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Sialidases in biological systems

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Sialidases are very common in biological systems. They are found particularly in diverse virus families and bacteria, but also in protozoa, some invertebrates and mammalian. The enzymes differ in their biochemical properties, e.g., kinetics, binding affinity or substrate preference. Nevertheless, they have conserved domains and structural similarities. Their functions offer a wide spread spectrum in nutrition, pathomechanisms and communication in mammalian. In pathogen organisms sialidases give insight in coevolution and interaction between pathogen or commensale and host. As well the enzymes are of interest in drug targeting, like neuraminidase inhibitors of influenza viruses.

1. Introduction

1.1. Sialic acids

The family of sialic acids includes over 40 representatives of N- and O-acetylic derivates of neuraminidase (5-amino-3,5-dideoxy-D-glycero-D-galactononulonic acid) (Taylor 1996; Schauer 2000). The best known and most common compound is N-acetylneuraminic acid (Neu5Ac or NANA) (Fig. 1) (Traving et al. 1998). The aminosugar with 9 carbon atoms is biosynthesised from N-acetyl-mannosamin-6-phosphate, and phosphoenolpyruvate is bound on hydroxyl groups of galactose, N-acetylgalactosamin or another molecule of N-acetylneuraminic acid by glycosidic linkage (Löffler et al. 2007; Devlin 2006; Berg et al. 2007).

Sialic acids are located within or terminally both on macromolecules or surfaces of cells (Schauer 2000). They are natural parts of glycoproteins and -lipids. One can also find them in mucopolysaccharids, cell membranes, glycocalyx, membrane proteins (e.g. insulin receptors) and colominic acids of cell walls of *Escherichia coli* (Traving et al. 1998). Table 1 gives an overview about the functions and locations of neuraminic acids.

Sialic acids seem to be found only in the lineage of deuterostomia with the exception of some bacteria, viruses, protozoa and larvae of *Drosophila* (Schauer 2000; Traving et al. 1998; Roggentin et al. 1993). No sialic acids could be detected in plants or other metazoa until now (Schauer 2000; Roggentin et al. 1993; Zeleny et al. 2006). The sialic acids in deuterostomia have predominantly an alpha(2,3)- or alpha(2,6)-glycosidic linkage to sugars as oligosaccharines, glycoproteins or glycolipids. An alpha(2,8) linkage occurs mainly in gangliosides and glycoproteins (Roggentin et al. 1993).

Abbreviations: F, Fusion protein; GPI, Glycophosphatidylinositol; HA, Haemagglutinin; HPIV, Human parainfluenza virus; HN, Haemagglutinin-neuraminidase; LPS, Lipopolysaccharide; NEU, Neuraminidase in mammalian; NA, Neuraminidase; NAI, Neuraminidase inhibitor; NDV, Newcastle disease virus; RSV, Respiratory syncytial virus; SA, Sialidase; TS, Trans-sialidase.

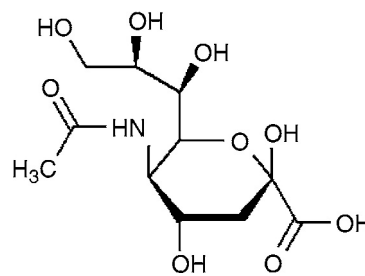


Fig. 1: Structure of N-acetylneuraminic acid

Table 1: Function and occurrence of neuraminic acids in mammalian (referred to Bundschuh et al. 1976)

Biological function	Occurrence
Charge carrier	cells, blood cells, tumor cells, gangliosides (CNS), glycoproteins
Masking of antigens ((Pseudo-)Friedrich-antigens)	antigens of erythrocytes, transplantation antigen, tumor antigen, normal cell antigen
Receptors	serotonin, alkaline polymers, tetanus toxin, viruses
Part of antigens	blood groups, bacteria
Biological activity of hormones	intrinsic factor, choriogonadotropin, erythropoietin

Terminal sialic acids determine the survival time of erythrocytes, thrombocytes and leucocytes. Furthermore, these terminal sialic acids mediate (immunological) distinction in between foreign and self (Traving et al. 1998; Rassow et al. 2006).

Sialic acids are parts of the membranes. This way, they enhance the cell surface's electronegativity. Thus, they have an influence on binding and transport of positive charges (for example

Ca²⁺) as well as on aggregation and segregation in between cells and molecules (Traving et al. 1998; Nakano et al. 2006). They stabilize molecule conformation and protect glycoproteins from degradation by proteases and endoglycosidases. Moreover, sialic acids have protective effects, for example by masking cell receptors from pathogens (Schauer 2000; Traving et al. 1998; Nakano et al. 2006; Wiggins et al. 2001). They are an important part of mucous and serum in higher animals mediating viscosity and elasticity (Traving et al. 1998). In prokaryotes, sialic acids seem to occur exclusively on the capsule and in LPS. There, they have an alpha(2,8), alpha(2,9) or an alternating homopolymer alpha(2,8) and alpha(2,9) linkage (Schauer 2000; Roggentin et al. 1993; Corfield 1992). Moreover, sialic acids serve as a receptor for various pathogens. Thus, they empower bacteria as *E. coli*, influenza viruses, trypanosomes and toxins (e.g., cholera toxin) to enter the host cell (Traving et al. 1998; Rinninger et al. 2006).

1.2. Neuraminidase

Neuraminidases belong to the family of exoglycosidases (EC 3.2.1.18). By hydrolysis, they cleave bonds between non-deoxidising terminal sialic acids and mono- or oligosaccharides of glycoproteins or glycolipids linked ketosidically with each other (Monti et al. 2002). They have high substrate specificity (Wiggins et al. 2001) and their affinity and activity depends mainly on the type of sialic acid derivative, the quality of the ketosidic bond and the penultimate saccharide-moiety (Roggentin et al. 1993). This specificity is of paramount importance for an effective control of the sialic acids' various functions *in vivo* (Roggentin et al. 1993).

