IDENTIFICATION AND CHARACTERIZATION OF LYSOSOMAL NEURAMINIDASE

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IDENTIFICATION AND CHARACTERIZATION OF LYSOSOMAL NEURAMINIDASE

IDENTIFICATIE EN KARAKTERISERING VAN HET LYSOSOMALE NEURAMINIDASE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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Aan Rita
Aan mijn ouders

CONTENTS:

Objectives

Neuraminidase catalyzes the removal of α-glycosidically linked sialic acids from a variety of sialylated substrates. The enzyme is produced by a number of bacteria and viruses and is commonly occurring in vertebrates. Bacterial and viral neuraminidases have been extensively studied as many of the neuraminidase containing microorganisms are pathogenic in man and the enzyme is thought to mediate infections. In contrast, comparatively little is known about the vertebrate neuraminidases. In mammals three different neuraminidases can be distinguished which, according to their subcellular localization, are defined as lysosomal, plasma membrane and cytosolic neuraminidase. Like the bacterial and viral enzymes, mammalian lysosomal neuraminidase is of medical interest as it is associated with disease. A genetically determined defect in lysosomal neuraminidase does result in the intracellular accumulation and urinary excretion of undegradable sialylated compounds and development of a lysosomal storage disorder. Lysosomal neuraminidase can be partially purified as a high molecular mass complex with lysosomal ß-galactosidase and its "protective protein". In contrast to the latter two proteins, knowledge about the neuraminidase component of the ß-galactosidase/neuraminidase/protective protein complex is limited. The protein has not been identified or purified thusfar, nor has the cDNA encoding the protein been cloned. The aim of the work presented in this thesis is to identify the lysosomal neuraminidase polypeptide in the ß-galactosidase/neuraminidase/protective protein complex, to study its interaction with other proteins for optimal functioning, and to further purify the protein for amino acid sequence analysis and subsequent cloning of the corresponding cDNA.

Chapter 1

INTRODUCTION TO NEURAMINIDASES

History, nomenclature and occurrence

Research on neuraminidases has been initiated half a century ago by the discovery of Hirst (1942) that hemagglutination of erythrocytes by influenza virus could be prevented by preincubation of erythrocytes with the same virus at 37 °C. Shortly afterwards, culture filtrates of the bacterial strains *Clostridium perfringens* and *Vibrio cholerae* were shown to contain a similar agent preventing erythrocytes from agglutination by influenza virus. Because of its enzyme-like properties and virus-receptor-destroying activity this agent was called receptor-destroying-enzyme or RDE (Burnet *et al.*, 1946; McCrea, 1947; Burnet and Stone, 1947). In 1949 Gottschalk and Lind demonstrated that mucoproteins are the natural substrates for RDE. In the following years RDE was shown to be an α-O-glycosidase (Gottschalk, 1956, 1957; Heimer and Meyer, 1956; Kuhn and Brossmer, 1958). The identification of N-acetylneuraminic acid, a member of a group of sugars known as sialic acids, as the chemical compound released from mucoproteins by RDE led Heimer and Meyer (1956) to introduce the name "sialidase", whereas Gottschalk (1957) independently suggested the name "neuraminidase" (E.C. 3.2.1.18). Although some scientists prefer the name sialidase arguing that neuraminic acid is not the product of the enzymatic reaction, and others use the name neuraminidase arguing that not all sialic acids are recognized by the hydrolase, both terms are generally accepted. The enzyme is defined as the α-glycosidase, specifically

cleaving the α-ketosidic bond linking the keto group of a terminal N-acylated neuraminic acid to an adjacent sugar residue (Gottschalk, 1958, Kuhn and Brossmer, 1958).

