result led us to conclude not only that the N- acetyl group is crucial for binding, but also it would appear that the reducing terminal sialic acid moiety (Sia$_{red}$) receives slightly more saturation due to a close proximity to and interaction with the protein. It is not immediately obvious why this would be the case, since it appears that all three sialic acid residues can be accommodated within the active site but it is not unreasonable to hypothesize that a less tight binding

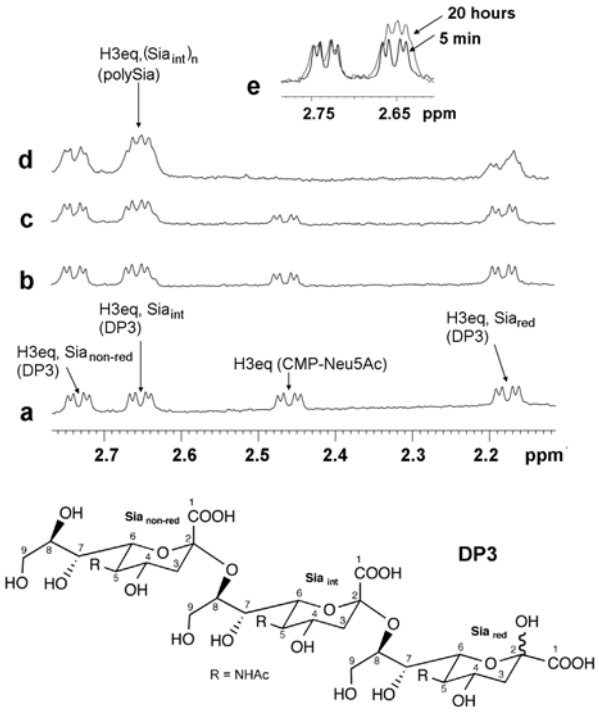

Figure 3. ^{1}H NMR experiments of a mixture containing 100 µM trimeric α(2,8)-linked polySia (DP3) and 100 µM CMP-Neu5Ac after 5 min (a), 30 min (b), 60 min (c) and 20 hours (d) incubation with 170 µg MBP-*Nm*B-polyST. (e) Superimposed ^{1}H NMR spectra at 5 min (black) and 20 hours (grey) to demonstrate increased line broadening and signal intensity of the H3eq proton of the internal sialic acid residue. All experiments were performed at 600 MHz and 293 K in 20 mM TRIS containing 6 mM $MgCl_2$, pH 8.0.

of the non-reducing terminal end of polySia residue facilitates a fast release of the polysialic acid product. These results may also provide a structural explanation for the non-processive nature observed in vitro for the *Nm*B-polyST suggesting an efficient dissociation of the polySia product after each addition of a sialic acid residue.[6]

To follow the biosynthesis of polySia over time, 1D ^{1}H NMR spectroscopy was used. The advantage of using 1D ^{1}H NMR spectroscopy in the study of enzyme reactions is that this non-invasive method does not require any radiolabelled or fluorescently labelled (GT3-FCHASE) substrates but allows a simultaneous monitoring of the consumption of native substrates and subsequent buildup of products. An essential requirement for investigating enzyme-mediated reactions by NMR spectroscopy is that the substrates and products must show at least one resolved signal. For the purpose of the present study well-resolved resonances (H3eq) for both sialic acid donor substrate (CMP-Neu5Ac) and acceptor [trimeric α(2,8)-linked polySia (DP3)] could be detected in the 600 MHz ^{1}H NMR spectrum. The progress of the reaction is shown in Figure 3. Figure 3a shows the ^{1}H NMR spectrum of 100 µM CMP-Neu5Ac and 100 µM DP3 after only 5 min incubation with 170 µg MBP-*Nm*B-polyST revealing no change in signal intensity compared to a spectrum without enzyme.

After 30 min incubation (Figure 3b) with MBP-*Nm*B-polyST a slight broadening of the H3eq proton of the internal (Sia_{int}) sialic acid moiety can be detected. This trend is further evident in the spectrum after 60 min incubation time (Figure 3c), but a longer incubation time (20 hours, Figure 3d) reveals a dramatic increase in signal intensity and broadening of the H3eq proton of the internal (Sia_{int}) sialic acid residue. Furthermore, it is immediately obvious that the signal intensity of the H3eq of the sialic acid donor substrate molecule CMP-Neu5Ac (2.46 ppm) decreases with increased incubation time leading to a complete disappearance of this signal after 20 hours reaction time, suggesting that the sialic acid donor substrate has been completely consumed after this time period. Simple hydrolysis of CMP-Neu5Ac would result in appearance of an additional signal at 2.18 ppm. (H3eq,Sia_{free}).

To highlight the increase in signal intensity and line broadening of H3 Sia_{int} a superimposition of the ^{1}H NMR spectrum after 5 min incubation time and after 20 hours is shown (Figure 3e). Interestingly, the signal intensity of H3eq associated with Sia_{nonred} after 5 min incubation time is almost identical after 20 hours incubation time. In contrast, the signal intensity of the H3eq Sia_{int} proton at 5 min incubation time and 20 hours is significantly different, as intensity and broadening of this signals increased dramatically. The addition of sialic acid residues to the terminal sialic acid residue of DP3 resulted only in an increase of intensity and line broadening of H3eq of the internal sialic acid residue [(Sia_{int})n] but this result clearly demonstrates that *Nm*B-polyST has transferred sialic acid moieties onto the DP3 sialic acid acceptor. We have previously reported that the degree of polymerisation depends on the concentration of the acceptor.[6] At an equimolar concentration of CMP-Neu5Ac and DP5 all polySia products remained under a degree of polymerisation of DP11.[6] A similar result was obtained when DP3 was chosen as acceptor molecule (see supporting information in[6]). However, at very low acceptor concentration only very large sialic acid polymers were detected with a degree of polymerisation of >100.

The STD NMR study reported here provides for the first time structural evidence that the sialic acid residue of the donor substrate CMP-Neu5Ac makes fewer contacts with the *Nm*B-polyST protein compared to the cytosine and ribose moiety. This loose binding to the protein enables a fast and efficient sialyl transfer to the acceptor substrate. In addition, the observed less tight binding of the non-reducing terminal end of the polySia acceptor might

facilitate a fast release of the polysialic acid product and supports the non-processive nature observed in vitro for the *Nm*B-polyST. We were also able to use ^{1}H NMR spectroscopy to directly study the biosynthesis of polySia without the requirement of radiolabelled or fluorescently labelled (GT3-FCHASE) substrate. Overall, our NMR analysis offers a close view of the structural determinants that are necessary for binding to *Nm*B-polyST that provide the basis for the development of novel *Nm*B-polyST inhibitors and consequently novel *Neisseria meningitidis* serogroup B drugs.

Experimental Section

General – CMP-Neu5Ac and D_2O (99.9%) were purchased from Sigma Aldrich (Australia). Trimeric α(2,8)-linked polySia (DP3) were purchased from Nacalai Tesque, Japan. All NMR experiments were performed on a Bruker Avance 600 MHz NMR spectrometer, equipped with a 5 mm cryoprobe using Shigemi tubes. The measurements were performed in 20 mM deuterated TRIS buffer containing 4 or 6 mM $MgCl_2$, pH 8.0.

Expression of soluble *Nm*B-polyST MBP fusion protein – The cloning and expression was done as previously described[6] with minor modifications. In brief: Expression was achieved by transforming BL21 (DE3) Gold (Stratagene) cells in PowerBrothTM medium (AthenaES) containing 125 µg l^{-1} carbenicillin at 37 °C and 225 rpm. The cells were grown to an optical density of OD_{600} = 0.9 before cooling to 15 °C. At OD_{600} = 1.8 induction with a final concentration of 0.1 mM IPTG was applied. Cells were harvested after 24 h induction. Recombinant proteins were purified as described[6] using a 100 mM TRIS, pH 7.5 buffer containing 1mM DTT. Bio-Spin® 30 Tris Chromatography Columns (BioRad) were used to exchange the standard biochemical buffer into a 20 mM deuterated TRIS (TRIS-d) buffer containing 4 mM $MgCl_2$, pH 8.0, suitable for NMR experiments. After initial centrifugation (4 °C, 170 xg, 1 min) the column was washed four times with 500 µL NMR buffer by centrifuging as before. Protein samples were loaded and then eluted by the same centrifugation protocol. The final protein concentration was determined using the Biorad Protein Assay (BioRad).

Saturation Transfer Difference (STD) NMR experiments – STD NMR experiments were performed at 283 K and pH 8.0. The protein was saturated on-resonance at −1.0 ppm and off-resonance at 33 ppm with a cascade of 40 selective Gaussian shaped pulses, of 50 ms duration with a 100 µsec delay between each pulse in all STD NMR experiments. The total duration of the saturation time was set to 2 s. CMP-Neu5Ac and polySia DP3 were added (molecular ratio protein: ligand of 1:100) to give a final concentration of 0.8 mM. A total of 1024 scans per STD NMR experiment were acquired and a WATERGATE sequence was used to suppress the residual HDO signal. A weak spin-lock filter with a strength of 5 kHz and a duration of 10 ms was applied to suppress protein background. Control STD NMR experiments were performed by adding an equimolar concentration of a non-binding spy molecule (galactose) to a *Nm*B-polyST:CMP-Neu5Ac and *Nm*B-polyST:DP3 complex.

^{1}H NMR-based *Nm*B-polyST activity test – All enzyme reactions were performed at 293 K in a 20 mM deuterated TRIS buffer, containing 6 mM MgCl2, pH 8.0. In a typical ^{1}H NMR experiment, a spectrum of each individual reaction mixture containing 100 µM CMP-Neu5Ac and 100 µM trimeric α(2,8)-linked polySia (DP3) was acquired at t = 0 min. After addition of 170 µg MBP-*Nm*B-polyST ^{1}H NMR spectra were recorded in 6 min intervals over a period of 1 hour. The reaction mixture was then incubated for 20 hours at room temperature. Suppression of the residual HDO signal was achieved by pre-saturation with a weak rf field for 2.0 s during the relaxation delay. Data acquisition and processing were performed with TOPSPIN software (Bruker).

Acknowledgements

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Keywords: Polysialyltransferase, *Neisseria meningitidis* Serogroup B, Saturation Transfer Difference (STD) NMR

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Chapter 4

A Cyclic Enzyme Reaction for the Production of Highly Pure α2,8-linked Polysialic Acid

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Manuscript in preparation

Preface – About the Manuscript

The increase of biotechnological and pharmaceutical applications of polySia during the past years demand for a sufficient supply of ultrapure material. In this study an enzymatic approach to establish polySia production *in vitro* has been achieved for the first time.
Firstly, I established an enzymatic production cycle for polySia, using the recombinant soluble polysialyltransferase from *Neisseria meningitidis* serogroup B combined with the CMP-Sialic acid synthetase and a CTP recycling system including the enzymes pyruvate-kinase, and myo-kinase. Starting from affordable substrates I succeeded in optimizing the cyclic reaction to produce polySia in sufficient amounts. In further experiments I could achieve directed synthesis leading to tailor-made polySia. Further, I carried out the development and optimisation of straight forward purification systems for ultrapure polySia as well as subsequent analysis of the reaction product. Finally, I wrote the paper together with Prof. Dr. Rita Gerardy-Schahn.

A Cyclic Enzyme Reaction for the Production of Highly Pure α2,8-linked Polysialic Acid

ABSTRACT

α2,8-linked polysialic acid (polySia) is a unique posttranslational modification primarily attached to the neural cell adhesion molecule (NCAM). The polymer is well known as an essential regulator of cellular contacts during neuronal development and as a substrate that maintains plasticity and regeneration potential in the adult brain. Particularly the favourable features that polySia has in neural regeneration, have stimulated experimental models in which forced re-expression of the polymer and its external supplementation were used to endorse repair processes in the central and peripheral nervous system, respectively. Moreover, because polySia has gained attention for vaccine production, as stabilizer of protein drugs and complement of infant formulae, a high demand for biocompatible material has evolved. In this study we describe an enzymatic production cycle for polySia, using the recombinant soluble polysialyltransferase (polyST) from *Neisseria meningitidis* serogroup B (*Nm*B) in combination with CMP-Sialic acid synthetase (CSS) and the CTP recycling enzymes pyruvate-kinase, and myo-kinase. Starting from cheap substrates the cycle produces polySia in a multi milligram scale. Exploiting the non-processive nature of *Nm*B polyST, oligo- and polymer fractions with a narrow degree of dispersity could be obtained and purified to homogeneity in a straight forward protocol. The described reaction chain can be easily adjusted to GMP conditions and thus provides an ideal platform for the synthesis of highly pure polySia suited for medical applications.

INTRODUCTION

Polysialic acid (polySia) as a polymer of α2,8-linked sialic acid units occurs in nature in a variety of organisms reaching from bacteria to men (Troy, 1992). In mammals polySia is shown to be associated with the neuronal cell adhesion molecule NCAM (Rutishauser, 2008), a voltage-sensitive sodium channel in rat brain (Zuber *et al*. 1992), a soluble form of the scavenger receptor CD36 in human milk (Yabe *et al*., 2003), and neuropillin-2 expressed on mature dentritic cells (Curelli *et al*., 2007). In prokaryotes polySia is found as the capsular polysaccharide of the neuroinvasive bacteria, *Echerichia coli* K1, the dipplococcus *Neisseria meningitidis* Serogroup B, *Pasteurella haemolytica* A2, and *Moraxella nonliqiefacien*. (Devi *et al.* 1991). The structural identity of the human and bacterial polySia (Finne *et al*. 1983) enables the pathogens to evade the human immune system by means of molecular mimicry. Therefore α2,8-linked polySia is an important virulence factor. In contrast α2,9-linked polysialic acid as well as other capsular polysaccharides occurring in *Neisseria* are high inflammatory agents, underlining the low immunogenicity of α2,8-linked polySia as a key function of this polymer. Due to this characteristic property, polySia in its native state is not capable of inducing antibody formation or opsonisation in mammals. Vaccination based on the capsular polysaccharide, as it is successfully used against *Neisseria meningitidis* serogroup A, C, Y and W135, is therefore not possible in the case of serogroup B. Recently de-N-acetyl sialic acid containing polySia produced from native polySia was used as a vaccine eliciting antibodies which showed cross-reactivity with bacterial capsular polySia, but not with human polySia (Moe *et al.* 2009).

Moreover, Yabe *et al*. (2003) showed scavenger receptor CD36 to be associated with polySia in human milk but not with human blood cells and macrophages, where CD36 receptors are also known. The polysialylation-state of CD36 is shown to be lactation stage-dependent and therefore polySia is suggested to be important for the development of neonates and should be considered as a nutrient supplement in formula. Subsequent studies carried out in piglets confirmed that acidic sugars including Neu5Ac have a beneficial effect for the development of the nervous tissue in neonates. (Wang *et al*., 2009)

The well documented function of polySia in neural development, plasticity, and repair (for review see Hildebrandt *et al*. 2007, Mühlenhoff *et al*. 2009, Rutishauser *et al*. 2008) has most recently stimulated efforts to use this substrate as an exogenous support in nerve repair models. To evaluate its suitability in this respect, Haile *et al.* (2007) first tested the impact of polySia on cell cultivation of primary neurons and glial cells. All cell types including neonatal and adult Schwann cells, spinal ganglionic, and neural progenitor cells, and neurons behaved on polySia substrates comparable as control cells kept under standard conditions. One year later the same group demonstrated that polySia based scaffolds represent promising new biocompatible and bioresorbable material for nerve tissue engineering and recommends it as an alternative for reconstructive therapeutic strategies (Haile *et al.* 2008).