To date, neuraminidases could be found in animals, microorganisms and viruses. They have an impact on pathogenity. In bacteria, they seem to serve mainly nutritional purposes by providing carbohydrates (Roggentin et al. 1993; Wiggins et al. 2001).

1.3. Origin of the neuraminidases

There is a big biochemical diversity in neuraminidases. This seems to exclude close relationships. Nevertheless, there are homologies on the molecular level, which support the idea of a common origin. It is thought that the origin of neuraminidases lies in higher organisms. These higher life forms might have incorporated genetic information from commensalic or pathogenic bacteria. Subsequently this genetic information might have been passed on in between bacteria via phages (horizontal gene transfer) (Roggentin et al. 1993). The diversity in sialic acid structure and glycosidic linkage might have fuelled the evolution of neuraminidases (Roggentin et al. 1993).

1.4. Goals of research

The neuraminidases got into the spotlight of current research at the latest with the search for effective drugs for the treatment of viral flu. Thus, two neuraminidase inhibitors (NAI) effective against influenza A and B got approved by the European Union in 2002 (McKimm-Breschin et al. 2003). Neuraminidases are also found in other virus families, several prokaryotes, protozoa and mammalia. This review gives an overview of the current knowledge of neuraminidases covering both of their occurrence and function as well as the possible application of NAIs in biological systems. We will focus on selected humanopathogenic organisms.

The literature research for the work at hand was done with the database PubMed. A total of 896 items was displayed. Of these, 51 articles were included in the current review.

2. Neuraminidases

2.1. Neuraminidases in viruses

So far, Neuraminidases with virulent qualities could be found in paramyxoviruses and orthomyxoviruses (Table 2).

2.1.1. Paramyxoviridae

In Paramyxoviridae, neuraminidases were identified in respirorubula- and avulaviruses. The mumps virus is classified as a rubulavirus. Morbilliviruses, though having haemagglutinin, possess no neuraminidase activity. Among the pneumoviruses (also comprising the respiratory syncytial virus [RSV]) neither haemagglutinin nor neuraminidase activity could be detected. In these viruses the G-protein mediates adsorption to the host cell (Modrow et al. 2003; Takimoto et al. 2004). Therefore, there is a huge heterogeneity within the family and even the genus. N-terminally, the haemagglutinin-neuraminidase molecule (HN) has a small cytoplasmatic region, followed by a short transmembrane region. The ektodomain with the C-terminal tail consists of a stick region and a globular head (Yuan et al. 2008; Moscona 2005). The latter contains the active centre. It is responsible for both the binding to the receptor (sialic acids) as well as the neuraminidase's enzymatic activity (Moscona 2005). The HN in mumps viruses and parainfluenza viruses type 1 is glycosylated and acetylated (Modrow et al. 2003). The monomeric neuraminidase domain stabilizes the folding and oligomerization of the tetrameric stick which is bound non-covalently. The tetrameric stick is made of two dimers. Depending on the virus, these dimers are linked with each other by two disulphide bridges each (Yuan et al. 2008). The HN-domain with neuraminidase activity in respiro- and rubulaviruses has a strong homology to the neuraminidase in influenza viruses (Modrow et al. 2003). Its catalyzation of neuraminic acid breakdown prevents virus particles from agglutination, inhibits cell superinfection and promotes viral detachment from the host cell during budding

Table 2: Overview of occurrence of viral neuraminidases (modified from Modrow et al. 2003)

Virus family	Genus	Human	Animal	Virulenz factor
Paramyxo-viridae	Respirovirus	Parainfluenzavirus type 1 & 3	Bovines Para-influenzavirus type 3 Sendaivirus	HN
	Rubulavirus	Mumpsvirus Parainfluenzavirus type 2 & 4a,b	Simian-Virus 5	HN
	Avulavirus		Newcastle-Disease-Virus	HN
Orthomyxo-viridae	Influenza-A-Virus	Influenza-A-Virus	Influenza-A-Virus	NA
	Influenza-B-Virus	Influenza-B-Virus	Influenza-B-Virus	NA

(Modrow et al. 2003; Takimoto et al. 2004; Moscona 2005; Huang et al. 2008). The majority of paramyxoviridae is transmitted by droplet infection (Flint et al. 2004), thus getting in touch with the mucous secretion in the respiratory tract. Therefore, a function of neuraminidase similar to influenza particles has to be taken into consideration: the detachment from mucous containing sialic acid (Matrosovich et al. 2004).

Both the binding activity and the enzymatic activity on the globular head of HN in human parainfluenzavirus type 3 (HPIV 3) can be inhibited by zanamivir and DANA (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid). Both drugs (the somewhat smaller DANA lacking the guanidine group) act by binding to the enzyme's active centre (Kellogg et al. 2007). A mutation in position 193 (threonin to isoleucin) in the catalytic centre causes a steric inhibition of zanamivir binding. Thus, the mutation leads to zanamivir-resistance. Substitution of asparaginic acid by asparagin in position 216 in the globular head results in a loss of neuraminidase activity (Moscona 2005). Above, we discussed the HN molecule's function of receptor-binding and neuraminidase activity. Apart from these immediate functions, its "stick" appears to be essential for the fusion protein (F) activation promoting viral fusion with the host membrane (Yuan et al. 2008; Palermo et al. 2007; Lamb et al. 2006). The exact mechanism remains unclear. Mutations within the HN-molecule, however, have an effect on the activation energy of F (Moscona 2005; Lamb et al. 2006).

In mumps viruses it was not possible to disclose the exact influence of HN, either. Nevertheless it is clear that the glycoprotein plays an important role in the virus-cell-fusion. Strains with lower neuraminidase activity showed a higher fusion activity than strains with high neuraminidase activity (Lemmon et al. 2007).