After the discovery of neuraminidase, the enzyme has been shown to occur in a great variety of other organisms (reviewed by Gottschalk and Drzeniek, 1972; Müller, 1974; Rosenberg and Schengrund, 1976). With the exception of microorganisms and viruses, the distribution of neuraminidases in nature largely parallels that of sialic acids. In vertebrates, in which sialic acids are generally observed, the enzyme performs an indispensable role in the catabolic reactions of the sialic acid metabolism. Occasionally, neuraminidases and sialic acids have been observed in invertebrates. Plants, fungi and algae contain neither sialic acids nor neuraminidases. In microorganisms, mostly lacking the capacity to synthesize sialic acids, a correlation between the occurrence of enzyme and sugar is absent. For example, amongst bacteria, *Escherichia coli* has been shown to contain sialic acids (colominic acid) without possessing a neuraminidase, whereas the opposite situation is observed in species like *Bacteroides*, *Pseudomonas* and *Streptococcus*. The flagellate protozo *Trypanosoma cruzi* has been shown to contain both neuraminidase and sialic acids. Viral neuraminidases have been observed in ortho- and paramyxoviruses. To illustrate the importance of neuraminidases, the composition, appearance and function of sialic acids will be described.

Sialic acids and sialoglycoconjugates

The term "sialic acids" comprises a group of N- and O-acyl derivatives of the 9-carbon acid amino sugar neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranos-1-onic acid; Blix *et al.*, 1957). Neuraminic acid in its unsubstituted form does not occur in nature as the free amino group at C-atom 5 will form an internal Schiff-base, the resulting product being rapidly degraded. Instead, as shown in figure 1, natural neuraminic acid derivatives are stabilized by acylation of the amino group with either an acetyl or glycolyl group, giving rise to N-acetylneuraminic acid (NeuAc) or N-glycolyl-neuraminic acid (NeuGc) respectively. In addition, the hydroxyl groups at C-atoms 4,7,8, and/or 9 can be methylated or esterified with acetyl groups (maximally three O-acetyl groups per sialic acid molecule), lactyl, sulphate or phosphate groups. The potential diversity of sialic acids is reflected by the occurrence of at least 23 different neuraminic acid derivatives in nature (Schauer, 1982).

Although sialic acid can be present in low concentrations as a free molecule in cells, tissues with an active sialic acid metabolism and in serum and urine, most sialic acids are α-glycosidically linked to other sugars at terminal position(s) of oligosaccharides, polysaccharides, and the oligosaccharide moiety of glycoproteins and gangliosides (for review see Corfield and Schauer, 1982). As shown in figure 1, linkage can be α(2-3), α(2-4), or

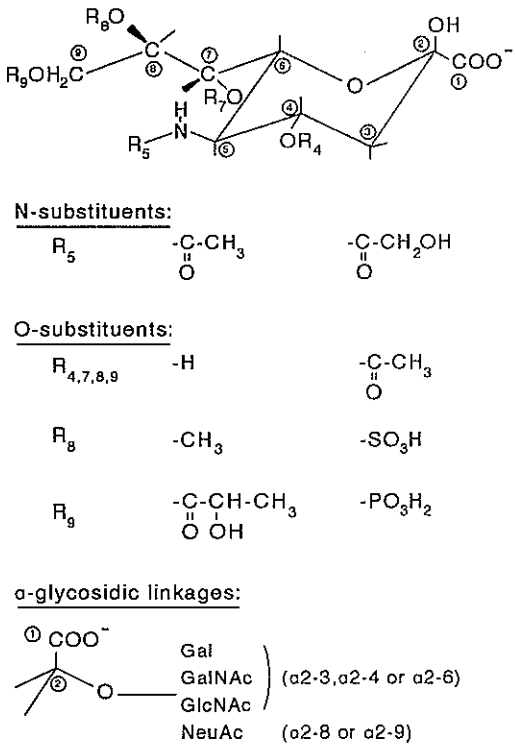

Figure 1. Structure of naturally occurring sialic acids

α(2-6) with galactose (Gal), N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) serving as accepting sugars, although α(2-3)Gal, α(2-6)Gal and α(2-6)GalNAc linkages are predominating. In addition, sialic acids can be mutually linked via α(2-8) or α(2-9) bonds. In contrast to α-glycosidically linked sialic acids, only one naturally occurring ß-glycosidic linkage is observed in the form of cytidine-mono-phosphate (CMP) sialic acids (Haverkamp *et al.*,1979).