Polysialylation of protein therapeutics has shown potential to increase the serum half-live and reduce immunogenicity of this important class of drugs. The impact of protein polysialylation was first evaluated in 1997 by Fernandez *et al*. who found asparaginase, a cytostatic drug in cancer treatment, to have increased circulation half-live following polysialylation. Further experiments widened the scope to reduced antigenicty which occurs in terms of chronic treatment (Fernandez and Gregoriadis 2001). These findings could be confirmed with Insulin, α2b-interferon, and Factor VIII of the blood

clotting cascade, a therapeutic drug for haemophilia (Jain *et al*., 2003).
In summary, these findings open important applications for polySia in nutrition, regenerative medicine and pharmacy, which exact an abundant supply of this polymer for experimentation and clinical trials in the near future. To date several companies offer colominic acid in a mixture of chain lengths harvested from bacteria expressing polySia as a capsular polysaccharide (K1 antigen). The chemical synthesis of oligoSia, up to tetramer, has been reported (Ando *et al*., 1999) but costs and effort of the multi-step procedure conflict with the need for production at preparative scale. To our knowledge, there are no chemical approaches to synthesize long chain polySia. With the cloning and recombinant production (CSS: Gilbert *et al*., 1997) of the enzymes involved in the terminal steps of polySia synthesis, foremost the polysialyltransferase (polyST: Freiberger *et al*., 2007), a new situation has been established that enables large scale polySia production under conditions that meet the specifications for medical application. The reaction cycle described in this study, leans on a cycle previously described by Wong and co-workers (Ichikawa *et al.* 1991) for the production of mono-sialoglycoconjugates. Intermediates like CTP are regenerated to overcome inhibitory effects. Profiting from the non-processive nature of the *Nm*B polyST tailor shaped, small range disperse polySia can be produced at multi milligram scale and purified to homogeneity.

EXPERIMENTAL PROCEDURES

Materials

Sialic acid (Neu5Ac) and oligosialic acid (DP3) were purchased from Nacalai Tesque, Japan. Phosphoenolpyruvate was purchased from ABCR GmbH & Co. KG. Myokinase and pyruvate kinase from rabbit muscle Type I, ammonium sulphate suspension were purchased from Sigma Aldrich.

Expression and purification of recombinant proteins

The cloning and expression of the MBP-polysialyltransferase was done as previously described (Freiberger *et al*. 2007) with minor modifications. In brief: Expression was achieved by transforming BL21(DE3)Gold (Stratagene) cells in PowerBroth™ medium (Athena ES) containing 125µg l^{-1} carbenicillin at 37°C and 225 rpm orbital shaking. The cells were grown to an optical density of OD_{600} = 0.9 before cooling to 15°C. At OD_{600}= 1.8 induction with a final concentration of 0.1mM IPTG was applied. Cells were harvested 24 h after induction. Recombinant proteins were purified as described previously (Freiberger *et al*. 2007) but with buffer containing 100 mM Tris-HCl pH 7.5 containing 1 mM DTT for desalting, size exclusion chromatography, and storage of protein.
The CMP-sialic acid synthetase form *Neisseria meningitidis* was cloned as described earlier (Gilbert *et al*., 1997) containing an N-terminal Step-tag (IBA) and a C-terminal hexahistidine-tag. Expression was achieved by transforming BL21(DE3)Gold (Stratagene) cells in PowerBroth™ medium (Athena ES) containing 125µg l^{-1} carbenicillin at 37°C and 225 rpm. The cells were grown to an optical density of OD_{600} = 0.9 before cooling to 15°C. At OD_{600}= 1.8 induction with a final concentration of 0.1mM IPTG was applied. Cells were harvested 24 h after induction. Recombinant Protein was purified by a Strep-column.
EndoNF was purified as previously described (Schwarzer *et al*., 2009).

Size-exclusion Chromatography

Size-exclusion chromatography was carried out on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 100 mM Tris-HCl pH 7.5, 1 mM DTT. The column was calibrated using the Gel Filtration Molecular Weight Markers (MW-GF-200) from Sigma.

Production of polysialic acid

Reaction conditions for the linear reaction:
The reaction mix contained 200 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, serial dilutions of CTP (5 to 20 mM) and 0.01 mM DP3. Neu5Ac was added in concentrations of 5 to 20 mM after being neutralised with sodium hydroxide. The enzymatic reaction was performed at 25°C and started by adding 37 µg/ml MBP-polysialyltransferase and 30 µg/ml CMP-Neu5Ac synthetase. Samples were taken from the solution at different time steps by transferring 50 µl of the reaction mix to 50µl of pure EtOH.
Reaction conditions for the cyclic reaction:
The reaction mix contained 200mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, 1mM CTP, 1mM ATP and 20 mM phosphoenolpyruvate. The acceptor DP3 was added in concentrations of 0.3 mM if not stated other in the text. Neu5Ac was added in a final concentration of 10 mM after being neutralised with sodium hydroxide. The reaction was started with 37 µg/ml MBP-polysialyltransferase, 30 µg/ml CMP-Neu5Ac synthetase, 7 µg/ml myo-kinase (Sigma), and 20 µg/ml pyruvate-kinase. The reaction was incubated at 15°C, 25°C or 37°C for 48 h before purification of polySia from the reaction mix.

Purification and analysis of poly sialic acid by anion exchange chromatography

Anionic exchange chromatography was done with a MonoQ HR 5/5 (Amersham / GE Healthcare) column and an Äkta FPLC system (GE Healthcare). Samples were filtered (0.22 µm) before loading. The column was equilibrated with running buffer (10 mM Tris-HCl pH 8.0) and elution was performed using different gradients: For analysis of chain length distribution a segmented linear gradient of: 2 ml at 0%, 2 ml at 0–8%, 7 ml at 8–20%, 26 ml at 20–45%, 1 ml at 45–100%, and 5 ml at 100% of 10 mM Tris-HCl, pH 8.0, 1 M NaCl was applied. For purification of polySia by anionic exchange chromatography a three-step gradient of: 6 ml at 0% (equilibration), 4 ml at 30% (washing step), 1.3 ml at 100% (polySia elution), 4.5 ml at 0% (re- equilibration) of 10 mM Tris-HCl, pH 8.0, 1 M NaCl was applied, instead. Absorbance of polySia was monitored at 214 nm.

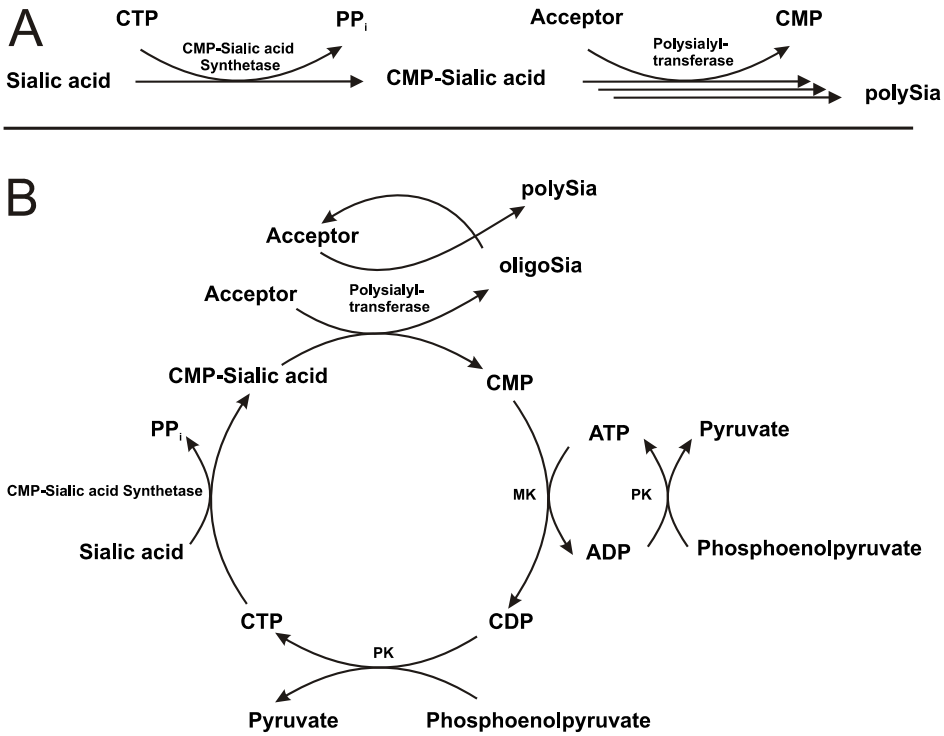

FIG. 1. **Schematic of linear and cyclic reaction for polysialic acid synthesis.** A, linear one-pot two enzyme reaction, using CMP-sialic acid synthetase which activates sialic acid and polysialyltransferase for elongation of a staring acceptor (e.g. DP3). Eqimolar amounts of CTP and sialic acid are required. B, one-pot cyclic reaction using additionally pyruvatekinase (PK) and myokinase (MK) from rabbit muscle for the regeneration of CMP to CTP. Twice as much phosphoenolpyruvate to sialic acid is needed.

The elution profiles were processed with MS Excel and Prism 4.03 (GraphPad Software Inc.). PolySia containing fractions were combined for further purification.

Digestion of polySia by endoNF

100 µg purified polySia was incubated with and without 125 ng endosialidaseNF (Stummeyer *et al*., 2005) at pH 5.7 in 100 mM sodium phosphate buffer for 10 h at 25°C. The reaction mixtures were analysed by anion exchange chromatography before and after incubation.

Protein and Phosphate quantification

Protein quantification was carried out with the Pierce® BCA Protein Assay Kit according to the manufacturer's guidelines. Phosphate was detected using Upstate biotechnology Tyrosine Phosphatase Assay Kit 2 following the manufacturer's instructions. Samples throughout the purification were treated the same way as the samples of the standard curve described in the manual.

Gel chromatography and staining of oligo and polysialic acid

To analyse the products formed by the cyclic reaction in more detail, reaction mixtures were analysed in a TBE-buffered (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) electrophoresis system described for analysis of acidic capsular polysaccharides (Pelkonen *et al.* 1988). 50 µg of oligoSia or polySia containing reaction volumes were treated with equal volumes of sample buffer (2 M sucrose in TBE) before loading to the gel. The following dyes were used as standards and correspond to polySia chain length given in brackets: trypan blue (DP100), xylene cyanol (DP52), bromphenol blue (DP19), bromcresol purple (DP11). Electrophoresis was performed at 400 V and 4 °C for 3 h. Subsequently gels were stained by alcian blue and silver staining following the protocol from Tsai (1982).

NMR analysis of polySialic acid

^{1}H-NMR-spectroscopy was carried out with a Bruker AM-400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 400MHz and room temperature. ^{13}C NMR-spectroscopy was carried out with a Bruker AM-400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 100 MHz and room temperature The solid samples were dissolved in D_2O. and calibrated to HDO, 4.79 ppm

RESULTS

Polysialic acid production by linear reaction

In the first attempts to produce polysialic acid (polySia) in an enzymatic reaction, the CMP-Neu5Ac synthetase (CSS) from *Neisseria meningitidis* serogroup B (*Nm*B) and the polysialyltransferase

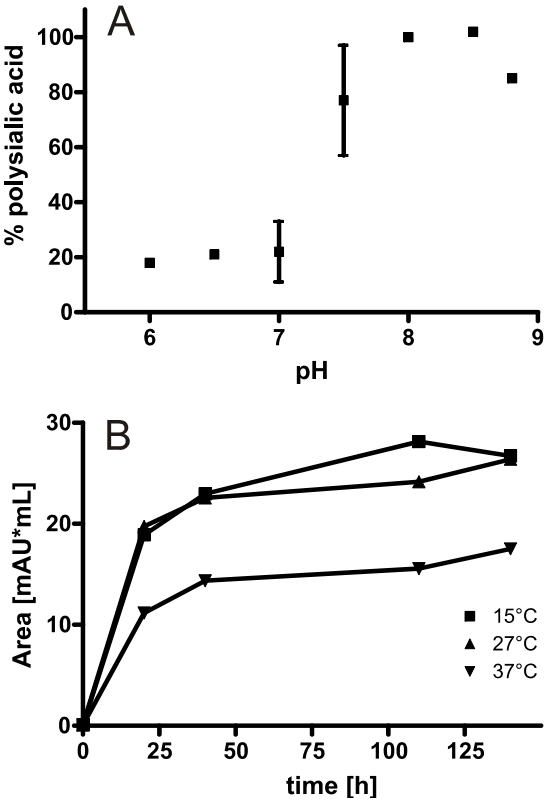

FIG. 2. Evaluation of reaction conditions of a cyclic reaction. A, pH dependency: Several reactions were carried out at different pH. Reactions from pH 6 to pH 8 were buffered in MES, reactions from pH 7.0 to 8.8 were Tris buffered. The amount of polysialic acid at pH 8.0 is set to 100%. Error bars show the standard deviation of two independent experiments.
B: Temperature sensitivity: Three cyclic reactions were carried out at different temperatures for 24 h. Subsequently, product formation was investigated by anion exchange chromatography.

(polyST) of *Nm*B were used to produce polySia from N-acetyl neuraminic acid (Neu5Ac) and CTP. The activating enzyme, CSS transfers CMP from CTP on Neu5Ac in C-2 position (Fig 1A). Consecutively, the activated sugar CMP-Neu5Ac is transferred by the polysialyltransferase onto trimeric sialic acid (DP3) as acceptor to form α2,8-linked polySia.
Reaction products were analysed by anion exchange chromatography at different time points to monitor the formation of polySia. To determine the maximum possible yield of polySia, with this linear reaction setup, the initial concentration of substrates Neu5Ac and CTP was increased. Concentrations above 20 mM CTP inhibited the reaction and resulted in no product formation. Further, a maximum Neu5Ac concentration of 2 mM could be used in this linear reaction format.

PolySia production by a cyclic reaction

To economise the production of polySia and to avoid inhibitory effects of CMP, the high energy phosphate donor CTP which was consumed by CSS in the CMP-Neu5Ac activation step, was regenerated *in situ* by action of myosin kinase and pyruvate kinase (Fig. 1B). The myosin kinase transferred the γ-phosphate of ATP onto CMP forming ADP and CDP. Whereas pyruvate kinase regenerates ATP and CTP from ADP and CDP by transferring phosphate residues of phosphoenolpyruvate (PEP) releasing pyruvate. Initial trials revealed the importance of an efficient buffer system to maintain a stable pH. Therefore, the pH profile of the cyclic reaction was assayed by monitoring the total yield by anionic exchange chromatography. Samples were taken after 20 h (Fig 2A). The pH optimum of each involved enzyme is depicted in Tab. 1 and suggests an optimal reaction pH between pH 7.5 and pH 8.0. In fact the pH profile of the cyclic reaction shows a rapid decrease of product formation with pH below pH 7.5. At pH 7 only 20% overall activity could be observed. In contrast, reactions carried out at higher pH (up to pH 9.0) were less affected. Subsequent experiments were, therefore, carried out at pH 8.0 in a solution buffered with 100 mM Tris-HCl.
To further improve the yield of the cyclic reaction, the reaction temperature was varied between 15°C and 37°C were performed and product formation quantified (Fig. 2B). Interestingly, at 37°C approximate 50% product was formed compared to the reaction temperature 25°C. The incubation at 15°C brought no further benefits to the reaction.

Tailor-made reaction products

Recently, we have shown that the recombinant *Nm*B polyST acts in a non-processive manner if tested *in vitro*. To make use of this feature, trials were undertaken to focus the polymerization degree of generated products. As shown in Fig. 3 this goal was reached by starting the polySia production cycle in the presence of variant acceptor (DP3) to donor (Neu5Ac) ratios. As expected, the major product formed with DP3 and Neu5Ac in equimolar concentrations was DP4, which due to the higher diffusion rate of small molecules during the developing procedure of the alcian-blue and silver staining could not be visualized on the polyacrylamid gel. In contrast, products formed in reaction cycles started with acceptor/donor ratios of 1:17, 1:33, 1:333, and 1:3330 are displayed in Fig. 3 and yielded polySia chains with a narrow size distribution. In detail, chains were ranging from DP4

Tab. 1: Reaction optima of used enzymes.

	pH optima	Temperature optima
CSS (NmB)	8.5*	37°C*
polyST (NmB)	7.5 – 8.0#	37°C#
MK (rabbit)	7.6+	37°C+
PK (rabbit)	7.5+	25°C+

*Mizanur *et al*., 2008; # Willis *et al*., 2008; +corresponding data sheet of Sigma-Aldrich.