Similar to the newcastle-disease-virus (NDV), Kellogg et al. (2007) postulate for HPIV 3 a second active domain within the HN-protein. This domain, however, does not have neuraminidase activity, promoting only the receptor-binding and reportedly being resistant to zanamivir. The domain is supposed to be located at the intersection of the two dimers around position 552 (Kellogg et al. 2007). A mutation at this position (H552Q) is thought to result in higher receptor affinity (Moscona 2005). With zanamivir, both NDV and HPIV 3 infections could be inhibited markedly. Contrary to what would be expected, zanamivir treatment of HPIV infection does not inhibit the release of virus particles from the host cell's surface (as in influenza viruses). In fact, the interaction between viral HN and cellular receptor is blocked. Therefore, NAI therapeutic effect is limited to the beginning of the infectious cycle (Moscona 2005). Blockage of HN-receptor-interaction inhibiting the necessary fusion prevents virus entry into the cell. Yet, this seems to work only for HPIV to a satisfactory degree (Moscona 2005). It is possible that the second receptor binding site not blocked by zanamivir accounts for this.

2.1.2. Orthomyxoviridae

In Orthomyxoviridae, the neuraminidase is encountered only in influenza viruses. Because of its importance in flu epidemics and pandemics, it is studied extensively. Therefore it is arguably the most thoroughly investigated neuraminidase in biological systems.

The neuraminidase in influenza viruses is build by four identical monomers, which are associated in a mushroom-like tetramere. To date, nine antigen-subtypes of the influenza neuraminidase have been identified (N1 to N9). Each monomer consists of six quadruple chain-, antiparallel beta sheets, whose arrangement is evocative of a beta propeller. There is a nearly six fold symmetry axis running through the centre of the monomer. It

links the six beta sheets to the active centre of the enzyme, which lies next to these pseudo symmetry axes (Taylor 1996; von Itzstein et al. 1993). The neuraminidase's head is connected to the transmembrane domain by a stick. The stick's function is unknown (Ferraris et al. 2008). There is an excavation in the active centre close to the O4 of the sialic acid, flanked by two glutamine acids. This catalytic centre and its framing amino acids (centre: R118, D151, R152, R224, E276, R292, R371 and Y406; framing amino acids: E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294 and E425) is highly conserved both between the different subtypes of influenza A as well as between type A and B (Taylor 1996; Yen et al. 2006). The neuraminidase is seen as an integral type II membrane-glycoprotein being anchored to the membrane with a hydrophobic sequence of 29 amino acids close to the N-terminus (Taylor 1996; Ferraris et al. 2008). The protein's cytoplasmatic portion consists of six amino acids. In this context it is noteworthy that one of these is a proline conserved both in type A and B. By means of the secondary structure, this proline seems to be important for the enzymatic function (Chen 2007). There are 1413 nucleotides in the gene coding for a protein of 454 amino acids which has five potential sites for glycosylation (Ferraris et al. 2008).

Russell et al. (2006) classified the neuraminidases into three clusters based on their three-dimensional structure. The first one contains the subtypes N1, N4, N5 and N8; the second one N2, N3, N6, N7 and N9; the third one the neuraminidase of influenza B. It could be shown that N1 and N2 differ in an excavation in the active centre close to the ligand binding site. This excavation cannot be found in N2. The neuraminidase in influenza viruses recognises terminal *N*-acetyl-neuraminic acids with alpha(2,3) or alpha (2,6) linkage depending on the subtype and subsequently cleaves them by hydrolysis. Substances as zanamivir, oseltamivir or peramivir (currently being developed) compete for the substrate. Therefore they are competitive inhibitors of the neuraminidases (Taylor 1996; Ferraris et al. 2008). Peramivir proved to be effective against H5N1 (A/Vietnam/1203/04) when administered intramuscularly in mice (Boltz et al. 2008).

The cleavage product, sialic acid, binds to the active centre in boat conformation. During this, the substrate's equatorial carboxyl group (at C2) interacts with three conserved arginines (R118, R292 und R371) in the enzyme (von Itzstein et al. 1993; Yen et al. 2006). Several studies suggest that it is R118 among the three arginines which plays the major role since strains with a mutation at this site can not replicate efficiently. The $N\epsilon$ group of R118 builds a salt bond with E425. It is via the $N\eta 1$ group that it also interacts with E119, which in itself is connected to R156. E227 stabilises the active centre's structure on several levels by hydrogen bonds: on the one hand between the oxygen atom of the carboxyl group (the O in S179) and the amide in D180, on the other hand between the carboxyl group's other oxygen atom and the amide in the substrate's *N*-acetyl-group as well as a water molecule in immediate vicinity. This water molecule on its part is connected by a hydrogen bond to E277. R152 interacts both with the oxygen in the substrate's *N*-acetylic group (sialic acids and neuraminidase inhibitors) and the asparaginic acid in position 198. The hydrophobic portions of R224, I222 and A246 form a hydrophobic pouch enclosing the glycerol chain of sialic acids and of zanamivir. The glutamine acid in 276 is connected by a hydrogen bond to the glycerol chain's O8 and O9 (O8 and O9 are the hydroxyl groups located at C6). In oseltamivir, by contrast, this connection is made by the amino acids on position 224 and 276 via the pentyloether group. D151 appears to work as an acidic catalyst for the initial step in the sialyl-enzyme interaction by forming a hydrogen bond with the glycosidic oxygen (Yen et al. 2006).