The structural diversity of sialic acids as well as their occurrence in terminal, thus exposed positions in oligosaccharide chains of many glycoproteins and glycolipids suggests an important biological role for this group of sugars (for review see Reutter *et al.*, 1982). Sialic acids have been shown to determine the physiological properties of glycoproteins such as viscosity, protease resistance and stability. In case of enzymes, sialylation has also been shown to influence kinetic parameters and substrate specificity. At the cellular level, the negative charge of sialyl residues in membrane sialoglycoproteins and sialoglycolipids is known to affect the process of cellular adhesiveness. The importance of sialic acids is also illustrated by their involvement in many biological recognition processes. Many receptors for viruses, peptide hormones and toxins have been shown to contain sialic acids as essential components. Sialic acids are also observed as part of antigenic determinants and in the latter case even small differences in the composition of the sugar may be sufficient to trigger antibody synthesis. For example, horse NeuGc containing gangliosides, in contrast to their NeuAc counterpart, are antigenic in man and may cause serum-sickness disease as a result of the synthesis of Hanganutziu-Deicher antibodies after immunization with horse sera (Merrick et al., 1978). Oppositely, sialic acid can mask antigenic or recognition sites. A well known example of this "anti-recognition" effect is the clearance of many serum sialoglycoproteins from the bloodstream by the galactose specific asialoglycoprotein receptor on mammalian hepatocytes. Removal of terminal sialic acid residues uncovers the Gal

residues and renders the glycoprotein accessible to the receptor (Ashwell and Harford, 1982). Similarly, sialic acids can mask antigenic sites, thus acting as an "anti-antigen" on a variety of proteins and cells, hereby regulating their life-time (Schauer, 1988). Removal of sialic acids by neuraminidases greatly alters the behaviour of many cells and glycoconjugates.

Role of neuraminidases in sialic acid metabolism

Neuraminidases are of pathophysiological importance since many pathogenic microorganisms and viruses express neuraminidase activity and a genetically determined deficiency of a mammalian neuraminidase has been associated with metabolic storage disorders. Of all neuraminidases, microbial and viral neuraminidases are best understood.

Most neuraminidase producing bacteria are symbiontic or pathogenic inhabitants of the respiratory and intestinal tracts of mammals. Bacterial neuraminidases are excreted in large amounts as extracellular or membrane associated enzymes, function as monomers, and have a molecular mass between 50 and 100 kDa. Synthesis of neuraminidase can be induced by addition of sialylated compounds like sialyllactose, sialoglycolipids or sialoglycoproteins to the culture medium (reviewed by Rosenberg and Schengrund, 1976). According to Müller (1974) the degree of pathogenicity of neuraminidase producing bacteria is proportionally linked to the amount of enzyme activity generated, and upon excessive multiplication or invasion of tissues, harmless symbionts might even turn into dangerous pathogens. Although bacterial neuraminidases are capable of altering the characteristics of sialoglycoproteins on the cell surface, in the intracellular matrix and in the circulation, the direct role of the enzyme in bacterial infections remains to be solved. The substrate inducible gene expression suggests a nutritional role for neuraminidase. It has been shown that *Escherichia coli* and other neuraminidase lacking species are able to utilize free sialic acids as only carbon source via the inducible *nan* system, consisting of a sialic acid transporting protein *nanT* and the intracellular N-acylneuraminate pyruvate lyase (aldolase) *nanA* (Vimr and Troy, 1985). The synthesis of an extracellular neuraminidase might be an evolutionary adaptation to consume α-glycosidically linked sialic acids, readily available in the mucin and glycoprotein rich respiratory and intestinal tracts. This hypothesis is supported by the observation that expression of the *Vibrio cholerae* neuraminidase gene *nanH* in *Escherichia coli* is under control of the host *nan* system (Vimr *et al.*, 1988).