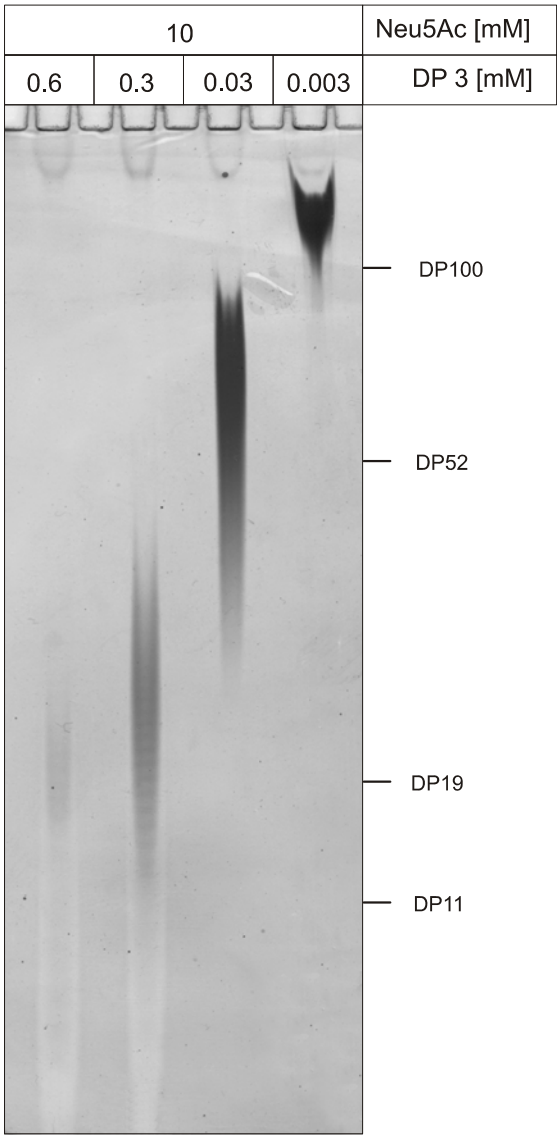

FIG. 3. Production of tailor shaped polysialic acid. By varying the acceptor concentrations, chain length directed synthesis is possible. Acceptor concentrations were varied as indicated with constant Neu5Ac concentrations. The following dyes were used as standards and correspond to polysialic acid chain length given in brackets: trypan blue (DP100), xylene cyanol (DP52), bromphenol blue (DP19), bromcresol purple (DP11).

to DP15, DP15 to DP30, DP30 to DP90, and > DP100, respectively. These results clearly show, that tailor shaped products are readily available by simple adjustment in the concentrations of reactants.

Purification and analysis of polySia

A fast protocol for purification of polySia has been established. After incubation times of 24 - 48 h the reaction mix was spun at 13.000 rcf for 5 min to precipitate denatured protein and magnesium phosphate. The polySia containing supernatant was harvested and treated with 80% ethanol for complete polySia precipitation. After centrifugation for 4 min at 3600 xg the pellet was washed in 100% ethanol for removal of water. This suspension was vacuum dried and resolved in water for further purification by anion exchange chromatography. The described salt gradient was established in order to separate polySia from residual educts, salts, and enzymes. PolySia containing fractions were harvested and polySia was precipitated by addition of 80% ethanol. The sodium chloride containing supernatant was discarded and the pellet vacuum dried producing a fine white powder. The yield of purified polySia throughout purification represents 70% of the original reaction product. All fractions were tested for polySia content by integration of the polySia signal, residual protein was determined by the BCA assay (Pierce) and phosphate content by Upstate biotechnology Tyrosine Phosphatase Assay Kit 2 throughout the purification process. After anion exchange chromatography the phosphate content could be removed below limit of detection < 0.86 µg/ml (8µM HPO_4^{2-}), as well as the protein concentration < 5 µg/ml. The total yield of purified polySia after the purification represents 70% of the original reaction product. Biochemical and physicochemical analysis were applied to further analyse the reaction product. Anion exchange chromatography detecting OD_{214} and OD_{280} revealed no detectable impurities but a strong signal at 40 ml retention volume and OD_{214} corresponding to polySia. To prove the integrity of the polySia structure, digestion by the α2,8-linkage specific endosialidase EndoNF was performed. As illustrated in Fig. 4B, the polySia was entirely degraded to small oligomers ranging from DP3 to DP7 after 10 h at 25°C incubation. The experiment was repeated twice with identical results. Further reaction time led to a complete degradation (DP1-DP3) by EndoNF as described in Schwarzer *et al*. 2009 (data not shown). NMR spectra provided final prove for of the structure and purity of the polySia product. The ^{13}C spectrum clearly demonstrates the presence of polySia. All 9 carbon atoms could be assigned. Additional peaks at 16.8 ppm and 57.4 ppm correlate with ethanol signals due to the last precipitation step. The H1 spectrum again reveals high purity of the product only traces of ethanol appear as residual solvent.

DISCUSSION

The production of polySia in large amounts is subject to research for a long time. Meanwhile the need of polySia as material for vaccine development and in biotechnological approaches like drug stabilisation and, tissue engineering has reached considerable attention. For instance, polySia has been demonstrated to be a versatile add-on in terms of peptide and protein drugs, a growing class of therapeutics (Bezuglov V.V 2009, 2009; Gregoriadis G. 1999, 2000, 2005, Fernandes 1996, 1997, 2001). Production of large sugar polymers like α2,8-linked polySia based on chemical synthesis is an extremely difficult task due to (i) stereo selectivity, e.g. the glycosidic linkage in α or in β position and (ii) regio selectivity, e.g. glycosidic linkage at different hydroxyl groups. While oligomers up to DP4 have been obtained by chemical means (Ando *et al*., 1999), recent approaches focus on the purification of

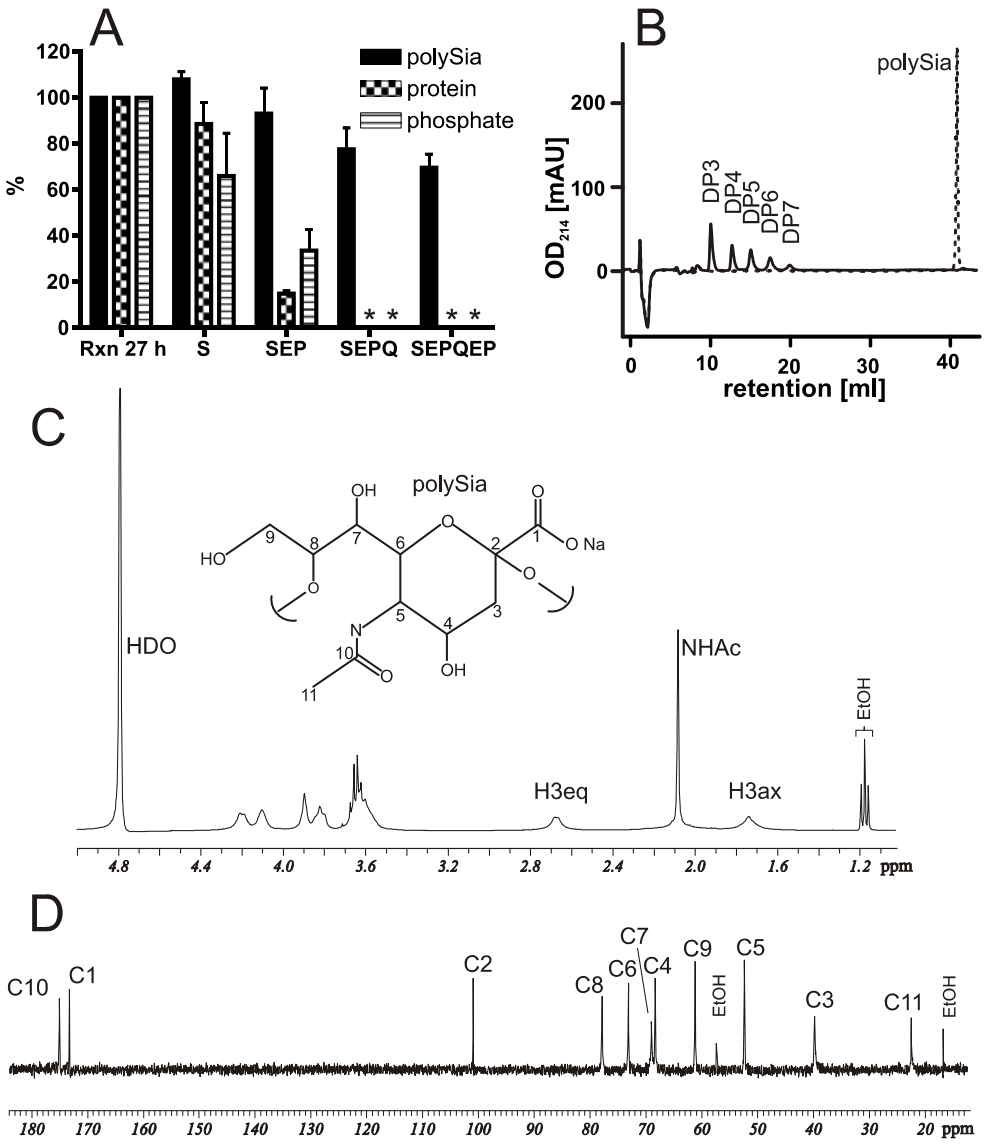

FIG. 4. Purification and analysis of polysialic acid produced by a in vitro cyclic reaction. A: After incubation of the reaction mix for 27 h (Rxn 27 h) the supernatant (S) was treated with 80% ethanol (v/v) for precipitation. The resulting pellet (SEP) was resolved and filtered (0.22 µm) before it was applied to a MonoQTM HR 5/5 column. PolySia containing fractions were combined (SEPQ) and again treated with 80% ethanol (v/v). The vacuum dried pellet was resolved in water to give a pure polySia solution (SEPQEP). The polySia was quantified by TBA assay, protein amount was detected by BCA Assay (Pierce), and phosphate was determined by Millipore Phosphate detection Kit. Asterisks indicate amounts below detection limit. Error bars indicate the standard deviation of three independent experiments. The produced polySia was either incubated with (——) and without (-----) a2,8-linkage specific endosialidase EndoNF before analysis by anion exchange chromatography (B) or resolved in D2O prior to ^{1}H (C) and ^{13}C (D) NMR analysis.

polySia produced by pathogenic bacteria in large scale fermenters (Rode *et al*. 2008). A shortcoming of the fermentation is the need for use of complex media and the production of numerous metabolic products. Purification of products thus remains a challenge in particular, if substrates for medical application are required.

A different approach is the chemo-enzymatic synthesis. It combines the advantages of chemical- and enzymatic synthesis by applying defined starting materials, defined regio and stereo-selectivity, and assuaged purification. Accordingly, the enzyme catalysed synthesis of sugar and nucleotide sugars (Lee *et al.* 2002, Hamamoto *et al.* 2005, Yu *et al.* 2004) has become a standard procedure in modern carbohydrate chemistry and protocols have been established that extend the use of enzymes for the chemoenzymatic production of oligo- and polysaccharides (Ichikawa *et al.* 1992; Kobayashi *et al.* 1999; DeAngelis *et al.* 2003). Improved protocols, thereby, take advantage of the regeneration of high energy intermediates by the establishment of cyclic reactions (Ichikawa *et al.* 1991). These loops are frequently also required to overcome inhibitory effects of products. While the synthesis of monodispers oligosaccharide structures by means of chemoenzymatic reactions has sporadically been achieved (Sismey-Ragatz *et al.* 2007; Muthana *et al.*

2009) the synthesis of size-defined polysaccharides remains a challenging task.
The polysialyltransferase of *Nm*B is to date the most efficiently expressible enzyme for the production of α2,8-linked polySia (Freiberger *et al*., 2007; Willis *et al*., 2008). Although, solubility of the active enzyme depends on the presence of a large fusion tag, the protein can be produced in mg scale per litre (12 mg/l). To our knowledge no other active recombinant polysialyltransferase is yet available at a comparable scale. To combine polyST with the Neu5Ac activating enzyme, it was important that the two enzymes act under similar conditions. CSS of *Nm*B, which fulfils this criteria (Mizanur *et al*. 2008), was therefore combined with the polyST and used in a cyclic reaction (Fig. 1B) in which the high energy phosphate donor CTP of the CMP-sialic acid synthesis was regenerated. This recycling system opened the possibility to produce polySia in higher quantities by using cheap starting material.

Moreover, because regeneration of CTP includes, the removal of CMP and CDP, that are known inhibitors of sialyltransferases (Cambron and Leskawa 1993), are not accumulating, the turnover could be highly increased. With our reaction setup we are able to produce α2,8-linked polySia in multi milligram scale 5 g/l, while isolation of the compound from fermentation yielded 0.2 g per 1 l (Rode *et al*., 2008). It is also worth mentioning that additional modification of polySia like O-acetylation (Orskov *et al.* 1979), or de-N-acetylation after polymerisation (Moe *et al*., 2009), as it occurs in some *E. coli* K1 strains, does not take place by enzymatic synthesis producing defined products.
The homogenous purification of chemo-enzymatically produced polySia was achieved in a three step purification. The *in vitro* system allows the production of polySia without the involvement of DNA *a priori*, another highly negative charged polymer. This avoids the pre-treatment by nucleases for degradation of DNA which might be necessary to enable homogenous polySia purification e.g. by size exclusion chromatography or anionic exchange chromatography.
To date, the formation of medium chain lengths (DP20 to DP70) of α2,8-linked polySia is achieved by either acidic (Roy *et al*. 1990, Cheng *et al.* 1999), thermal hydrolysis (Hayrinnen *et al*. 2002), or degradation by polySia specific endosialidases (Schwarzer *et al*. 2009, Stummeyer *et al*. 2005) of long polymer. All of them are producing either a great variety of different chain lengths or very short oligomers ranging only from DP2 to Dp6 due to complete hydrolysis (Patane *et al.* 2009). Especially the enzymatic degradation by endosialidases favours the formation of DP3 due to its processivity (Schwarzer *et al*. 2009). Instead the demand on more defined polySia, especially medium chain lengths is increasing, supported by findings about the influence of the chain length to filament formation (Toikka *et al.*, 1998), and antibody or endosialidase recognition (Häyrinnen *et al*. 2002, Aalto *et al*., 2001). Also the immunogenicity of related polysialic acid structures as there are α2,9 linked polysialic acid or α2,8- α2,9- alternating linkages are chain length dependent (Gregoriadis *et al*., 2000). Now it is possible to direct the shape of the produced chains in advance by adjusting the donor acceptor ratio of the cyclic reaction. We showed the production of chains ranging from DP4 to DP15, DP15 to DP30, DP30 to DP90, and chains >DP100. This is the first demonstration of a one step production of tailor-shaped small range disperse polySia.
In vitro synthesis of polySia additionally allows the production of radiolabelled (e.g. ^{14}C or ^{3}H) or fluorescently labelled oligo or polysialic acid which might be of great use for investigations of drug clearance and biochemical and histological staining. Extention of the cyclic reaction by synthesis of sialic acid from N-Acetyl mannosamine (ManNAc) and N-Acetyl glucosamine (GlcNAc) or their derivatives is part of future investigations as there are initial trials done by Yu *et al*., 2004 and Hamamoto *et al*., 2005.

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Chapter 5

Proteolytic Release of the Intramolecular Chaperone Domain Confers Processivity to Endosialidase F

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Preface – About the Manuscript

In this study, the processivity of endosialidase EndoNF, as subject to the proteolytic release of the intramolecular chaperone domain was investigated. Therefore, wild type, a non-cleavable mutant, and a mutant containing a disrupted polySia binding site "b" in the stalk domain, were analysed for their catalytic properties. Activity tests showed an increased enzymatic efficiency for the mutants towards soluble polySia, but at the same time an impaired processivity compared to wild type. My contribution to this study comprised the establishment and optimisation of a test system to investigate the cleavage products with regard to chain length separation by means of anion exchange chromatography. With this system, I could determine the influence of the integrity of the binding site "b" on processivity in wild type and mutant endosialidases.