The neuraminidase is essential for the virus's excretion from the host cell and the prevention of superinfection by viruses

Table 3: Overview of neuraminidases in pathogenic bacteria

Family	Genus	Species	Disease	Virulenz factor
Actinomycetaceae	<i>Actinomyces</i>	<i>A. viscosus</i>	Periodontitis	NA
Clostridiaceae	<i>Clostridium</i>	<i>C. perfringens</i>	Gas gangrene Enterocolitis	NA
		<i>C. septicum</i>	Gas gangrene	NA
		<i>C. sordelli</i>	Gas gangrene Enterocolitis	NA
		<i>C. haemolyticum</i>	Sepsis	NA
Corynebacteriaceae	<i>Corynebacterium</i>	<i>C. haemolyticum</i>	Sepsis	NA
Enterobacteriaceae	<i>Bacterioides</i>	<i>B. fragilis</i>	Sepsis Enterocolitis Peritonitis	NA
		<i>Escherichia</i>	Peritonitis	NA
		<i>Salmonella</i>	Gastroenteritis	NA
Enterococcaceae	<i>Enterococcus</i>		Peritonitis	NA
Helicobacteraceae	<i>Helicobacter</i>	<i>H. pylori</i>	Ulcus ventriculi Ulcus duodeni	NA
Pasteurellaceae	<i>Haemophilus</i>	<i>H. influenzae</i>	Periodontitis Sepsis, Otitis media	NA
		<i>Pasteurella</i>		NA
Pseudomonadaceae	<i>Pseudomonas</i>	<i>P. aeruginosa</i>	Respiratory ailments at cystic fibrosis	NA
Staphylococcaceae	<i>Staphylococcus</i>	<i>S. aureus</i>	Pyoderma Enteritis	NA
			Toxic shock syndrom Endocarditis	
Streptococcaceae	<i>Streptococcus</i>	<i>S. pneumoniae</i>	Pneumonia, Otitis media	NA
		<i>S. sanguis</i>	Periodontitis Endocarditis	NA
			Sepsis	
Vibrinoaceae	<i>Vibrio</i>	Group B, strain 122	Meningitis	
		<i>V. cholerae</i>	Cholera	NA
without classification	<i>Erysipelothrix</i>	<i>E. rhusiopathiae</i>	Erysipeloid	NA

environments and substrates. The proteins differ in form and size in between the species. The size ranges from 40 kDa up to 120 kDa. Usually the enzyme is a monomer, but there are descriptions of oligomeric structures. Neuraminidase is often secreted as a soluble protein. Partly it is bound to the bacterial surface. Sometimes it is not secreted at all. The sequential accordance in bacterial neuraminidases is around 30% and hence very low. Nevertheless there are two conserved motives: the RIP/RPL-motive (Arg-Ile/Leu-Pro) and the Asp-Box-motive (Ser/Thr-X-Asp-[X]-Gly-X-Thr-Trp/Phe [X representing any amino acid]). The Asp-Box may appear several times in the chain. The catalytic centre is also conserved. The arginine in the RIP/RPL motive interacts with the substrate's carboxyl group. The Asp-Box always has the same position in the beta-propeller and might be involved in the secretion process (Taylor 1996). The bacterial neuraminidase's pH optimum is between 5 and 7. Therefore it differs from the pH optimum of neuraminidases in mammalian lysosomes (Corfield 1992).

Bacterial sialidases in *V. cholerae*, *C. perfringens* and *Arthrobacter ureafaciens* are among the most scrutinely investigated. They have a broad substrate specificity and are widely used. The sialidase's substrate specificity is determined particularly by the aglykone, along with the type of its glycosidic linkage (alpha(2,3), alpha(2,6), or alpha(2,8) among others) and the substitution of the hydroxyl group in C7-9. An increased O-acetylation at these hydroxyl groups leads to decreased sialidase activity (Table 4). This O-acetylation was detected especially in the sialic acids of the sputum and the colon. These are sources where most sialidase-producing bacteria are found. Blockage of the carboxyl group stops the sialidase's activity. The carboxyl group is essential for the enzyme's function (Corfield 1992).

2.2.1. Neuraminidases as virulence factor

Sialidases are among the hydrolytic enzymes involved in the bacterial invasion into the host and the spread within the host. The enzyme activity belongs to the initial step in the degradation of bonds containing sialic acids. It leads to general and specific functional damage (Table 5). In addition, a direct toxic

effect on the host tissue and immunologic interactions could be observed. The sialidase activity is inducible by sialic acids, N-acetylmannosamines and other glycoconjugates and polysaccharides (Corfield 1992).

2.2.2. Neuraminidases in *Pseudomonas aeruginosa*

Currently, very little is known about the neuraminidase in *Pseudomonas aeruginosa*. It is clear, however, that it mainly splits alpha(2,3) bonds. Unlike some other micro-organisms the bacterium cannot use sialic acids as a source of carbohydrates. An

Table 4: Chemical properties of neuraminic acid substrates influencing the neuraminidase activity of *V. cholerae*, *C. perfringens* and *Arthrobacter ureafaciens* (according to Corfield 1992)

Chemical Group/Moiety	Influence on sialidase action
Aglycone	Activity related to: nature of the glycosidic linkage nature of the linkage monosaccharide nature of the glycoconjugate arrangement of the oligosaccharides
Carboxyl	Activity depends on an intact, unblocked carboxyl group
C4-Hydroxyl	Activity inhibition by substitution with acetyl group
C7-C9-Hydroxyl	Decreased activity by substitution with acetyl group (dependent on number and position of substitutions)
N-Acyl	N-Acetyl-group usually effects a higher activity than N-glykosyl-group
C7-9 Side Chain	Shortening of the side chain reduces likely activity (without physiological influence)

Table 5: Pathologic effects of bacterial neuraminidases (Corfield 1992)

Target Structure	Effect
blood cells	Erythrocyte pan-agglutination, haemolytic anaemia, reduction of circulating and half-life time of erythrocyte and leukocyte, thrombocytopenia
soluble blood glycoconjugates	Increased blood viscosity, reduction of circulating half-life time of glycoconjugates, loss of circulating cytokines, e.g. erythropoietin, increased formation of immune complexes, increased titre of auto-antibodies, e.g. anti-T-antigen connected with autoimmune diseases
vascular cells	Loss of negative charge on endothelial cells, loss of cell surface receptor specificity for hormones, enzymes and cellular signalling structures
mucosal surfaces	Loss of viscoelasticity and defensive properties of mucus, unmasking of bacterial binding sites on epithelial cells connected with increased invasion
common phenomena	Unmasking of crypt antigens

investigation of the cell wall did not indicate that the sialic acids are part of the LPS. Soong et al. (2006) rather suppose that neuraminidases account for biofilm building. Especially patients with cystic fibrosis suffer from pseudomonadic biofilms evading antibiotic treatment. *In vitro* studies showed that approved viral neuraminidase inhibitors could also inhibit the bacterial enzyme, thus preventing biofilm formation. In general, an increased neuraminidase expression improves bacterial adhesion to the epithelium (Pastoriza Gallego et al. 2006).