Viral neuraminidases are present as spike-like projections protruding from the viral envelope. Together with hemagglutinin (HA), neuraminidase (NA) belongs to the major surface antigens of myxoviruses like Influenza A virus (Laver and Valentine, 1969; Rosenberg and Schengrund, 1976). Both proteins are subject to antigenic variation and allow the virus to escape from immunity caused by infection of the host with an earlier strain

(Webster et al, 1983). In paramyxoviruses the hemagglutinin and neuraminidase activities are contained within one protein (HN) (Scheid and Choppin, 1974). Under control of the viral genome, viral neuraminidases are synthesized by the host cell as glycoproteins with a molecular mass around 60 kDa, which aggregate into complexes in the range of 100-200 kDa (Rosenberg and Schengrund, 1976). Whereas the function of the hemagglutinin in viral infection is to bind with sialic acid containing receptors on the host cell membrane, it is thought that neuraminidase facilitates the transport of the virion through the mucin layer to the site of infection, as well as the release of budding virus from the infected host cell. In the latter case, the removal of sialic acids from the cellular plasma membrane and viral surface glycoproteins is supposed to prevent self-association (Palese *et al.*, 1974; Griffin *et al.*, 1983).

In *Trypanosoma cruzi*, a flagellate protozo causing Chagas' disease, neuraminidase resembles the viral enzyme in that it is present as a cell surface antigen. At least two protein families demonstrating neuraminidase activity have been observed: the TCNA (*Trypanosoma cruzi* neuraminidase) and the SA85-1 (surface antigen) protein family. The TCNA proteins are highly polymorphic, ranging in molecular mass from 120 to 220 kDa (Cavallesco and Pereira, 1988; Pereira *et al.*, 1991) whereas proteins from the SA85-1 family have a molecular mass of 85 kDa (Kahn *et al.*, 1990, 1991). TCNA and SA85-1 proteins are differentially expressed during distinct developmental stages of the parasite (Kahn *et al.*, 1990; Rosenberg *et al.*, 1991). The expression of neuraminidase in the intracellular trypomastigote and loss of activity upon release in the extracellular space suggests that neuraminidase plays a role in parasite exiting from infected cells (Rosenberg *et al.*, 1991). A unique feature of *Trypanosoma cruzi* is the occurrence of a trans-sialidase, responsible for the generation of a sialic acid containing epitope on the parasite cell surface, involved in adhesion to target cells (Schenkman *et al.*, 1991). The trans-sialidase acts as a sialyltransferase by transfering sialic acid directly from sialylated host cell macromolecules (rather than CMP-NeuAc) to the acceptor molecule on the parasite. Recently it has been demonstrated that the Shed-Acute-Phase-Antigen (SAPA), a member of the TCNA family, contains "normal" as well as trans-sialidase activity (Parodi *et al.*, 1992).

In comparison to microbial and viral neuraminidases, knowledge about the mammalian neuraminidases is limited (for review see Conzelmann and Sandhoff, 1987). As a result of their lability, low abundancy, and in many cases tightly membrane-bound character purification till homogeneity has been difficult to achieve. Genes encoding these proteins have not been cloned yet. Mammalian neuraminidases form a heterogeneous group with respect to substrate specificity, solubility and subcellular distribution. According to the subcellular localization of the enzyme, three mammalian neuraminidases can be distinguished: a membrane bound lysosomal neuraminidase, thought to act mainly on water soluble

substrates such as sialooligosaccharides (Mahadevan *et al.*, 1967; Horvath and Touster, 1968; Tulsiani and Carubelli, 1970), a plasma membrane neuraminidase, degrading ganglioside substrates (Schengrund and Rosenberg, 1970) and a cytosolic neuraminidase (Schengrund and Rosenberg, 1970; Tulsiani and Carubelli 1970). Lysosomal neuraminidase is responsible for the desialylation of substrates in the intracellular degradative pathway. The enzyme has been shown to be deficient in patients with the lysosomal storage disorders sialidosis and galactosialidosis. The mammalian neuraminidases, and in particular the lysosomal neuraminidase, will be discussed in detail in the next chapter.