Proteolytic Release of the Intramolecular Chaperone Domain Confers Processivity to Endosialidase F

Endosialidases (endoN), as identified so far, are tailspike proteins of bacteriophages that specifically bind and degrade the α2,8-linked polysialic acid (polySia) capsules of their hosts. The crystal structure solved for the catalytic domain of endoN from coliphage K1F (endoNF) revealed a functional trimer. Folding of the catalytic trimer is mediated by an intramolecular C-terminal chaperone domain. Release of the chaperone from the folded protein confers kinetic stability to endoNF. In mutant c(S), the replacement of serine-911 by alanine prevents proteolysis and generates an enzyme that varies in activity from wild type. Using soluble polySia as substrate a 3-times higher activity was detected whilst evaluation with immobilized polySia revealed a 190-fold reduced activity. Importantly, activity of c(S) did not differ from wild type with tetrameric sialic acid, the minimal endoNF substrate. Furthermore, we show that presence of the chaperone domain in c(S) destabilizes binding to polySia in a similar way as did selective disruption of a polySia binding site in the stalk domain. The improved catalytic efficiency towards soluble polySia observed in these mutants can be explained by higher dissociation and association probabilities, while inversely, an impaired processivity was found. The fact that endoNF is a processive enzyme introduces a new molecular basis to explain capsule degradation by bacteriophages, which until now has been regarded as a result of cooperative interaction of tailspike proteins. Moreover, knowing that release of the chaperone domain confers kinetic stability and processivity, conservation of the proteolytic process can be explained by its importance in phage evolution.

All endosialidases (endo-*N*-acylneuraminidases, endoN) known so far are specialized tailspike proteins of bacteriophages infecting encapsulated *Escherichia coli* strains. Phages infecting the neuroinvasive bacterium *E. coli* K1, a major cause of meningitis and sepsis in neonates have been intensively studied. The dense capsule of these bacteria consists of α2,8-linked polysialic acid (polySia) (1, 2) and provides an efficient infection barrier for coliphages lacking polySia degrading tailspikes (3).

PolySia is widely expressed also in the vertebrate system, where it forms a posttranslational modification on some cell surface proteins. The most prominent polySia carrier is the neural cell adhesion molecule (NCAM) (for review see *Ref.* 4 and 5). In vertebrates polySia is essential for regular ontogenesis and for maintenance of neuronal plasticity into adulthood (for review see *Ref.* 6). Since phage-borne endosialidases are the only known enzymes that specifically degrade polySia, they are widely applied in polySia research and particularly in neuroscience (for review see *Ref.* 4).

In contrast to exosialidases which cleave terminal α-ketosidically linked sialic acid residues, endosialidases require α2,8-glycosidically linked sialic acid oligo- or polymers. Five endosialidase genes have been cloned from different *E. coli* K1 bacteriophages (7–13). The proteins encoded by these genes share a common architecture with three linearly organized domains. (i) An N-terminal capsid binding domain is required to anchor the endosialidase tailspike to the phage particle. This domain varies in length in different phages and is dispensable for enzymatic activity (11, 12). (ii) A highly conserved central catalytic domain that comprises the polySia binding and cleavage activities (14), and (iii) a short C-terminal domain (CTD) that functions as an intramolecular chaperone and is released from the matured enzyme (11, 15; cf. Fig. 1*A*).

We have recently solved the crystal structure of the catalytic domain of endosialidase F isolated from the phage K1F (endoNF) (14).The functional enzyme forms a homotrimer with a mushroom-like outline (see

also Fig. 1*B*). The 'mushroom cap' is formed by three six-bladed β-propellers each harboring an active site '*a*'. Additionally, a lectin-like β-barrel domain extends from each β-propeller and contains a di-sialic acid binding site. The 'mushroom stalk' is built by the intertwining C-terminal portions and is composed of a triple β-helix that is interrupted by a short triple β-prism domain. Both folds are found in other phage tailspike proteins and have been suggested to mediate the unusual stability of these protein complexes (16). Interestingly, a sialic acid binding site '*b*' was identified in the stalk domain, indicating that this domain functions not only in stabilizing the endoNF trimer but directly participates in substrate binding (14).

In contrast to the catalytic domain, no structural information for the CTD is available. This intramolecular chaperone is known to be essential for folding of the functional catalytic trimer (11). Interestingly, the CTD is conserved in a number of otherwise unrelated tailspike and fiber proteins (15). Furthermore, the CTD is proteolytically released in all cases at a highly conserved serine residue post-folding (11, 15; '*c*' in Fig. *1*). We have recently demonstrated that proteolytic release of the CTD confers kinetic stability to the trimeric enzyme (15).

In this study, we demonstrate that release of the CTD also fundamentally influences the mode of endosialidase activity. We show that the wild type endoNF (*control*, Fig. 1*A*) is processive meaning that the enzyme remains attached to the polymeric substrate for several rounds of cleavage after an initial association event. Introduction of mutations that either prevent the release of the CTD or destroy polySia-binding site b drastically interfere with processivity. Concordant with other processively active enzymes (17–19) the mutant forms of endoNF gained catalytic efficiency towards the soluble polySia substrate. With these data we provide new insight into the molecular basis underlying processivity of endoNF. Moreover, we indorse the notion that the proteolytic release of the chaperone CTD not only confers kinetic stability to the endoNF-tailspike, but is also essential for an optimal balance of catalytic and binding functions of this receptor destroying enzyme.

EXPERIMENTAL PROCEDURES

Materials – pBlueScript SK- was purchased from Stratagene. pET expression vectors were obtained from Novagen. Polysialic acid (polySia) for ELISA based assays was purified from *Escherichia coli* K1 membrane fractions according to Decher and coworkers (20). PolySia isolated according to this protocol has been shown to contain a lipid anchor at the reducing end (20) which allows efficient immobilization of polySia on microtiter plates (14). A recently developed protocol (21) was used to isolate lipid free polySia (termed 'soluble polySia' in this study) from the supernatants of *E. coli* K1 cultures. PolySia chains of this preparation contains >130 residues (21). Sialic acid and oligomeric α2,8-linked sialic acid (oligoSia) with a degree of polymerization of 2 to 4 were obtained from Nacalai Tesque (Kyoto, Japan)

Bacteria – The wild type strain *E. coli* B2032/82 serotype K1 is an original clinical isolate obtained from the Department of Medical Microbiology of the Medizinische Hochschule Hannover (Hannover, Germany) (22). *E. coli* BL21-Gold(DE3) were purchased from Stratagene.

Site-directed mutagenesis – Mutagenesis was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's guidelines with the following primer pairs (mutated nucleotides are shown in upper case): KS084 5'-c ccg gca ggg cag GCA atc ata ttt tgc ggg gg-3' and KS085 5'-cc ccc gca aaa tat gat TGC ctg ccc tgc cgg g-3' for endoNF-R837A; KS080 5'-t gaa ggc acc agt Gca acg act ggc gc-3' and KS081 5'-gc gcc agt cgt tgC act ggt gcc ttc a-3' for endoNF-S848A; KS082 5'-tca acg act ggc gca GCg att acg cta tat ggt gc-3' and KS083 5'-gc acc ata tag cgt aat cGC tgc gcc agt cgt tga-3' for endoNF-Q853A. Plasmids containing the corresponding sequence encoding for endoNF protein lacking the N-terminal capsid binding domain (ΔN-endoNF, cf. *Ref*. 15) were used as a template. The sequence identity of all PCR products was confirmed by sequencing. PCR products were ligated into BamHI/XhoI sites of the expression vector pET22b-Strep, a modified pET22b vector containing the

sequence encoding an N-terminal Strep-tag II followed by a Thrombin cleavage site (WSHPQFEKGALVPR'GS) and a C-terminal His_6 tag. Enzymatically inactive endoNF variants were generated by exchanging the 1009 bp endoNF NdeI/XhoI-fragment in pET22b-Strep containing the gene encoding for ΔN-endoNF-R596A/R647A (14) with the corresponding fragment from the respective endoNF binding site mutant.

Protein expression and purification – Proteins were expressed in *E. coli* BL21-Gold(DE3) in the presence of 100 µg/ml Carbenicillin. Bacteria were cultivated in PowerBroth (Athena Enzyme Systems) at 30°C. Expression of endoNF was induced by adding 0.1 mM IPTG at an optical density (A_{600}) of 1.5 and bacteria were harvested 6–7 h after induction. For the analysis of soluble and insoluble proteins, bacteria were lysed by sonication. Soluble and insoluble fractions were obtained after centrifugation (22,000 x *g*, 20 min, 4°C). Protein purification was performed as described previously (15).

Size exclusion chromatography – Size exclusion chromatography was carried out on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 10 mM sodium phosphate buffer, pH 7.4. The column was calibrated using the Gel Filtration Molecular Weight Markers (MW-GF-200) from Sigma.

SDS-PAGE – SDS-PAGE was performed at 85 V and 20°C. Proteins were incubated in the presence of 1% SDS for 5 minutes at 95°C prior to loading to the gel. For the detection of SDS-resistant complexes, the incubation step was omitted. Proteins were stained using RotiBlue (Carl Roth GmbH) according to the manufacturer's guidelines. Scanning was performed with LI-COR Odyssey Infrared imaging system.

Nuclear magnetic resonance – All NMR experiments were performed on a Bruker Avance 600 MHz spectrometer, equipped with a 5 mm TXI probe with triple axis gradients. Deuterated phosphate buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) was used for all NMR experiments. The measurements were performed at 298 K without sample spinning. ^{1}H NMR spectra were acquired with 32 scans, a 2 s relaxation delay over a spectral width of 6,000 Hz. Solvent suppression of the residual HDO peak was achieved by low power presaturation during the relaxation delay. Data acquisition and processing were performed using XWINNMR software. Chemical shift assignment of DP4 oligoSia was achieved by COSY, TOCSY, NOESY and HSQC NMR experiments. Digestion of 1 mg DP4 oligoSia was monitored by acquiring ^{1}H NMR spectra in 10 min time intervals over 16 h post-incubation with 55 pmol of endoNF.

Analysis of OligoSia and PolySia Cleavage Products by Anion-Exchange chromatography – To monitor cleavage of DP3 or DP4 oligoSia 400 µg of the oligomer were incubated with 2 pmol endoNF (control or mutants) in 100 mM sodium phosphate buffer, pH 5.1 for 16 h at 37°C. To stop the reaction 1 volume of cold (-80°C) absolute ethanol was added and samples were frozen. Protein precipitates were removed by centrifugation (3 min at 19.000 x *g*) and supernatants were filtered (0.22 µm) before chromatographic separation. One half volume of the supernatant was loaded onto an ÄKTA design system equipped with a MonoQ HR 5/50 column (GE healthcare) using 10 mM Tris-HCl, pH 8.0 as running buffer. Elution was performed using a segmented linear gradient: 2 ml 0%, 2 ml 0-8%, 7 ml 8-20%, 8 ml 20-28%, 1 ml 28-100%, and 5 ml 100% of 10 mM Tris-HCl, pH 8.0, 1 M NaCl at a flow rate of 1 ml/min. Absorbance of oligoSia was monitored at 214 nm. The elution profiles were processed with MS Excel and Prism 4.03 (GraphPad Software Inc.). For the analysis of soluble polySia cleavage, 400 µg of substrate were incubated with 2 pmol of endoNF (control or mutants) for appropriate incubation times. Samples were processed as described above for oligoSia cleavage, but separation on MonoQ HR 5/50 column was performed using a different segmented linear gradient: 2 ml 0%, 2 ml 0-8%, 7 ml 8-20%, 26 ml 20-45%, 1 ml 45-100%, and 5 ml 100% of 10 mM Tris-HCl, pH 8.0, 1 M NaCl.

Determination of endosialidase activity using soluble polySia as substrate – The enzymatic activity of purified endoNF variants was determined by means of the thiobarbituric acid assay as described previously (11, 23). Serial dilutions of sialic acid monomer were used as a standard. Kinetic parameters K_M and k_{cat} values were determined globally in

Prism 4.03 (GraphPad Software Inc.).

Determination of endosialidase activity using surface bound polySia as substrate – Cellstar 96 well plates (Greiner BIO-ONE) were coated with 40 ng of polySia in PBS (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl) per well for 1 h. Blocking was performed with 200 µl/well 1% (w/v) BSA in PBS. Coating and blocking were each followed by three washing steps with PBS to remove unbound material. Purified active endoNF variants were applied in 40 µl per well in serial dilutions in 1% BSA/PBS, and incubated for 30 minutes at 37°C. EndoNF and cleavage products of polySia were removed by three washing steps, each with 200 µl PBS for 5 minutes with orbital shaking at 1,000 rpm. Residual polySia was detected using 200 ng/well monoclonal antibody 735 (mAb 735 (24) in 1% BSA/PBS) and subsequently goat anti-mouse antibody conjugated to horse radish peroxidase (Southern Biotechnology Associates, 1:2,000 in 1% BSA/PBS). Each antibody was incubated for 1 h at room temperature with orbital shaking at 1,000 rpm and followed by three washing steps with PBS. Chromogenic reaction was performed with ABTS (Roche) according to manufacturer's guidelines. Data were analyzed using Prism 4.03 (GraphPad Software Inc.).

In vitro Binding Assay – Cellstar 96 well plates (Greiner BIO-ONE) were coated as described in the previous section. Serial dilutions of inactive endoNF variants were applied in 40 µl per well to polySia coated microplates, and incubated for 1 h at room temperature with orbital shaking at 1,000 rpm. After washing three times each with 200 µl PBS for 5 minutes at 1,000 rpm on a shaker the N-terminal Strep-tag II of EndoNF was detected incubating Strep-Tactin conjugated to horse radish peroxidase (IBA, 1:5.000 in 1% BSA/PBS) for 1 h at room temperature with orbital shaking at 1,000 rpm followed by three PBS washing steps. Color development was performed with ABTS (Roche). Data were analyzed using Prism 4.03 (GraphPad Software Inc.).

RESULTS

The enzymatic activity of proteolytically processed and un-processed endoNF – Previous work has shown that proteolytic processing of endoNF is not a prerequisite for enzymatic activity (11). However, it remains unknown whether the release of the C-terminal chaperone domain (CTD) affects the kinetics of polySia digestion.

To address this question, the enzymatic activities of the mature, proteolytically processed endoNF (ΔN-endoNF*, *control*) and the non-cleavable mutant ΔN-endoNF-S911A (*c(S)*, Fig. 1*A*) were compared. All endosialidases used throughout this study are lacking the N-terminal capsid binding domain (indicated by ΔN). As shown previously, this does not influence complex formation, activity, and thermostability (11, 14, 15). The scheme presented in Fig. 1*A* describes the nomenclature and *Table 1* summarizes the details of endoNF variants generated in the course of this study. For efficient purification of unprocessed (c(S)) and processed enzymes, the proteins were expressed with N-terminal Strep-tag II and C-terminal His_6 tag as previously published (14).

Enzymatic activities of purified proteins were determined in two assay systems. The thiobarbituric acid (TBA) assay was used to monitor enzymatic activities on soluble polySia (lipid anchor free polySia prepared according to *Ref.* 21) (Fig. 2*A*). An end-point ELISA-based assay was applied to measure enzymatic activity on surface bound polySia (Fig. 2*B*). Microtiter plates were coated with polySia containing a lipid anchor (20) to allow efficient attachment of polySia to the solid phase (14). After endoNF digestion and removal of the enzyme, remaining substrate was detected with the polySia-specific mAb 735 which binds to α2,8-linked polySia ≥8 residues (22, 24, 25).