2.2.3. *Neuraminidases in Streptococci*

The maximal production of sialidase in strains of streptococci was determined during the late exponential or the early stationary phase with a steep decrease when protease production began. Within group B the strains of serotype III have the highest enzyme activity. Unlike other groups, the neuraminidase within this group is highly substrate-specific. It works only on mucines. The enzyme in *S. pneumoniae* has a broader spectrum and is also effective on serum-glycoproteins. It leads to a reduction in the mucous viscoelasticity. As a result it gets more difficult to transport the mucous over the tracheobronchial epithelium, which is part of the pathological mechanism of infection. The specific role of neuraminidases as virulence factor remains unclear. To date, no clear correlation between virulence and neuraminidase-activity could be established. Nevertheless an easier bacterial spread based on the enzyme's activity seems probable. The role of sialidase in the onset of group B and serotype III streptococcal meningitis remains unclear. Supposedly, the desialination of mucines results in an exposition of receptors on the meningeal surface. The bared receptors enable bacterial colonization. The neuraminidase activity of group A streptococci correlates with the desialination of IgG. This results in a higher IgG affinity to IgM and the building of autologous immune complexes (Corfield 1992).

There are at least two forms of bacterial neuraminidase, NanA and NanB, with molecular weights of around 108 kDa and 75 kDa, respectively. NanA is expressed on the bacterial surface

and has an around 100fold higher activity than NanB. Moreover, NanA c-terminally has a sequence with LPXTGX-motive which lacks NanB. The pH-optima differ in both forms. NanA reaches its optimum at pH 5, NanB at pH 7. These variations suggest a specialization in different environments and thus different stages of host invasion and infection. The three-dimensional structure of NanA and NanB are unknown. Nevertheless, both enzymes are known to be monomers (Jedrzejas 2001).

2.2.4. *Neuraminidases for carbohydrate provision*

There are non-pathogenic prokaryotes with known sialidase expression. These are, among others, the environmental bacteria *Arthrobacter ureafaciens* and *Micromonospora viridifaciens* and the lactic acid producing bacterium *Bifidobacterium bifidus*. Some are human endosymbiontes in the oral cavity and the intestinal tract. In the latter also *Bacteroides spec.*, *E. coli*, *Enterococcus faecalis* and *Ruminococcus spec.* can be found. They meet their energy demands partly by mucine breakdown. They do not only have sialidases but also permeases for sialic acid transport. Sialic acids are also a carbohydrate source for the pathogenic *Salmonella enterica ssp. enterica* LT2. In this species sialidase seems to be of minor importance as virulence factor. It is expressed only facultatively. Neuraminidase plays a nutritional role both in non-pathogenic and pathogenic bacteria (Corfield 1992).

2.3. *Neuraminidases in Protozoae*

In Protozoae, neuraminidase is found in the genera *Acanthamoeba*, *Eimeria*, *Endotrypanum* and *Trypanosoma* (Table 6) (Pellegrin et al. 1991, 1993; Medina-Acosta et al. 1994; Schenkman et al. 1994).

2.3.1. *Neuraminidases in Trypanosoma*

The name "trans-sialidase" refers to the enzyme's ability to transfer sialic acids directly from one carbone framework to another, without the detour of CMP-sialic acids. The donor molecules used by the enzyme are disaccharides as sialyllactose or fetuine bound by alpha(2,3) linkage to beta-galactose (Cazzulo et al. 1992; Colli 1993). Other good donors are sialic acid containing gangliosides and glykopeptides with O- or N-linkage. On the other hand, alpha(2,6), alpha(2,8) or alpha(2,9) linkages, CMP-sialic acids containing beta bonds or free sialic acids can not be processed (Colli 1993). The acceptor is beta-galactose in glykoproteins or glykolipids. Macromolecules on the surface of the parasite's membrane are sialylated, because trypanosomes cannot synthesize sialic acids themselves. Where no acceptor molecules are available, the sialic acids are hydrolysed (neuraminidase activity) (Cazzulo et al. 1992). Trans-sialidase, however, works more efficiently than neuraminidase (Colli 1993).

The enzymatically active glycoprotein is encoded by 12 genes, which belong to the huge superfamily of trans-sialidases (Neres et al. 2008). It is located on the protozoon's surface (anchored by glycoposphatidylinositol [GPI]) and is secreted into the environment. The molecular mass ranges from 12 to 200 kDa per subunit. In the amino acid sequence of the N-terminus (around 640 to 1.000 amino acids) two copies of the Asp-Box of the bacterial neuraminidase (Ser-X-Asp-XGly- X-Thr-Trp) are found. This is the catalytic part of the protein. The markedly hydrophobic C-terminus made up almost entirely by 44 12-amino acid long tandem repeats (SAPA repeats) (D-S-S-A-H-G-T-P-S-T-P-V) helps with the oligomerization and the aggregation of the heterogeneous subunits (Schenkman et al. 1994; Cazzulo et al. 1992; Colli 1993; Briones et al. 1994; Frasch 2000).