References

Ashwell, G. and Harford, J. (1982) Carbohydrate specific receptors of the liver. *Ann. Rev. Biochem.* **51**:531-554

Blix, G., Gottschalk, A. and Klenk, E. (1957) Proposed nomenclature in the field of neuraminic and sialic acids. *Nature* **179**:1088

Burnet, F.M., McCrea, J.F. and Stone, J.D. (1946) Modification of human red cells by virus action. I. The receptor gradient for virus action in human red cells. *Br. J. Exp. Path.* **27**:228-236

Burnet, F.M. and Stone, J.D. (1947) The receptor destroying enzyme of V. cholerae. *Austr. J. Exp. Biol. Med. Sci.* **25**:227-233

Cavallesco, R. and Pereira, M.E.A. (1988) Antibody to Trypanosoma cruzi neuraminidase enhances infection in vitro and identifies a subpopulation of trypomastigotes. *J. Immunol.* **140**:617-625

Conzelmann, E. and Sandhoff, K. (1987) Glycolipid and glycoprotein degradation. *Adv. Enzymol.* **60**:89-216

Corfield, A.P. and Schauer. R. (1982) Occurence of sialic acids. In: Sialic acids. Chemistry, metabolism and function (Schauer, R. ed) pp. 5-50, Springer-Verlag, New York

Gottschalk, A and Lind, P.E. (1949) Product of interaction between influenza virus enzyme and ovomucin. *Nature (Lond.)* **164**:232-233

Gottschalk, A. (1956) The linkage of sialic acid in mucoprotein. *Biochim. Biophys. Acta* **20**:560-561

Gottschalk, A. (1957) Neuraminidase, the specific enzyme of influenza virus and vibrio cholerae. *Biochim. Biophys. Acta* 23:645-646

Gottschalk, A. (1958) The influenza virus neuraminidase. *Nature* **181**:377-378

Gottschalk, A. and Drzeniek, R. (1972) Neuraminidase as a tool in structural analysis. In: Glycoproteins: their composition, structure and function (Gottschalk, A. ed.), BBA library, Vol.5 pp. 381-402, Elsevier, Amsterdam

Griffin, J.A., Basak, S. and Compans, R.W. (1983) Effects of hexose starvation and the role of sialic acid in influenza virus release. *Virology* **125**:324-334

Haverkamp, J., Spoormaker, T., Dorland, L., Vliegenthart, J.F.G. and Schauer R. (1979) Determination of the ß-anomeric configuration of cytidine 5'-monophospho-N-acetylneuraminic acid by ^{13}C NMR spectroscopy. *J. Am. Chem. Soc.* **101**:4851-4853

Heimer, R. and Meyer, K. (1956) Studies on sialic acids of submaxillary mucoid. *Proc. Natl. Acad. Sci. USA*

42:728-734

Hirst, G.K. (1942) Absorption of influenza haemagglutinins and virus by red blood cells. *J. Exp. Med.* **76**:195-209

Horvath, A. and Touster, O. (1968) On the lysosomal occurrence and the properties of the neuraminidase of rat liver and of Ehrlich ascites tumor cells. *J. Biol. Chem.* **243**:4380-4390

Kahn, S., Van Voorhis, W.C. and Eisen, H. (1990) The major 85-kD surface antigen of the mammalian form of Trypanosoma cruzi is encoded by a large heterogeneous family of simultaneously expressed genes. *J. Exp. Med.* **172**:589-597

Kahn, S., Colbert, T.G., Wallace, J.C., Hoagland, N.A. and Eisen, H. (1991) The major 85-kDa surface antigen of the mammalian-stage forms of Trypanosoma cruzi is a family of sialidases. *Proc. Natl. Acad. Sci. USA* **88**:4481-4485

Kuhn, R. and Brossmer, R. (1958) Die Konstitution der Lactaminsäurelactose, α-Ketosidase Wirkung von Viren der Influenza-Gruppe. *Angew. Chem.* **70**:25-26

Laver, W.G. and Valentine, B.C. (1969) Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology* **38**:105-119

Mahadevan, S., Nduaguba, J.C. and Tappel, A.L. (1967) Sialidase of rat liver and kidney. *J. Biol. Chem.* **242**:4409-4413