Strikingly, a 3-times higher molar activity of *c(S)* over *Control* was found using soluble polySia substrates (Fig. 2*A*), while the activity of c(S) using immobilized polySia was drastically diminished. As shown in Fig. 2*B*, 680 pM of *c(S)*, and only 6.8 pM of *Control* were required for half-maximal depolymerization of the solid-phase bound substrate. To further interpret these apparently contradictory results, we investigated the

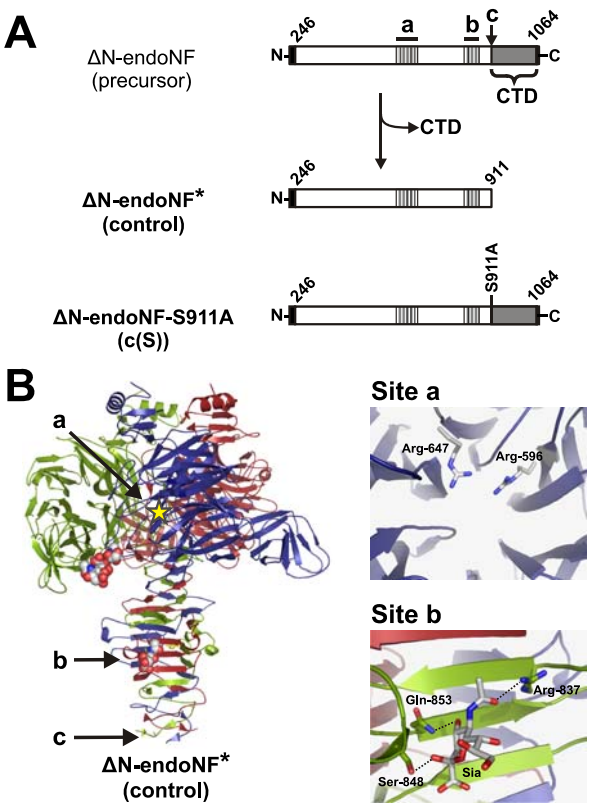

FIGURE 1. **Schematic representation of endoNF maturation and definition of polySia interaction sites in the ΔN-endoNF* structure** (adapted from *Ref.* 14). *A*, schematic representation of endoNF variants lacking the N-terminal capsid binding domain (ΔN): the endosialidase F *precursor* (*ΔN-endoNF*), the processed catalytic part *ΔN-endoNF** (*control*), and the non-cleavable mutant *ΔN-endoNF-S911A (c(S))*. The catalytic part is shown as an open bar, the C-terminal chaperone domain (*CTD*), which is released from the mature protein, in *grey*. The N-terminal Strep-tag II and C-terminal His_6 tag are given in *black*. The first and the last amino acid number of ΔN-endoNF are depicted *diagonally*. The active site, *a*, and the sialic acid binding site, *b,* are indicated as grey-black hatched boxes. The proteolytic cleavage site Ser-911 is indicated as *c*. The amino acid substitution *S911A* is written *vertically*. *B*, ribbon diagram of trimeric endoNF, with the monomers colored in *red*, *green*, and *blue*. The active site (*a*) of the *blue* subunit is schematically depicted with a *yellow asterix*. *Spheres* represent di-sialic acid and sialic acid bound in the β-barrel domain of the *green* subunit and the β-prism domain of the *red* subunit *(*site *b)*, respectively. The proteolytic cleavage site (Ser-911) is indicated as *c*. Catalytic amino acids (*Site a*, view along the pseudo six-fold axis of the β-propeller) and residues involved in *Sia* binding in the stalk domain (*Site b*) are highlighted.

possibility of CTD-induced conformational changes in the catalytic site of the mutant c(S) in kinetic studies. However, since endoNF possesses secondary substrate binding sites (14; Fig. 1*B*), the determination of kinetic parameters required the use of the minimal substrate that is large enough to be bound and cleaved, but too short to interfere with secondary binding sites.

TABLE 1
Notation of EndoNF variants
The ΔN-endoNF variants listed below were analyzed in this study. A convenient notation is introduced to facilitate reading (*left column*), with *a*, active site (*lower panel*), *b*, binding site in the β-prism domain, and *c* proteolytic cleavage site at Ser-911 (according to Fig. *1*). The respective amino acids exchanged to alanine (see right column) are given in brackets. All variants contain an N-terminal Strep-tag II. The non-cleavable mutants c(S) and a(RR)/c(S) additionally contain a C-terminal His_6 tag.

Protein	Amino acid substitutions
Control	-
b(R)	R837A
b(S)	S848A
b(Q)	Q853A
b(RS)	R837A/S848A
b(RQ)	R837A/Q853A
b(SQ)	S848A/Q853A
b(RSQ)	R837A/S848A/Q853A
c(S)	S911A
Control-a(RR)	R596A/R647A
a(RR)/b(R)	R596A/R647A/R837A
a(RR)/b(S)	R596A/R647A/S848A
a(RR)/b(Q)	R596A/R647A/Q853A
a(RR)/b(RS)	R596A/R647A/R837A/S848A
a(RR)/b(SQ)	R596A/R647A/S848A/Q853A
a(RR)/c(S)	R596A/R647A/S911A

Sialic acid tetramer is the minimal endoNF substrate – Previous studies by Troy and co-workers (26) have shown that endosialidase purified from bacteriophage K1F cleaves α2,8-linked sialic acid oligomers with a minimum degree of polymerization of five (DP5 oligoSia).

To confirm the minimal substrate for the purified recombinant ΔN-endoNF* (control) we developed a sensitive and robust ^{1}H NMR-based assay. In this system the distinct chemical shifts of the H3 equatorial protons (*H3eq*) of the four sialic acid units of DP4 oligoSia were monitored (Fig. 3*A* and 3*B*). In general, sialic acid moieties that constitute the reducing end of polySia chains are predominantly β-configured resulting in a chemical shift of the corresponding *H3eq* (β_e) at 2.22 ppm. Non-reducing end residues exhibit pure α-configuration with a chemical shift for the H3eq proton at 2.78 ppm (*H3eq* (α_e)). The H3eq protons of the two internal α-configured sialic acid residues (*H3eq* (α_i)) that form the major part of α2,8-linked polySia, are overlapped with an identical chemical shift of 2.70 ppm. The relative integrals obtained for the H3eq signals of DP4 oligoSia at t = 0 min revealed a ratio of 1:2:1 for H3eq (α_e) : H3eq (α_i) : H3eq (β_e) (see Fig. 3*B*). Addition of ΔN-endoNF* to DP4 oligoSia results in a 1:1:2

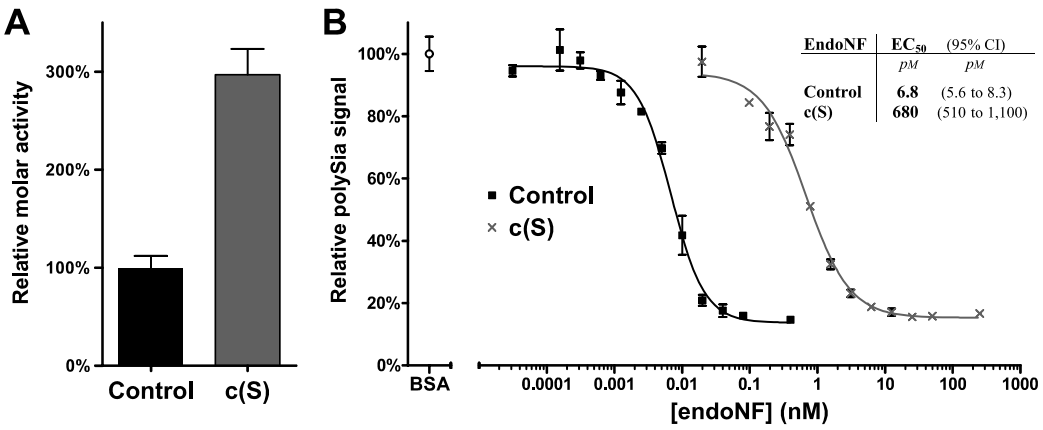

FIGURE 2. **EndoNF activity assays.** *A*, relative molar activities of ΔN-endoNF* (*Control*) and ΔN-endoNF-S911A (*c(S)*) as determined in the thiobarbituric acid assay. Data are means ± S.D. of two independent experiments performed in duplicates. The control was set to 100%. *B*, enzymatic activity of ΔN-endoNF* (*Control*) and ΔN-endoNF-S911A (*c(S)*) determined in an ELISA based assay. In each experiment, maximal loading of microtiter plates with polySia was determined after *BSA* blocking and set to 100%. Samples are plotted as relative values and represent surface bound polySia remaining after treatment with serial enzyme dilutions (for details see *Experimental Procedures*). Each value represents the mean of two independent experiments performed in duplicates. The concentration (x-axis) is scaled logarithmically. Error bars are representing S.D. *Inset table*: EC_{50} values determined for the respective endoNF variant are giving the protein concentration of half maximal polySia removal.

integral ratio for the H3eq protons after 1 hour incubation. This observation leads to the assumption that the sialic acid tetramer was cleaved by the endosialidase. However, an H3eq integral ratio of 1:1:2 did not correspond to a symmetrical cleavage of DP4 oligoSia into two DP2 oligoSia molecules (expected proton integral ratio 2:0:2). Instead, the observed proton integral ratio of 1:1:2 is a strong indication that DP4 oligoSia was cleaved into one DP3 oligoSia (one H3eq (α_e) and one H3eq (α_i) proton) and one monomeric sialic acid molecule (one H3eq (β_e) proton). Moreover, the analysis of the spectral data that were collected after 1 and 16 hours clearly excluded a potential cleavage of DP3 oligoSia (expected H3eq integral ratio 1:0:3). The results obtained by ^{1}H NMR spectroscopy were corroborated in a parallel anion-exchange chromatography experiment, that showed the DP4 oligoSia was cleaved into monomer and DP3 oligoSia after incubation with the active control enzyme (+ *Co*, Fig. 3*C*), while no cleavage products were found when this experiment was carried out with DP2 (data not shown) and DP3 (Fig. 3*D*) oligoSia .

Control and mutant endoNF exhibit similar kinetics with the minimal substrate DP4 oligoSia – Kinetic parameters were determined using the TBA assay and DP4 oligoSia as the minimal substrate. As shown in Fig. 4*A*, DP4 oligoSia (but not DP2 or DP3 oligoSia) was efficiently hydrolyzed by both control endoNF and c(S) mutant. Importantly, identical kinetic parameters (Fig. 4*B*; $K_M = 0.85$ mM and $k_{cat} = 4.37$ sec^{-1}) were determined for the two enzyme variants. While this experiment clearly supported the notion that the presence of the CTD in c(S) has no influence on the conformation and/or stability of the catalytic center, it did not explain the dramatic differences in activity observed when the enzymes were tested with polySia in either solution or immobilized form (see Fig. 2*A* and *B*).

Proteolytic maturation and integrity of site b are essential for efficient polySia binding – One of the polySia interaction sites outside the catalytic center identified previously in the crystal structure (site *b*) is located in the stalk domain in close proximity to site *c* where the CTD is proteolytically released (see Fig. *1A*). Binding studies were carried out to address the question of whether the presence of the CTD may affect polySia binding to site *b*, thus leading to a reduction in the stability of the enzyme/polySia complex and consequently in the observed changes in the kinetics of polymer cleavage. Essential in this study was the use of an enzymatically inactive variant of endoNF. A respective mutant termed a(RR) for active site mutant R596A/R647A (see *Table 1* and inset *Site a* in Fig. 1*B*) has previously been generated based on structural information (14). While the enzymatic activity is abolished in a(RR), the maturation of the protein into SDS-resistant

trimeric complexes and binding to polySia is unaffected (14).

The c(S) mutation was introduced into the a(RR) background giving clone a(RR)/c(S). Purified proteins produced from both clones (a(RR) and a(RR)/c(S)) were tested for binding to immobilized polySia. The half maximal effective concentration (EC_{50}) of 1.9 nM demonstrated high affinity binding of a(RR) to polySia. In contrast, the mutant a(RR)/c(S) showed a drastically reduced binding with an EC_{50} of 360 nM (see Fig. 5; *black* and *red curves*).

To further investigate whether the reduction in binding affinity is a consequence of interference with binding site b, this site was selectively disrupted in the a(RR) mutant. The information provided by the crystal structure (14) was used to guide the production of mutants. Amino acid residues that build up binding site b (Arg-837, Ser-848, Gln-853) are depicted in the inset *Site b* in Fig. 1*B*. These residues were individually and in combination exchanged to alanine resulting in a total of seven b-mutants (see *Table 1)*. In an attempt to produce the purified recombinant proteins the double b-mutant R837A/Q853A (a(RR)/b(RQ)) as well as the triple b-mutant R837A/S848A/Q853A (a(RR)/b(RSQ)) were found to be insoluble (data not shown), while all other variants were soluble. Moreover, analyses carried out by size exclusion chromatography (*Table S1*) demonstrated that all soluble b-mutants formed proteolytically matured homotrimers. However, homotrimers formed from b-mutants, did not attain SDS-resistance, with the single exception of a(RR)/b(S) as determined by SDS-PAGE (Fig. *S1*). SDS-resistance was described previously as a criterion for the kinetically stabilized ΔN-endoNF* (11, 15).

Binding of inactive b-mutants to surface bound polySia was analyzed in the ELISA-based assay (Fig. *5*). EC_{50} values varying between 4.1 nM for the SDS-resistant mutant a(RR)/b(S) (*grey curve*, cf. Fig. *S1*) and 30 nM for the double b-mutant a(RR)/b(RS) (*orange curve*) clearly demonstrated that the integrity of the binding site of the stalk domain is essential to optimize binding between endoNF and polySia chains. Interestingly, the single point mutation R837A of the mutant a(RR)/b(R) already increased the EC_{50} by a factor of 5 (*green curve*) and the defect was potentiated in

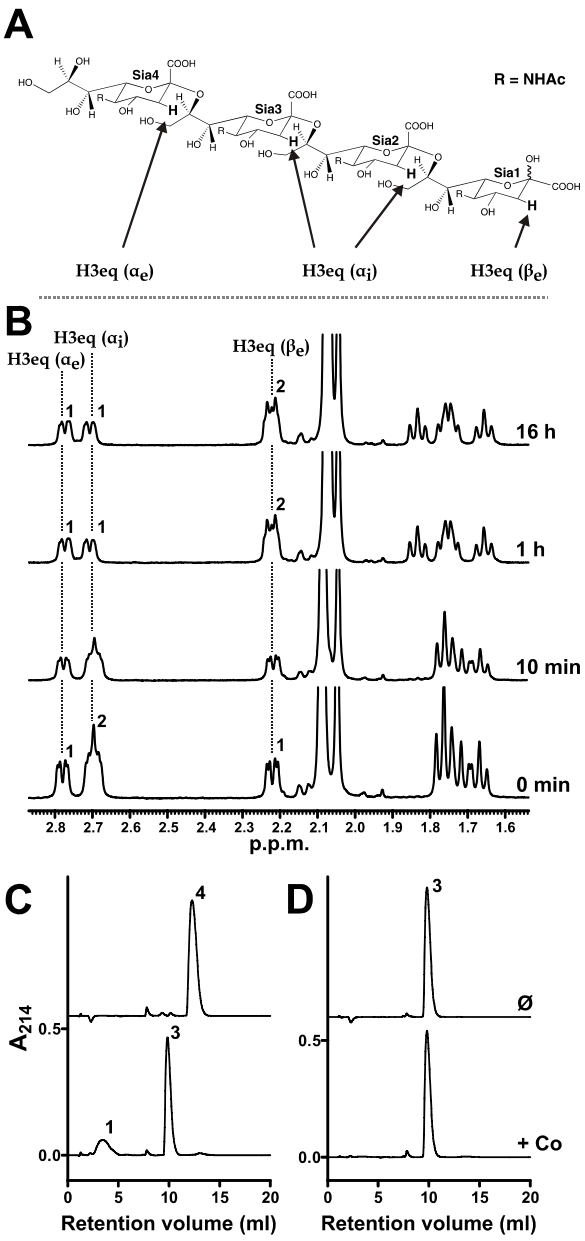

FIGURE 3. **Determination of the minimal endoNF substrate.** *A*, structure of DP4 oligoSia. The equatorial protons at position C3 of the individual sialic acid units of DP4 are depicted. *B*, ^{1}H NMR spectra of 1 mg DP4 oligoSia incubated with 55 pmol of ΔN-endoNF* (control). ^{1}H NMR spectra were acquired at different time intervals in deuterated 10 mM phosphate buffer, 150 mM NaCl, pH 7.4 in D_2O at 298 K. Numbers above peaks indicate the calculated relative integrals. *C–D*, monitoring of oligoSia cleavage products by MonoQ anion exchange chromatography. Samples of 200 µg DP4 (*C*) or DP3 oligoSia (*D*) were incubated either with buffer (*Ø*) or 1 pmol of ΔN-endoNF* (*+ Co*), respectively for 16 h at 37°C. Cleavage products were separated on a MonoQ HR 5/50 column. Elution profiles are separated by an appropriate y-axis offset. Numbers above peaks indicate the DP of oligoSia. Experiments were carried out in duplicates with identical results.