Table 6: Overview of neuraminidases in protozoae

Family	Genus	Species	Pathogenic to Humans	Virulenz factor
Acanthamoebaceae	<i>Acanthamoeba</i>	<i>A. castellani</i>	yes	SA?
		<i>A. polyphaga</i>		
		<i>A. culbertsoni</i>		
		<i>A. astronyxis</i>		
		<i>A. hatchetti</i>		
		<i>A. palestinensis</i>		
Eimeriidae	<i>Eimeria</i>	<i>E. tenella</i>	no (domestic fowl)	SA
		<i>E. maxima</i>	no (domestic fowl)	SA
		<i>E. necatrix</i>	no (domestic fowl)	SA
Trypanosomatidae	<i>Endotrypanum</i> <i>Trypanosoma</i>	<i>E. spec.</i>	no (sloths)	TS
		<i>T. cruzi</i>	yes	TS
		<i>T. brucei</i>	yes	(TS)
		<i>T. rangeli</i>	no	SA

TS, Trans-Sialidase; SA, Sialidase

Located upstream of the C-terminus is a subterminal motive (-Val-Thr-Val-X-Asn-Val- Phe-Leu-Tyr-Asn-Arg-) not found in the bacterial neuraminidases (Colli 1993). All amino acids in the catalytic centre of the *Salmonella enterica* spp. *enterica* sialidase are conserved in the trans-sialidase of *T. cruzi* and the sialidase of *T. rangeli* (Schenkman et al. 1994). Contrary to the trypomastigote form, the epimastigote form's enzyme is a monomer and lacks the C-terminal repeat of the 12 amino acids (Briones et al. 1994). The sialidase in *T. rangeli*, too, lacks the SAPA-repeat. It ends instead with a hydrophobic sequence after the Fn3 domain, a degenerated Asp-Box (Schenkman et al. 1994). Enzymes containing this repeat have an eight to ten-fold higher half-life in the blood than enzymes lacking this repeat (Frasch 2000). Little is known about the enzyme of the metacyclic trypomastigote form (Briones et al. 1994). Just like the trypomastigote form's enzyme in the blood it has a weight of 120-200 kDa, is expressed with the help of around 70 genes and has a tyrosine in position 342 in the catalytic N-terminal part. Mutation in the histidine moiety leads to a loss of function in the trans-sialidase (Frasch 2000). The crystalline structure of a mutant without the C-terminal SAPA domain is a globular N-terminus. This globular N-terminus is formed by a beta-propeller with six sheets containing the active centre. A long alpha-helix connects this part to the C-terminal lectin-like domain. The active centre is similar to bacterial structures. It comprises three arginines (Arg35, Arg245 und Arg314) interacting with the sialic acid's carboxyl group, an aspartat (Asp59) which is essential for catalysis and a hydrophobic pouch binding the N-acetyl group built –among others– by tryptophan 120, threonin 121, glutamin 195 and valin 203. Tyrosine 119 and tryptophan 312 are indispensable for the trans-sialidase's activity and the selectivity for alpha(2,3) bonds (Neres et al. 2008; Buchini et al. 2008). The sialidase and trans-sialidase have their maximal effectivity in the trypomastigote form. There, it has ten to twenty-fold higher activity than in the epimastigote form (compared to no activity in the amastigote form, see Table 7). The enzyme's pH optimum is 7. Changes in pH modulate the sialylation in the phagolysosomes. At pH 5.5 the trans-sialidase's enzyme activity drops to 12-13%, while the neuraminidase's activity drops to 42%. In *T. brucei* and *Endotrypanum* trans-sialidase activity could be detected only in the procyclic trypomastigote form. No activity could be found in the bloodstream-bound trypomastigote form encountered in vertebrates. The sialidase activity in *T. rangeli* is limited to the stages in invertebrates (Colli 1993; Briones et al. 1994). Possibly, *T. brucei* was the first within the family of trypanosomatidae that evolve the trans-sialidase and

did this in the insect stage. However, it seems unlikely that the enzyme was acquired by horizontal gene transfer from the mammalian host (unlike in bacteria) (Schenkman et al. 1994; Briones et al. 1994).

On the surface of *T. cruzi* around 2×10^7 sialic acid molecules are found. They lead to a strong negative charge. The parasites form the trans-sialidases within the host cell. Then, they are secreted by the help of phospholipase C and accumulate in the cytoplasm. Cell rupture equalling parasitic contact to the extracellular medium leads to complete sialylation of the parasite surface glykoproteins (Schenkman et al. 1994; Briones et al. 1994). These sialic acids influence the cell invasion by hampering both macrophage phagocytosis and complement recognition (Schenkman et al. 1994; Cazzulo et al. 1992; Colli 1993; Briones et al. 1994). There is no complement activation, neither by the classical nor by the alternative way. This is due to the sialic acids binding C3b and C4b, thus preventing the assembly of the active C3-convertase (Frasch 2000). Trans-sialidases secreted into the bloodstream remove sialic acids from the thrombocyte surface. This way, they cause the thrombocytopenia during the acute phase of the Chagas disease. Moreover, the enzyme induces apoptosis in spleen, thymus and periphery ganglia (Neres et al. 2008). Cells deficient in sialic acids hardly get invaded by trypomastigotes. Sialic acids seem to facilitate parasite escape from phagosomes (Schenkman et al. 1994). In the insect stage an influence of sialic acids on differentiation during the parasite development and/or migration into the insect's intestine is conceivable (Briones et al. 1994).

The protozoan sialidases and trans-sialidases got into focus as a target of therapeutic intervention by enzyme inhibition. Especially the trans-sialidase is not found in mammalia. The classical bacterial sialidase inhibitor DANA showed only little potency (Schenkman et al. 1994; Frasch 2000). This is surprising because DANA fills the enzyme's active centre entirely. Zanamivir and *N*-(4-nitrophenyle)-joxamic acid, the non-competitive sialidase inhibitor in *Vibrio cholerae*, had little to no effect. GM3 gangliosides with modified side chains and the pentasaccharid alditol showed higher effect. Nevertheless there is no promising compound for the development of an inhibitor until now (Neres et al. 2008).