McCrea, J.F. (1947) Modification of red-cell agglutinability by Cl. welchii toxins. *Austr. J. Exp. Biol. Med. Sci.* **25**:127-136

Merrick, J.M., Zadarlik, K. and Milgrom, F. (1978) Characterization of the Hanganutziu-Deicher (serum-sickness) antigen as gangliosides containing N-glycolylneuraminic acid. *Int. Arch. Allergy Appl. Immunol.* **57**:477-480

Müller, H.E. (1974) Neuraminidases of bacteria and protozoa and their pathogenetic role. *Behring Institute Mitteilungen* 55:34-56

Palese, P., Tobita, K., Ueda, M. and Compans, R.W. (1974) Characterization of temperature sensitive influenza mutants defective in neuraminidase. *Virology* **61**:397-410

Parodi, A.J., Pollevick, G.D., Mautner, M., Buschiazzo, A., Sanchez, D.O. and Frasch, A.C.C. (1992) Identification of the gene(s) coding for the trans-sialidase of Trypanosoma cruzi. *EMBO J.* **11**:1705-1710

Pereira, M.E.A., Mejia, J.S., Ortega-Barria, E., Matzilevich, D. and Prioli, R.P. (1991) The Trypanosoma cruzi neuraminidase contains sequences similar to bacterial neuraminidases, YWTD repeats of the low density lipoprotein receptor, and type III modules of fibronectin. *J. Exp. Med.* **174**:179-191

Reutter, W., Köttgen, E., Bauer, C. and Gerok, W. (1982) Biological significance of sialic acids. In: Sialic acids. Chemistry, metabolism and function (Schauer, R. ed) pp. 263-306, Springer-Verlag, New York

Rosenberg, A. and Schengrund, C.L. (1976) Sialidases. In: Biological roles of sialic acid (Rosenberg, A. and Schengrund, C.L. eds.) pp. 295-359, Plenum, New York

Rosenberg, I.A., Prioli, R.P., Mejia, J.S. and Pereira, M.E.A. (1991) Differential expression of Trypanosoma cruzi neuraminidase in intra- and extracellular trypomastigotes. *Infect. Immun.* **59**:464-466

Schauer, R. (1982) Chemistry, metabolism, and biological functions of sialic acids. *Adv. Carbohydr. Chem.* **40**:131-234

Schauer, R. (1988) Sialic acids as antigenic determinants of complex carbohydrates. *Adv. Exp. Med. Biol.* **228**:47-72

Scheid, A. and Choppin, P.W. (1974) Identification of biological activities of paramyxovirus glycoproteins. Activation of fusion, hemolysis and infectivity by proteolytic cleavage of an inactive precursor protein of

Sendai virus. *Virology* **57**:475-490

Schengrund, C.L. and Rosenberg, A. (1970) Intracellular location and properties of bovine brain sialidase. *J. Biol. Chem.* **245**:6196-6200

Schenkman, S., Jiang, M.S., Hart, G.W. and Nussenzweig, V. (1991) A novel cell surface trans-sialidase of Trypanosoma cruzi generates a stage specific epitope required for invasion of mammalian cells. *Cell* **65**:1117-1125

Tulsiani, D.R.P. and Carubelli, R. (1970) Studies on the soluble and lysosomal neuraminidases of rat liver. *J. Biol. Chem.* **245**:1821-1827

Vimr, E.R. and Troy, F.A. (1985) Identification of an inducible catabolic system for sialic acids (nan) in Escherichia coli. *J. Bacteriol.* **164**:854-860

Vimr, E.R., Lawrisuk, L., Galen, J. and Kaper, J.B. (1988) Cloning and expression of the Vibrio cholerae neuraminidase gene nanH in Escherichia coli. *J. Bacteriol.* **170**:1495-1504

Webster, R.G. and Laver, W.G. (1975) Antigenic variation of influenza viruses. In: The influenza viruses and influenza (Kilbourne, E.D. ed.), pp. 269-314, Academic, New York

Chapter 2

MAMMALIAN NEURAMINIDASES

Lysosomal neuraminidase

Lysosomal neuraminidase distinguishes itself from the plasma membrane and cytosolic neuraminidase in that it is associated with inborn errors of metabolism. Impaired lysosomal degradation of sialylated compounds due to genetically determined aberrations in neuraminidase functioning will result in massive accumulation of its substrate and appearance of a lysosomal storage disorder.