the double b-mutant a(RR)/b(RS) (*orange curve*). Combined with the reduced protein stability found in SDS-PAGE analysis (Fig. *S1*) these data emphasize that Arg-837 plays an

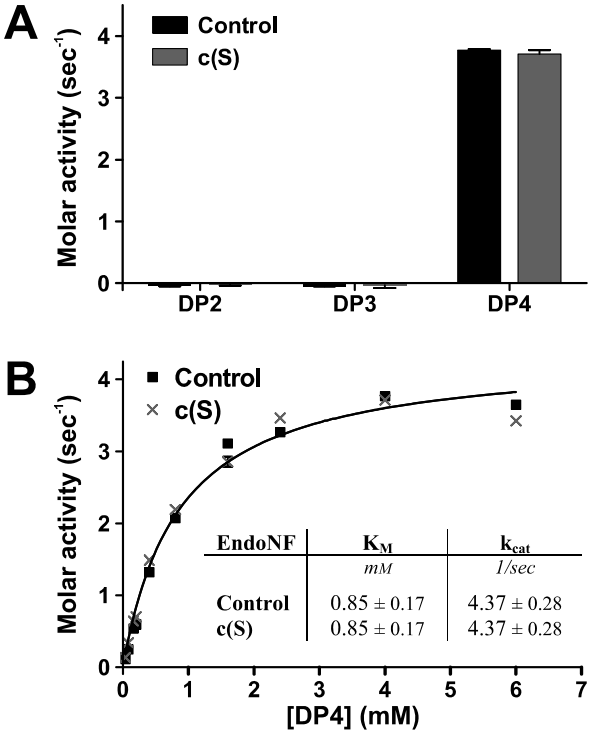

FIGURE 4. **Control enzyme (ΔN-endoNF*) and mutant c(S) cleave the minimal substrate DP4 with identical kinetics.** *A*, molar activities on DP2–*4* oligoSia were determined by the thiobarbituric acid assay in two independent experiments. *B*, kinetic parameters of endoNF variants on the minimal substrate DP4 oligoSia were determined by the thiobarbituric acid assay (in two independent experiments). The curves were best fitted globally with shared parameters. *Inset table* in *B*, kinetic parameters determined for both endoNF variants ± 95% confidence interval. Values in *A* and *B* express the amount of released reducing ends of sialic acid in mol per mol enzyme and sec (1/sec). Error bars are representing S.D.

important role for both protein folding (cf. *Ref.* 15) and formation of the Sia binding site.

In total, mutations introduced into polySia binding site b decreased polySia binding. This effect is similar to the effect caused by lack of proteolytic processing in c(S). Therefore, the CTD preserved in c(S) masks or destabilizes site b and weakens the 'lectin properties' of endoNF.

Depletion of binding site b increases endoNF activity towards soluble substrate – As shown for c(S), the decreased binding to polySia results in an increased enzymatic activity using soluble polySia (Fig. 2*A*). To test whether this is also the case for proteins carrying mutations in site b we generated enzymatically active forms of the soluble b-mutants (see *Table 1*). Purified proteins were analyzed in the TBA assay in comparison to the control (Fig. *6*). In accordance with our hypothesis, protein variants with mutations that significantly affected polySia binding (cf. Fig. *5*) showed significantly increased molar activities (*b(R)* and *b(RS)*), while proteins with only minor binding deficits (b(S), b(Q), b(SQ)) were active within the control range.

Mutations in the stalk domain influence processivity of endoNF – Based on the observation that bacteriophages infecting encapsulated bacteria create narrow paths through the thick capsules, indicating that capsule degradation could occur in a processive manner (27–29). A suggestion for the molecular mode of action has recently been provided in line with the reconstruction of the coliphage K1E (30). However, the question of whether processivity is an intrinsic feature of the tailspike or imposed upon the phage particle due to the interplay of six copies of the tailspike protein has not been investigated as yet. To address this point, soluble polySia was digested with equimolar amounts of control endoNF and the mutants c(S) and b(RS). Reaction products were monitored by anion exchange chromatography in a time dependent manner (Fig. *7*). The control enzyme released short oligoSia species with DP3 as main product. The long chain starting material was still detectable after 10 min (Fig. 7*A*). Conversely, in digests performed with the stalk domain mutants c(S) and b(RS) long chain polySia was cleaved more rapidly. After less than 5 min all starting material was digested into fragments with DPs ranging from 3 to >15. Interestingly, the production of DP3 oligoSia appeared to have similar kinetics in digests carried out with the control and c(S) protein (compare Fig. 7*A* and *B*), while cleavage products generated by b(RS) were more random (Fig. 7*C*). After 16 hours the digestions were complete with free Sia, DP2 and DP3 oligoSia representing the end products of endoNF digestion (cf. Fig. *3* and *4*). The results of this experiment prove processivity for ΔN-endoNF* and confirm that multipoint binding of the polySia chain is crucial to maintain processivity.

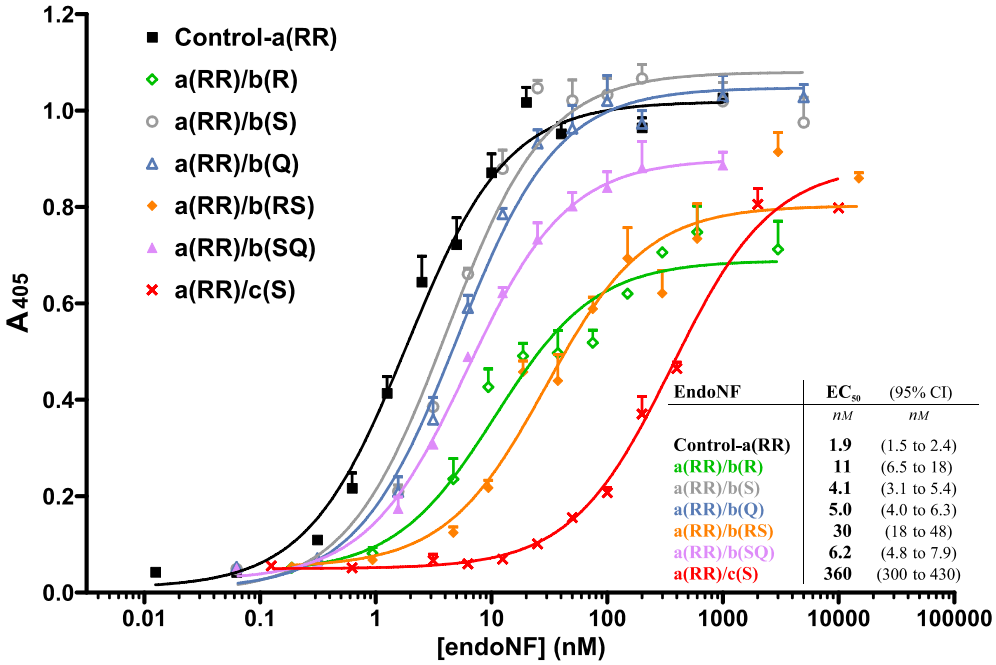

FIGURE 5. **Binding of enzymatically inactive ΔN-endoNF-a(RR) variants to surface bound polySia.** The binding assay was performed as described in Experimental Procedures in four independent experiments. The concentration (x-axis) is scaled logarithmically. Error bars above the curves are representing S.E.M. *Inset table*: EC_{50} values determined in the *in vitro* binding assay of the respective inactive endoNF variant are giving the protein concentration of half maximal binding. *95% CI* is giving the 95% confidence interval.

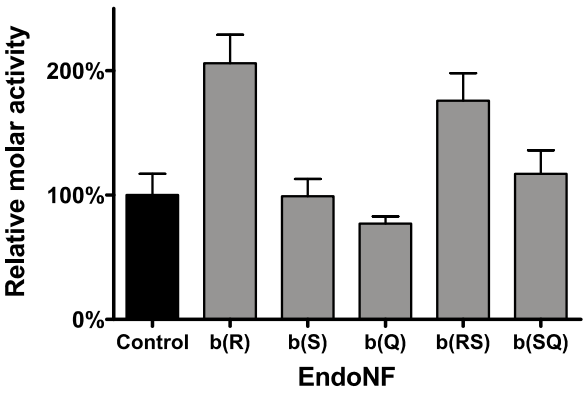

FIGURE 6. **Relative molar activities of ΔN-endoNF* variants with single and double amino acid exchanges in the sialic acid binding site b.** Activities were determined by the thiobarbituric acid assay using soluble polySia as substrate. Data are means ± S.D. of two independent experiments performed in duplicates. The molar activity of control was set to 100%.

DISCUSSION

Many pathogenic bacteria are decorated by a dense polysaccharide capsule which protects the bacterium from desiccation and immune surveillance (31). The capsular polysaccharide layer can be up to 400 nm thick and provides an efficient shield against bacteriophage infections (3, 32). However, in parallel to the evolution of bacterial capsules, specialized phages emerged with tailspike proteins that carry polysaccharide hydrolase or lyase activities to allow depolymerization of the respective host capsule. Electron micrographs of phages attacking encapsulated bacteria revealed that penetration of the capsule by the phage particle mostly occurs in a processive and unidirectional manner leading to the formation of a narrow tunnel (27–29). Based on the 3D cryo-EM reconstruction of the *E. coli* K1 specific phage K1E, processive degradation of the capsule was suggested to proceed by an intermolecular interplay of the six copies of endosialidase tailspikes which are symmetrically arranged around the tail (30).

Using endoNF, the endosialidase of coliphage K1F, we now show that the isolated tailspike protein exhibits processivity by itself, giving new insight into the molecular machinery enabling phages to infect encapsulated bacteria. We demonstrate that endoNF processivity is based on its unique multi-domain structure and depends on an intact substrate binding site b located in the β-prism of the stalk domain.

Previously, we have postulated that site b plays an important role in fixing the phage particle to the outer membrane of the host by binding the oligomeric remnants of the depolymerized capsular polysaccharide (14).

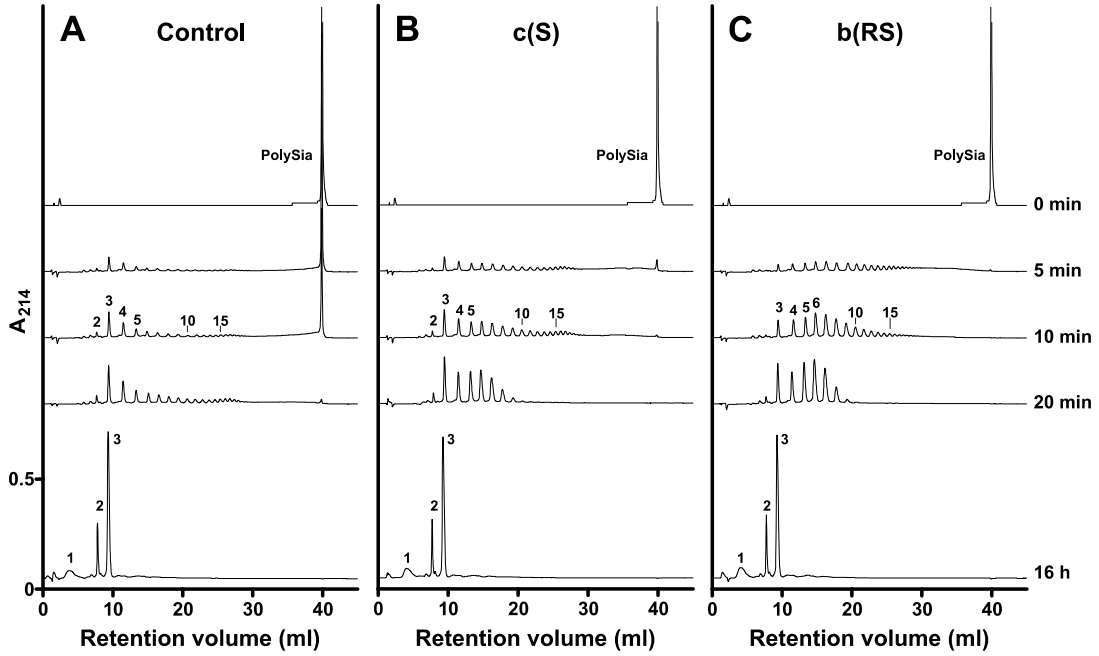

FIGURE 7. **Cleavage characteristics of control (ΔN-endoNF*) and mutant endoNF variants.** Anion exchange chromatography was used to directly monitor the cleavage products generated by control and mutant endoNF. *A-C* After digest of 200 µg of polySia with 1 pmol of the respective endoNF variant for the indicated time points cleavage products as well as the polymeric substrate (*PolySia*, *0 min*) were separated on a MonoQ HR 5/50 column. Elution profiles are drawn at the same scale and separated by an appropriate y-axis offset. Numbers above peaks indicate the DP of oligoSia. The experiment was repeated twice with identical results.

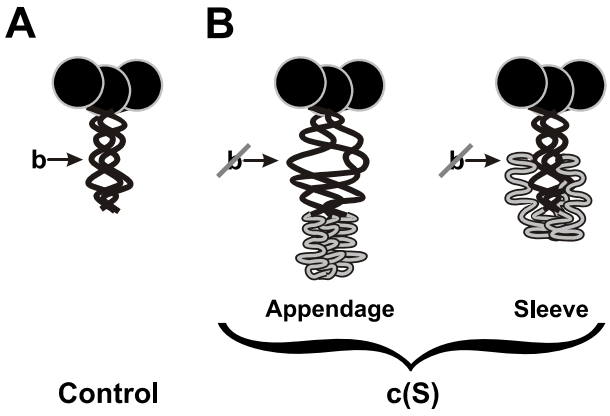

FIGURE 8. **Two proposed folding models of structural influence of the CTD on site b.** Schematic representations of endoNF variants with the β-propeller shown as *black circle*, the stalk domain as *black curve*, and the CTD is shown in *grey* (adapted from *Ref.* 15). *A,* ΔN-endoNF* (*Control*) with proper folded stalk domain (location of site b is indicated by an *arrow*). *B*, two possible folding models of the CTD (*grey*) in (*c(S)*) in which site b is affected: in the '*Appendage'* model the preserved CTD induces a conformational change in the stalk domain, which abolishes the binding site b. The '*Sleeve'* model suggests that the CTD interacts with regions of the stalk domain and hides site b.