2.4. Neuraminidases in fungi

Trimble, Pavia, and Pereira M. describe a trans-sialidase in *Pneumocystis carinii*, an ascomycotum. This sac fungus causes a

Table 7: Expression of the trans-sialidase in *Typanosoma cruzi* depending on the life cycle

Host	Form	Replication	TS Form	TS Activity	TS Secretion
insects (bug)	Epimastigote	Yes	Monomer without GPI	(+) (low)	no
	Trypomastigote (metacyclic)	No	Oligomer with GPI	+ (high)	yes
humans	Amastigote (intracellular)	Yes	-	-	no
	Trypomastigote (bloodstream)	No	Oligomer with GPI	+ (high)	yes

TS, Trans-Sialidase; GPI, Glykophosphatidylinositol

pneumocystis pneumonia especially in toddlers and immunosuppressed individuals (Schenkman et al. 1994).

Fungal neuraminidases are mentioned in the introductions of several publications, but there is no mention of specific families, genera or species (Miyagi et al. 2008; Achyuthan et al. 2001). Schenkman et al. (1994) cite a group describing neuraminidases in *Pneumocystis carinii* (ascomycota). A focused research in PubMed remained without success. With *Pneumocystis jirovecii* and not *P. carinii* having been identified in the meantime as causative organism of the human pneumocystis pneumonia, we searched for this species, but did not get any results. Therefore the presence of neuraminidases in fungi remains unclear.

2.5. Neuraminidases in invertebrates

Sialidases were described in the invertebrates *Macrobodella decora*, *Penaesus japonicus* and *Triatoma infestans* (Table 8) (Amino et al. 1998; Chou et al. 1996; Chuang et al. 1990).

Amino et al. (1998) reported a sialidase in the salivary glands of the predatory bug *Triatoma infestans*. In South America this bug transmits the Chagas-disease. The enzyme predominantly hydrolyses alpha(2,3) linked sialic acids at pH 4 to 8. It shows a high specific activity comparable to other sialidases. There is only speculation on the function of *Triatoma infestans* sialidase, however. Amino et al. (1998) suggested that a desialylation during the insect’s blood meal inhibits host thrombocyte aggregation, inflammatory processes, and activates fibrinolysis.

The sialidase in *Macrobodella decora*, too, preferentially works on alpha(2,3) bonds. It is secreted. N-terminally it has a sequence resembling the sequence in *Clostridium septicum*. Above that there is little resemblance to the amino acid sequence in other sialidases. Nevertheless, it has a FRIP region and four repeating Asp boxes. The catalytic centre has conserved amino acids like *Salmonella enterica*. The sialidase probably works similar to the bacterial enzyme (Chou et al. 1996). The enzyme’s function is not clear. It is conceivable that it has a similar function as in *Triatoma infestans* since clotting inhibition is advantageous for the North American leech, too.

2.6. Neuraminidases in mammalia

To date there are four sialidases known in mammalia, labelled Neu1, Neu2, Neu3 and Neu4. They differ in subcellular location, enzymatic properties and chromosomal location. They are also expressed differentially in different tissues (Miyagi et al. 2008). Beyond this, there seem to be further isoenzymes. In these, it

remains to be determined whether they are coded by different genes or if they are subject to posttranslational modification (Achyuthan et al. 2001).

Neu1, Neu2 and Neu3 are found mainly in lysosomes, cytosol and plasma membrane. Neu4, by contrary, is held to be found mainly in lysosomes, mitochondria and intramembraneous components. All enzymes have in common several Asp-boxes and the Arg-Ileu-Pro-sequence found in microorganisms (see above and Table 9) (Miyagi et al. 2008; Achyuthan et al. 2001; Yamaguchi et al. 2005; Monti et al. 2004). The homology in amino acids in between Neu1 and other sialidases is only 19–24%. Neu2, Neu3 and Neu4 have an accordance of at least 34–40% with each other. Located C-terminally in Neu1 there is an YGTL-motive not found in the other enzymes. This might be associated with the location in the lysosomes. Neu3 has a hydrophobic region which is supposedly a transmembrane domain (Miyagi et al. 2008). There are two isoforms of Neu4 (Neu4L and Neu4S), differing in a 12 amino acid long sequence located N-terminally (Neu4L having and Neu4S lacking this sequence) (Yamaguchi et al. 2005). The total size ranges from 380 amino acids (Neu2) to 496 amino acids (Neu4L). There is a homology in the sialidase’s primary sequence in humans, mice and rats with the bovine genes of 83%, 79% and 78%, respectively (Miyagi et al. 2008).

The human sialidase Neu1 has a similar substrate specificity within the oligosaccharides and the glycopeptides. Neu2 and Neu4 hydrolyse glykoproteins and gangliosides both at almost neutral pH and at pH 4.6 (Miyagi et al. 2008). Neu3 converts almost exclusively gangliosides. Contrary to the bovine and mural enzymes which have a pH optimum near 4.6, the human Neu3 has optima both at pH 4.5–4.8 and pH 6.0–6.5. It is not always possible to detect Neu3 on the cellular surface. Therefore, an existence in other intracellular components is conceivable, such as in endosomal structures (Miyagi et al. 2008).

The sialidases in mammalia seem to have also regulatory functions beyond their enzymatic functions as hydrolases. This is especially true for Neu3 which is supposed to be involved in transmembrane signalling via epidermal growth factor receptor and insulin receptor. Thus it has an influence on apoptosis (Miyagi et al. 2008). Another interesting aspect in human sialidases is that they are occasionally found as multiprotein-complexes. Mutations in accompanying proteins may lead to a loss of function in the sialidase without direct change in the sialidase (Achyuthan et al. 2001).