By abrogating site b we now show that this site is not only important for efficient binding of endoNF to polySia but also converts endoNF into a processive enzyme. The crystal structure of endoNF in complex with oligoSia (14) suggested that polySia is wrapped around the enzyme with the non-reducing end pointing towards the active site and the reducing end towards the C-terminus of the stalk domain. During processive cleavage of polySia, site b acts in advance of the active site and guides endoNF along the polySia chain in a directed manner from the non-reducing to the reducing end. Since *in vivo* polySia is fixed by its reducing end to the outer membrane of *E. coli* K1, the orientation of site b towards the reducing end ensures that endoNF and thereby the whole phage is not detached from the bacterium during capsule depolymerization. Two experimental approaches have provided clear evidence for this model. Firstly, the binding studies displayed in Fig. *5* clearly show reduced binding affinity to surface bound polySia for the mutant b(RS) lacking a functional site b. Secondly, the assay shown in Fig. 7 directly monitored processivity (19, 33, 34) and highlighted significant differences between control enzyme and the mutant b(RS). When long polySia chains (DP >130) were digested by the control enzyme the predominant initial cleavage product was found

to be DP3 oligoSia and highly polymeric substrate persisted over >10 min. In contrast, when digests were performed with the mutant b(RS), long polySia chains disappeared rapidly (<5 min) paralleled by the appearance of random-sized cleavage products. The cleavage pattern observed for the control enzyme indicates a prolonged association between enzyme and substrate that allows several cleavage steps before dissociation, which results in short cleavage products. By contrast, mutant b(RS) exhibited an increased dissociation probability resulting in an increased random fragmentation of the polymeric substrate. Thus, the reduced binding affinity caused by alanine substitution of two critical amino acid residues (Arg-837 and Ser-848) within site b is directly translated in a loss of processivity.

Similarly, reduced binding to surface bound polySia and loss of processivity was also observed for the non-cleavable endoNF variant c(S) which mimics the un-processed precursor protein. The possibility that the presence of the CTD induces conformational changes within the active site could be excluded by the fact that identical kinetic data were obtained for control and mutant enzyme with the minimal substrate DP4 oligoSia. Consequently, the 190-fold decrease in binding affinity observed for c(S) could be interpreted due to loss of secondary polySia binding sites, i.e. binding sites that are located outside the catalytic center. Since site b is located in close proximity to the CTD, a direct interference between CTD and site b is most likely.

As depicted schematically in Figure *8*, we propose two models to explain this interference. In the '*Appendage*' model (Fig. 8*B*), the CTD is shown as a linear extension that keeps the stalk domain in an immature state which is incompatible with proper binding of polySia (cf. Fig. 8*A* schematically showing the mature protein). Only cleavage and release of the CTD provides the folding enthalpy necessary to attain the mature conformation of the stalk and to generate binding site b. In the '*Sleeve*' model, the CTD is wrapped around the maturely folded stalk, thereby masking site b.

Both previous and current data support the *Sleeve* model. Firstly, preliminary data obtained for crystals of the c(S) mutant (Stummeyer *et al*., unpublished data) revealed no significant difference in the N-terminal portion of endoNF (aa 246–911) compared to the previously described structure of the processed enzyme (14). Additional electron density corresponding to the CTD was found not only at the C-terminal end of the stalk but also surrounding the stalk domain. These findings argue against the *Appendage* model which is based on a misfolded stalk domain.

Secondly, we have previously shown that the chaperone function of the CTD essentially depends on the integrity of a series of highly conserved amino acid residues. Single point mutations of these positions were sufficient to prevent generation of folded functional endosialidases (11, 15). It is likely that CTD-assisted folding involves the formation of protein-protein interactions between CTD and the N-terminal protein portion, which, as proposed in the *Sleeve* model, could take place between CTD and stalk domain. Proteolytic cleavage may be required to terminate these interactions for instance by enabling conformational changes in the CTD that facilitate dissociation from the stalk.

A third argument for the *Sleeve* model is the abrogation of SDS-resistance observed for site b-mutants (cf. Fig. *S1*). Single mutations of Arg-837 and Gln-853 resulted in SDS-sensitive protein complexes. This observation was unexpected since the targeted residues protrude out of the stalk, do not interact with other amino acids, and thus should not play a crucial role in stabilizing the rigid trimeric stalk domain (14). However, if these amino acids contribute to the interaction between stalk and CTD during folding, alanine substitutions could interfere with CTD-assisted folding, resulting in misfolded and therefore less stable enzyme complexes. In line with this hypothesis, the site b double mutant b(RQ) and triple mutant b(RSQ) were expressed exclusively as insoluble proteins.

Noteworthy, the unprocessed variant c(S) showed a 12-fold lower affinity towards solid-phase bound polySia than the b(RS) mutant. One explanation for this might be that binding of polySia to the stalk is not restricted to interactions with residues Arg-837 and Ser-848 but mediated by a number of weak protein-

carbohydrate interactions that were not detected in endoNF crystals soaked with DP5 oligoSia (14). In the c(S) variant, the CTD might conceal a large part of the stalk domain, as proposed by the *Sleeve* model, and thus prevent polySia binding more efficiently than abrogation of site b by two amino acid exchanges.

Interestingly, endoNF mutants that showed reduced binding to surface-bound polySia exhibited increased enzymatic activity towards soluble polySia, strongly arguing that neither CTD release nor integrity of site b is essential for efficient degradation of soluble substrate. However, interference with binding of polySia to the stalk seems to facilitate dissociation from the polymer, thereby allowing faster re-association with new substrate. A gain of cleavage efficiency towards soluble substrate at the expense of processivity and activity towards solid-phase bound substrate has been described for other enzymes that degrade biopolymers like cellulose (cellulases) and chitin (chitinases) (19, 33, 35–38). Processivity in these studies was measured by the fast appearance of short oligomeric cleavage products and a simultaneous slow decrease of long polymer chains (17–19). In the present study, this assay system was adapted to provide direct proof for the processive activity of endoNF.

In line with the fact that endoNF is an *endo*-acting sialidase oligomeric cleavage products were found for both processive and non-processive variants. By contrast, if the minimal acceptor substrate DP4 was used, a sialic acid monomer was released, opening the question whether this residue is released from the reducing end by *endo*-activity or from the non-reducing end by a not yet described *exo*-activity. However, cleavage of DP4 also produced DP3, which is the predominant cleavage product released by processive endoNF from long polySia chains. Therefore, it is quite likely that endoNF acts exclusively in an *endo*-manner and that a glycosidic linkage within oligo- or polySia can be hydrolyzed only if the cleavage site is followed by at least three sialic acids at the non-reducing end.

Based on the observed frequent horizontal gene transfer the concept of modular evolution of bacteriophages has been developed (39–41). In line with this concept, endosialidase genes seem to have evolved by genomic insertion of a bacterial exosialidase into a tailspike gene (12). The polySia binding site b within the tailspike-borne stalk domain might have evolved subsequently by a series of point mutations. The multifunctional structure generated in this way gave rise to an effective adhesin for attachment to the host surface and to a processive degradation machinery for unidirectional penetration of the capsule. In addition, the multidomain structure confers high protein stability by combining the sialidase β-propeller with an intertwining β-helix (14, 15), which, in summary, crucially impacts phage fitness.

Structural data at atomic level are also available for the tailspike endoglycosidases of *Salmonella* phage P22, *Shigella flexneri* phage Sf6 and coliphage HK620 (42–44) that all infect bacterial hosts decorated with a dense layer of lipopolysaccharide. Similar to endosialidases, the tailspike proteins of these phages combine receptor-destroying activity with substrate binding subsites. Although no direct proof is available yet, the organization of binding subsites in these tailspikes implicates a contribution in sliding along the polymeric substrate. Therefore, it is conceivable that processivity is an inherent feature of tailspike proteins with a polysaccharide depolymerase activity.

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FOOTNOTES

[#] The abbreviations used are: aa, amino acids; BSA, bovine serum albumin; CI, confidence interval; CTD, C-terminal domain; DP, degree of polymerization; EC_{50}, half-maximal effective concentration; ΔN-endoNF, endo-*N*-acylneuraminidase or endosialidase (E.C. 3.2.1.129) of coliphage K1**F** lacking the N-terminal 245 amino acids (*ΔN*) of the capsid binding domain (14, 15); ΔN-endoNF*, catalytic part (aa 246-911) proteolytically released from ΔN-endoNF (11, 15); oligoSia, oligosialic acid; polySia, polysialic acid; TBA, thiobarbituric acid. Further abbreviations are listed in *Table 1*.

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SUPPLEMENTARY MATERIAL

Figure S1

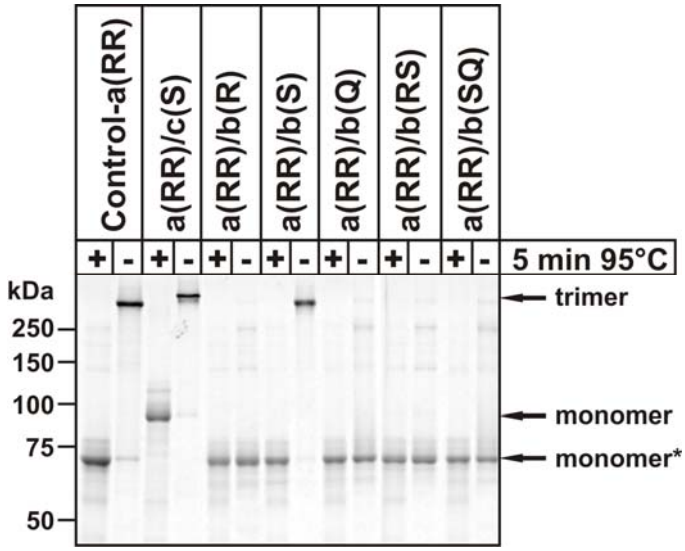

FIGURE S1. **Complex formation of enzymatically inactive ΔN-endoNF-a(RR) variants.** Affinity purified proteins were analyzed in 7% SDS-PAGE. To determine SDS-resistance (*trimer*) of the respective variant, the initial boiling step (+) in the presence of 1% (w/v) SDS was omitted (–). *Monomer* indicates the monomeric non-cleavable mutant c(S), whereas all other mutants were found proteolytically processed (*monomer**). Numbers on the left side represent protein standards.

TABLE S1
Quaternary structure of ΔN-endoNF variants. The oligomeric state of the indicated endoNF variants was determined for affinity-purified proteins by size-exclusion chromatography. Of each protein 480 µg were loaded in 300 µl onto the column. The oligomeric state was calculated from the values obtained by size-exclusion chromatography divided by the mass calculated for the monomer from the amino acid sequences including the N-terminal Strep-tag II (76.1 kDa).

EndoNF variant	Molecular mass (size-exclusion chromatography)	Oligomeric state
	kDa	
Control-a(RR)	225.7	3.0
a(RR)/b(R)	256.8	3.4
a(RR)/b(S)	252.6	3.3
a(RR)/b(Q)	239.5	3.1
a(RR)/b(RS)	245.3	3.2
a(RR)/b(SQ)	231.2	3.0

Chapter 6 - General Discussion

6.1 - Characterisation of a polysialyltransferase of *Neisseria meningitidis*

A major cause of bacterial meningitis in children and adolescents is the diplococcus *Neisseria meningitidis*. Sporadic outbreaks and recurring epidemic waves are registered not only in the so-called meningitis belt between Senegal and Ethiopia, but also in Asia and the developed countries of Europe, the Americas and Oceania. Current treatment of the bacterial meningitis is generally based on strong doses of antibiotics given intravenously. Recently observed resistances of *Neisseria meningitidis* against antimicrobial agents (Jorgensen *et al*., 2005), and a still high case-fatality of treated patients, necessitate the development of alternative therapeutics and healing strategies. A promising approach might be the interruption of capsule biosynthesis. In *Neisseria meningitidis* serogroup B (*Nm*B) this capsule is composed of α2,8-linked polysialic acid and is considered as an important virulence factor due to mediated resistance to phagocytosis and the inhibition of the alternative pathway of the hosts immune response. The key enzyme of capsule formation is the polysialyltransferase (polyST), which polymerises CMP-activated sialic acid units to form α2,8-linked polysialic acid (polySia), the serogroup B antigen.

At the beginning of this study, no information on the isolated polysialyltransferase and virtually no data on structure-function relationships, which are fundamental for the rational design of inhibitors, had been reported. In the following discussion data derived from the most homologous enzyme, the polyST of *E. coli* K1 is included, which shares 30 % homology with the amino acid sequence of the neisserial enzyme. Subcellular fractionation experiments argued for a membrane association of the protein at the inner side of the cytoplasmic membrane (Masson and Holbein 1983). Attempts to purify the enzyme in a soluble form led to inactivation, which again has been taken as poof for the necessity of membrane-attachment (Steenbergen and Vimr, 2003; Vionnet *et al.*, 2006). Our screening of a variety of expression and purification systems, however, enabled production of soluble and active polyST, clearly demonstrating that membrane attachment is not necessary for activity *in vitro*. Despite that fact, it is worth hypothesising that membrane localisation is required *in vivo* for the regulation of polySia biosynthesis and translocation to the cell surface. Accordingly only fusion of large soluble N-terminal tags as such as NusA and Maltose Binding Protein (MBP) fused to the polyST resulted in active and stable protein in the soluble fraction. This again points out the

per se hydrophobicity of the polyST, which had already been taken into account in the capsular biosynthesis complex model provided by Steenbergen and Vimr (2003).

A continuous spectrophotometric polyST assay adopted from Gosselin *et al*. (1994) allowed the permanent tracking of activity. This assay allowed us for the first time to biochemically characterise a purified polyST. A K_m value of 0.42 mM for the donor substrate (CMP-Neu5Ac) was observed and found to be five-fold higher than the obtained K_m of membrane bound enzyme from *E. coli* K1 polyST (Kundig *et al*., 1971; Vijay and Troy, 1975). This difference might be due to the different enzyme source and preparation methods, accompanied by different assay and buffer conditions. Later studies done by Willis *et al*. (2008) with a comparable *Nm*B polyST enzyme preparation, found the apparent K_m to be 1.4 mM. This aberrance might be caused by the different assay system they used (capillary electrophoresis), or more likely by the usage of GT3-FCHASE, a fluorescently labelled tri-sialyllactose as acceptor which might not reflect the native situation. The authors also report that better acceptor substrates were generated by elongation of the synthetic GT3-FCHASE which confounds their kinetic characterisation.

In our study, the determination of the minimal acceptor structure of the polyST revealed the necessity of three α2,8-linked sialic acid residues for an efficient chain elongation. This is in agreement with investigations done with membrane fractions containing polyST of *E. coli* K1 and K92. There the acceptor dependence is illustrated by radiochemical and HPLC analysis (Steenbergen and Vimr, 1990; Ferrero *et al*., 1991; Chao *et al*., 1999).

To elicit more detailed information about product formation, gel electrophoresis of the produced polySia has been performed followed by alcian blue and silver staining. These experiments show the generation of long polysialic acid chains with a degree of polymerisation of more than 100 residues. Similar chain lengths occur naturally at the surface of the bacterium of *E. coli* K1 (Rohr and Troy 1980) which in terms of capsular polysaccharide is most similar to *Nm*B. For this reason, a stand-alone synthesis of the capsular polysaccharide by the polyST can be hypothesised. In contrast, the identity of the original acceptor present in the bacterium is not yet known, but might include a sialyl monophosphorylundecaprenol as it is discussed for *E. coli* K1 (Troy *et al*., 1975). Likewise the termination of elongation remains to be explored as there is a defined polysaccharide chain length at the surface of *E. coli* reported (Rohr and Troy 1980).

With the available active polyST a detailed investigation of structure-function relations could be started. To narrow down the catalytic domain of the polysialyltransferase, N- as well as C-terminal truncations of the polyST domain in the NusA-polyST fusion protein were carried

out. Foregoing considerations including alignments of the polyST of *Neisseria meningitidis, E. coli* K1, and K92 revealed a C-terminal extension of 94 amino acids (aa) present in the neisserial enzyme. Interestingly, truncation of this complete domain resulted in increased solubility of the enzyme preparations. The activity, however, was completely abolished by the truncation of only 22 aa, 60 aa or the complete C-terminal domain (94 aa 95 aa 97 aa). On the contrary, only slight effects on solubility and activity could be observed in the case of short N-terminal truncations of 23 aa or 33 aa. But removing the first 64 aa resulted in an inactive and more insoluble protein. These findings clearly demonstrate that the C-terminal 94 aa have an essential function in catalytic activity of recombinant *Nm*B polyST, though not conserved in the homologous *E. coli* enzymes. Reasonable conjectures for the function of this domain includes therefore the involvement in substrate binding, protein folding or oligomerisation and remains to be investigated.

The eukaryotic sialyltransferases are grouped into a single CAZy glycosyltransferase family 29 (GT-29) and are described to contain four highly conserved and extensively described sialylmotifs (Drickamer, 1993; Lvingston and Paulson, 1993; Geremia *et al.,* 1997; Jeanneau *et al*., 2004). In contrast, bacterial sialyltransferases are grouped into four different CAZy families. GT-42, GT-52 and GT-80 contain bacterial LOS-sialyltransferases and GT-38 includes bacterial polysialyltransferases. Additionally, no conserved sialylmotif had been identified for the bacterial enzymes. Refined sequence alignments, carried out in this study, included members of the above described families and the pfam family 05855. As a result, two highly conserved short motives could be identified and are named D/E-D/E-G motif and HP motif. Mutagenesis of one single amino acid in the D/E-D/E-G motive to alanine (E153A and G154A) resulted in completely inactive protein *in vitro* as well as *in vivo*. This demonstrates the importance of this motif for enzymatic activity. In the case of the HP motif, a double mutation to alanine, (HP278, 279AA) is necessary to have the same effect. Single mutation of either the histidine or proline to alanine (H278A or P279A) resulted in reduced capsule formation and lowered catalytic efficiency. Most interestingly i*n vitro* studies of these mutants revealed an increased K_m value for CMP-Neu5Ac but not for colominic acid, which suggests the involvement of the HP motif in CMP-Neu5Ac binding.

Further information and confirmation of the functional significance of the D/E-D/E-G and HP motifs is provided by the crystal structure of the sialyltransferase of *Pasteurella multocida* (*Pm*ST1) which is grouped into the CAZy family GT-80 and belongs to the structural superfamily B of glycosyltransferases (Ni *et al*., 2006). This fold was also predicted for CAZy family GT-38 for bacterial polySTs including the *Nm*B polyST (Breton *et al*., 2006).

Interestingly, the two short motives D/E-D/E-G and HP could also be found in the active site of the *Pm*ST1 and the pertained histidine H311 forms a hydrogen bond to the phosphate group of CMP. Similar to our studies, mutation of the H311 resulted in an increase of the K_m value and was therefore hypothesised to stabilise the CMP leaving group of the *Pm*ST1 catalysis (Ni *et al*., 2007). The residual activity of the histidine mutants in both enzymes argue for the involvement in substrate binding rather than involvement in catalysis where a more severe effect would be expected. Combined, the data strongly indicate that histidine H278 of the *Nm*B-polyST also functions in binding the CMP moiety of CMP-Neu5Ac.

Our STD-NMR investigations of the MBP-polyST fusion construct revealed that binding of CMP-Neu5Ac is predominantly mediated through the ribose and cytosine moieties. The largest amount of saturation was observed to be associated with the ribose and cytosine moieties of CMP-Neu5Ac. In detail, H1 of the ribose received 100 % of saturation whereas the protons of the cytosine received 89% and 44% for the H5 Cyt and H6 Cyt, respectively. In contrast, the sialic acid residue received an average saturation of 36% indicating a less tight association with the enzyme. This data is in good agreement with the earlier STD-NMR studies of Biet and Peters (2001) done with a β(1,4)-galactosyltransferase T1. They showed that the galactose moiety of UDP-Gal, which is transferred by the enzyme to β–D-*N*-acetylglucosamine, received less saturation than the UDP moiety. Both studies revealed that the nucleotide moiety of the nucleotide sugars are the major interaction partners of the respective enzyme. This weaker binding of the transferred glycosyl residue in both cases, the UDP-Gal and CMP-Neu5Ac, presumably ensures an efficient transfer. The crystal structure of the *Pm*ST1 with bound 3-Fluoro(α)Neu5Ac (pdb code: 2IY7.pdb) exemplifies these findings. A deep cleft of the protein buries the complete CMP moiety while the sialic acid residue is more exposed to the solvent.

Detailed information on the mode of polySia synthesis was gained by gel chromatography of the produced polySia. The results clearly show the dependence of the product chain-length on the starting acceptor concentration. That proves a non-processive mechanism of the polyST, as in the case of a high processivity there would only occur long chains, irrespectively of the acceptor concentration. This finding however, might only be relevant to the *in vitro* situation with purified enzyme as there are studies carried out *in vivo* with *E. coli* K1 which suggest a processive manner of elongation (Steenbergen and Vimr, 2003). There, a large biosynthesis complex is proposed which tightly connects the biosynthesis and translocation of the polySia across the inner and outer bacterial membrane. Additionally, Vionnet *et al.* (2006) stated the functional polyST complex to be larger than a monomer in *E. coli* K92. Thus, an *in vivo*

situation including potential interaction partners of the polyST could increase its biosynthetic efficiency and thereby increase processivity. Membrane preparations of *E. coli* K92 were also demonstrated to be non-processive *in vitro* (Vionnet and Vann, 2007), but did not contain all natural factors involved in capsular biosynthesis. Even intact membrane preparations of *E. coli* K1 which most probably contain all relevant factors were found to be not as efficient with exogenously added sialyl-oligomers as with endogenous acceptors (Cho and Troy, 1994; Chao *et al*., 1999; McGowen *et al*., 2001). This might argue for a more enclosed binding of endogenous acceptors that lead to a higher processivity.

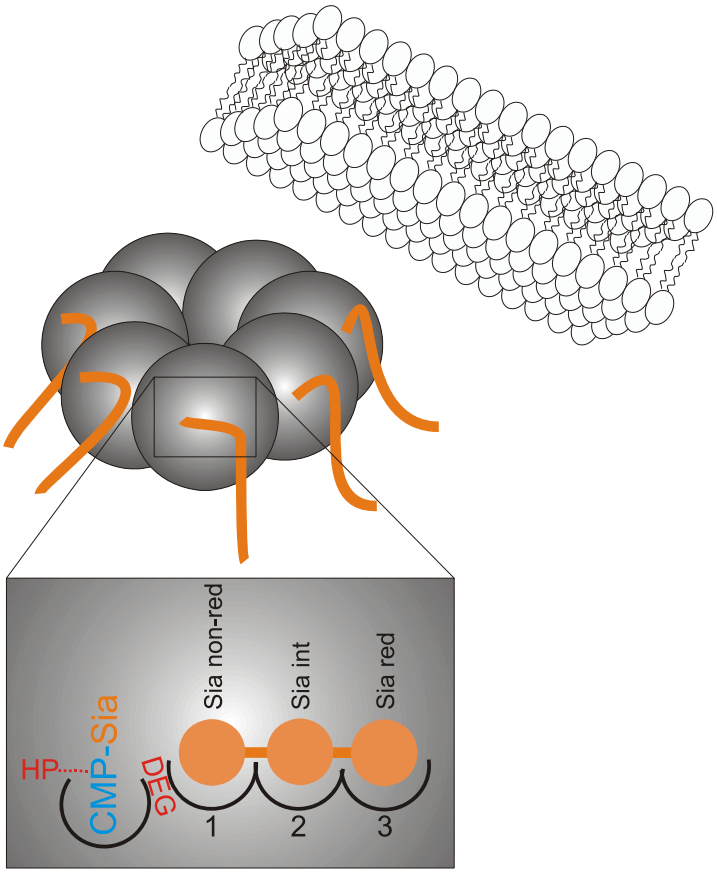

Fig. 6-1: Model of polySia Synthesis.

Based on gained data, a model of polySia synthesis has been established: (i) polyST is highly oligomerised, (ii) polyST is not attached to the membrane, (iii) CMP-Neu5Ac is mainly bound by CMP moiety, (iv) DP3 is in total attached to the enzyme, (v) DEG-motif is involved in the active site, (vi) HP-motif is involved in binding to the CMP moiety of CMP-Neu5Ac, (vii) non-prozessive, illustrated by external attachment of polySia. (poly)Sia is depicted in orange, polyST as grey sphere.

Binding studies carried out with the minimal sialic acid acceptor DP3, by STD-NMR analysis, however, showed a complete binding of all three sialic acid units. Close observation of the STD-NMR spectrum revealed similar signal intensities of the resolved signals of the H3 in equatorial position (H3eq) namely H3eq,$Sia_{non\text{-}red}$, H3eq,Sia_{int}, and H3eq,Sia_{red}. This argues for similar binding effectiveness irrespectively of the position in the oligomer DP3.

Additionally, integration of the partially resolved NHAc signals showed a slightly increased signal in the case of NHAc,Sia_{red} than the combined signals of NHAc,$Sia_{non\text{-}red}$, and NHAc,Sia_{int}. This excludes a predominant binding of the non reducing end of the polySia chain, where elongation takes place. Maybe this characteristic of the *Nm*B-polyST facilitates a fast release of the polysialic acid product and may therefore provide a structural explanation for the non-processive nature observed *in vitro*. This also may explain why DP2 is not an efficient acceptor (Freiberger *et al*., 2007). DP2 lacking the third sialic acid unit may be bound to sites 2 and 3 (Fig. 6-1) and therefore too distant to interact with the active site.

6.2 - Using polysialic acid active enzymes for biotechnological applications

During the last decade, polySia has been found to contribute to a variety of application fields including biotechnology and pharmacology. Recent publications describe polySia as a beneficial conjugate with protein and peptide drugs, which is an upcoming class of therapeutics (Bezuglov V.V. 2009, 2009; Gregoriadis G. 1999, 2000, 2005, Fernandes 1996, 1997, 2001). PolySia was also shown to be a suitable cell culture substrate for a variety of neurons and glial cells (Haile *et al*., 2007; 2008). Additionally Berski *et al*. (2007) investigated the production of a polySia based hydro-gel which offers attributes for a promising new biomaterial as it biodegradable by a bacteriophage derived endosialidase EndoNF. Therefore the need of polySia, by means of assembly as well as controlled degradation, is increasing. Chemical synthesis of this polymer is hindered due to challenging stereo-selectivity and regio-selectivity. Industrial supply of polySia concentrates therefore on purification of the polymer from cell culture supernatants as described by Rode *et al.* (2008). However, for the use of cell culture derived polySia in pharmaceutical purposes additional guidelines of Good Manufacturing Practice (GMP) apply. Enzymatic synthesis of polySia instead would combine advantages of chemical and biotechnological synthesis, namely highly stereo- and regio-selective synthesis, the application of defined starting materials, and simplified purification.

6.2.1 – *In vitro* production of polysialic acid

As discussed in Chapter 6.1 the polyST of NmB is the most efficiently expressible α2,8 sialic acid transferase available (Freiberger *et al*., 2007; Willis *et al.*, 2008), which can be produced in milligram scale. No other polysialyltransferases are available in reasonable amounts to date. Therefore, in this study a production system was established based on the neisserial

polysialyltransferase (MBP-polyST *Nm*B) and the CMP-Sialic acid Synthetase (CSS) of *Neisseria meningitidis*. The latter was chosen because of its comparable optimum reaction conditions (Mizanur and Pohl, 2008) to MBP-polyST *Nm*B. Additional enzymes, the pyruvate-kinase and myo-kinase, were added to form a cyclic reaction in which the CTP is regenerated. The high energy phosphate donor CTP is then replaced by phosphoenolpyruvate, which allows employing cheaper starting materials, and circumvents the accumulation of CMP which might have inhibitory effects on the polyST as it is shown for other silayltransferases (Cambron and Leskawa 1993). Finally, we could produce 5mg per 10ml reaction volume.

The enzymatic *in vitro* production of polySia accommodates the additional advantage of highly defined products in terms of absent side products like O-acetylation (Orskov *et al*., 1979), or de-N-acetylation after polymerisation (Moe *et al*., 2009).

Purification of the produced polySia to homogeneity could be achieved in a three step protocol and was facilitated by defined staring material and reduced contamination by using vitro synthesis.

To date, acidic or thermal hydrolysis (Roy *et al*., 1990; Cheng *et al*., 1999; Häyrinnen *et al*., 2002) of capsular polysaccharide are possibilities for the generation of medium chain lengths. They lead to large distribution of chain-lengths or in the case of complete hydrolysis to very short oligomers ranging from DP2 to DP6 (Patane *et al*., 2009). Meanwhile the demand on more define polySia chain-lengths, especially medium sized chains ranging from DP15 to DP90 is increasing, as a result of findings on the influence of the chain-length to filament formation (Toikka *et al*., 1998) and antibody or endosialidase recognition (Hayrinen *et al*., 2002; Aalto *et al*., 2001). Moreover, immunogenicity of α2,9-linked or alternating α2,8 - α2,9 linked polysialic acid alters with growing chain-length (Gregoriadis *et al*., 2000). As shown in Chapter 2 and 4, it is possible to predetermine the shape of the produced chain length by adjusting the donor acceptor ratio in the reaction mix. In this study the production of tailor-shaped small range disperse polySia i.e. DP4 to DP15, DP15 to DP30, DP30 to DP90, and chains >DP100 could be shown.

Enzymatic synthesis of polySia using purified enzymes *in vitro* additionally allows the production of radiolabelled (e.g. ^{14}C or ^{3}H) or fluorescently labelled oligo or polysialic acid which might contribute to upcoming investigations of drug clearance and biochemical and histological staining. Prospective extensions of the cyclic reaction comprise the use of sialic acid derivatives and the inclusion of the sialic acid synthase and N-acetyl-glucosamine-6-phosphate-2-epimerase. This would allow starting the reaction from N-acetyl-glucosamine-6-

phosphate. Initial trials are already done by Yu *et al*. (2004 and 2006) and Hamamoto *et al*. (2005) who enzymatically generated CMP-sialosides in a multi-step enzymatic synthesis.

6.2.2 - Degradation of polysialic acid by endosialidase Endo NF

As already mentioned in section 6.2, polySia is degraded by bacteriophage derived endosialidases that are tailspike proteins specifically degrading α2,8-linked polySia which naturally occurs as capsular polysaccharide of their hosts. Recently the crystal structure of the catalytic domain of EndoN from coliphage K1F (EndoNF) was solved (Stummeyer *et al.*, 2005) and a C-terminal domain was found to function as an intra-molecular chaperone domain, which is proteollytically cleaved during maturation (Schwarzer *et al*., 2007). In the present study the cleavage modalities of wild type enzyme as well as a non cleavable mutant and a mutant lacking of the polySia binding site "*b*" were investigated. It could be shown that the processivity of the wild type depends on the integrity of the binding site "*b*" which can be negatively affected by either mutation of involved amino acids (R837A and S848A) or by prevention of cleavage of the C-terminal domain by exchanging serine 911 to alanine. As a consequence of both mutations processive degradation of polySia is affected resulting in more random-sized brake down products. In terms of biotechnological applications these endosialidases, the wild type as well as the mutants, are useful tools for trimming of enzymatically synthesised polySia.

Chapter 7 - References

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Abbreviations

2D	two dimensional
3D	three dimensional
aa	Amino acid
CMP	Cytidine 5`-monophosphate
C-terminal	Carboxy-terminal
CSS	CMP Sia synthetase
CTP	Cytidine 5`-triphosphate
DP	degree of polymerisation
E. coli	*Escherichia coli*
EndoNF	Endoneuraminidase F
Gal	Galactose
Glc	Glucose
LOS	Lipo-oligosaccharide
MBP	Maltose binding protein
N.	*Neisseria*
NCAM	Neural cell adhesion molecule
Neu5Ac	5-N-acetyl- neuraminic acid
*Nm*B	*Neisseria meningitidis* serogroup B
*Nm*C	*Neisseria meningitidis* serogroup C
NMR	Nuclear magnetic resonance
N-terminal	amino-terminal
OD	optical density
polySia	polysialic acid
polyST	polysialyltransferase
PP_i	pyrophosphate
sia	sialic acid
STD-NMR	saturation transfer difference-NMR
wt	wild type