To date, only Neu2 could be crystallized and was structurally investigated. Based on this, models were established for Neu1, Neu3 and Neu4. Neu2’s crystalline structure has a canonical six-sheet beta-propeller with a conserved catalytic centre which includes tyr334, glu111 and asp46. This corresponds to the viral and bacterial sialidases (Chavas et al. 2005).

The importance of sialidases in humans becomes clear when mutations lead to a loss in the enzyme’s function. There could be shown a connection between inactive sialidase and sialidosis and galactosidosis. There is a supposed connection to melituria, Tay-Sachs-disease and bacterial infections (Table 10). Moreover

Table 8: Sialidases in invertebrates

Family	Genus	Species	Protein size
Hirudinidae	<i>Macrobodella</i>	<i>M. decora</i>	42 kDa
Penaecidae	<i>Penaesus</i>	<i>P. japonicus</i>	32 kDa
Reduviidae	<i>Triatoma</i>	<i>T. infestans</i>	<20 kDa

Table 9: Characteristics of the four human sialidase groups

Enzyme	Gen locus	Gen size	Amino-acids	Molecular-mass	Asp-boxes	F/YRIP-motifs	N-Glyco-sylation sites
Neu1	6p21	1,9 kb	415	45,5 kDa	3-5	1	3
Neu2	2q37		380	42,0 kDa	2	1	1
Neu3	11q13	3,0 kb	428	48,3 kDa	3	1	1
Neu4L	2q37	2,65 kb	496	52,9 kDa	3	1	0
Neu4S	2q37	2,65 kb	484	51,6 kDa	3	1	0

Table 10: Sialidase-associated diseases

Disease	Type	Molecular Basis
Sialidosis	Type I Type II	Transversion, point mutation, missens-mutation, Frame-shift-mutation
Galactosialidosis		Unknown
Melituria?		Unknown
Tay-Sachs-Disease?		Unknown
Bacterial Infection?		Unknown

there is an attempt to use sialidase activity and concentration as a biomarker for cancer (Achyuthan et al. 2001).

3. Conclusion and perspectives

With the exception of Hummel (1965) nobody described neuraminidases in plants. The lack of this enzyme would correspond to the theory of sialidase origin and evolutionary development according to Roggentin et al. (1993).

It becomes clear that the diversity in sialic acids parallels the diversity in the cleavage enzymes, the sialidases. They differ in structure, substrate specificity, pH-optimum and kinetics.

Viral neuraminidases are found in Orthomyxoviridae and Paramyxoviridae. The neuraminidase is constituted of a tetrameric protein with a stick and a globular head. The influenza neuraminidase is folded like a beta-propeller and has a striking similarity to the bacterial enzymes. The catalytic centre, too, is highly conserved in the different subtypes. The sequence homologies in between the influenza and the bacterial enzyme is around 15%, in between the bacterial enzymes around 30%. Viral neuraminidases, unlike the bacterial ones, have no Asp-box. The REP-motive corresponds to the bacterial RIP-motive. Therefore, they have a common structural centre, while there are tiny differences in the active site resulting in differing kinetics, binding affinity and substrate preference. Especially the kinetics show pronounced differences; the bacterial enzyme works 1000x faster than the viral one (Taylor 1996). The binding affinity differs, too. Bacterial sialidases bind no *N*-acetylneuraminic acid. Moreover, the avian influenza neuraminidase prefers sialic acids with alpha(2,3) linkage to alpha(2,6) linkage. Bacterial neuraminidases are less specific in their choice of sialyl conjugates. These differences make pathogen-specific neuraminidase inhibitors possible.

The trans-sialidase in *Trypanosoma cruzi* has two copies of the Asp-box, as well. It works preferentially on substrates with alpha (2,3) linkage. The catalytic centre in the trypanosomal trans-sialidase is a hydrophobic pouch just like in the viral and bacterial neuraminidases. Unlike the eukaryotic neuraminidase located in lysosomes, the enzyme in *T. cruzi* is located on the membrane surface (Cazzulo et al. 1992).

Taylor (1996) mentions a trans-sialidase in *Cryptosporidium parvum* (Cryptosporidiidae). This, however, could not be confirmed by other scientists.

As suggested by the presence of sialic acids, sialidases can be found in mammalia, too. The four types of the enzyme found in humans to date have homologies with each other and with bacterial sialidases. The Asp-Box-motive is found as well as the canonical six-sheet beat-propeller. Thus, these structural characteristics are the same in all discovered sialidases regardless of the organism they are found in.

The sporadic and non-systematic appearance of sialidases in invertebrates excludes a common evolution of the enzyme. There is rather an indication of an adaptation of the bloodsucker to the host. The big phylogenetic distance of *Macrobodella decora* and *Triatoma infestans* suggests two independent events. The exact origin of the ability to express sialidases (from the host, from bacteria or, as in the predatory bug, from trypanosomes) remains to be determined by structural and genetic comparisons. The exact function, remains to be investigated, too. The same problem arises concerning the occurrence of the sialidase in *Penaues japonicus*; from where did this crayfish species acquire the enzyme and what is its function?

The functionally and structurally differing forms of the sialidase are interesting targets for drugs. Influenza neuraminidase inhibitors already showed the specificity, effectivity and the paucity of side effects of such drugs. In the future the neuraminidases as virulence factors in pathogenic bacteria have to be further investigated since their exact influence on pathogenesis remains unclear. The neuraminidase does not seem to be the main virulence factor, but appears to have an effect on the course of disease.

It is crucial to keep in mind that neuraminidase inhibitors with poor effect on viruses might have a significant effect on bacteria or protozoae (as it is the case for DANA the other way round). Altogether sialidases represent a vast area of research in very different organisms regarding their occurrence, function and structure. This has to be pursued further. Moreover, they give information on the interaction and coevolution of host and commensale/parasite.

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