Lysosomes and sialic acid catabolism

Intermediary metabolism is the net sum of a series of highly coordinated enzymatic reactions, providing the living cell a means to generate chemical energy, to synthesize and assemble the building blocks for macromolecular (extra)cellular components such as proteins, nucleic acids and lipids, and to degrade these macromolecules into their original building blocks. Within the compartimental architecture of the eukaryotic cell, the principal site for the intracellular digestion of toxic, damaged, or redundant intra- and extracellular macromolecules is the lysosome (vacuole in plant cells), an organelle first recognized by de Duve in 1955 (for reviews see de Duve, 1969, 1983; Bainton, 1981; Kornfeld and Mellman, 1989). A simple definition of a lysosome is that of an acidic, hydrolase-rich vacuole, capable

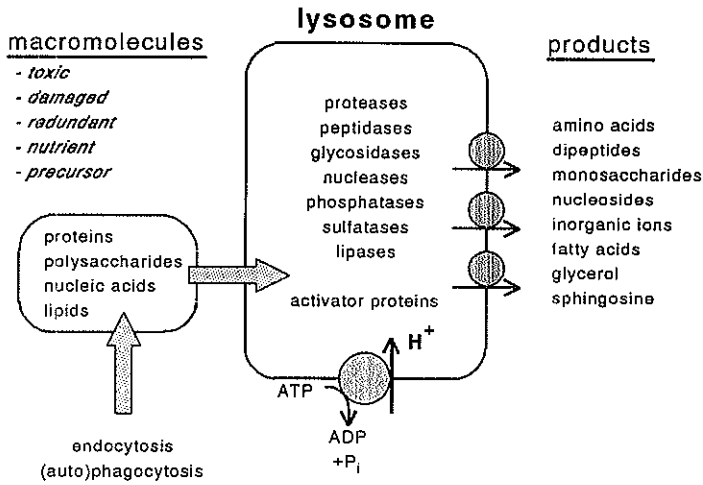

Figure 2. The lysosomal concept.

of degrading virtually all biological macromolecules. As shown in figure 2, to cover the full range of substrates presented for degradation, the lysosome is equipped with some 70 soluble and membrane-bound hydrolases (Holzman, 1989), demonstrating specificity towards the type of substrate (proteases, glycosidases, etc.) as well as type of linkage (α- and ß-glycosidases). Most lysosomal hydrolases are exolytic enzymes, often acting in a sequential manner. Some glycosidases require the assistance of so called activator proteins for the degradation of hydrophobic glycolipid substrates (Fürst and Sandhoff, 1992; chapter 3). To separate the hydrolytic enzymes from the cytosol and to conserve the acidic environment facilitating enzymatic hydrolytic reactions, the lysosome is lined by a membrane. The intralysosomal pH ($\approx$4.7) is maintained by a proton translocating ATPase (reviewed by Ohkuma, 1987). Following intralysosomal degradation, the reaction products are transferred to the cytosol for reutilization or further metabolization. Metabolite and inorganic ion efflux from the lysosome is mediated by a variety of solute specific (i.e. neutral sugars, acidic sugars, cationic amino acids, anionic amino acids, etc.) transport systems in some of which the proton pump also plays a role (for reviews see Mancini, 1991a; Pisoni and Thoene, 1991).

Intralysosomal degradation is also involved in the normal turnover of sialooligosaccharides, sialoglycoproteins and sialoglycolipids (figure 3). In line with the terminal position of the sialic acid residues, degradation of the oligosaccharide moiety of aforementioned compounds via sequential removal of sugars from the nonreducing end is initiated by lysosomal neuraminidase (Mahadevan *et al.*, 1967; Horvath and Touster, 1968). After cleavage, NeuAc is transported from the lysosome into the cytosol via a proton-driven

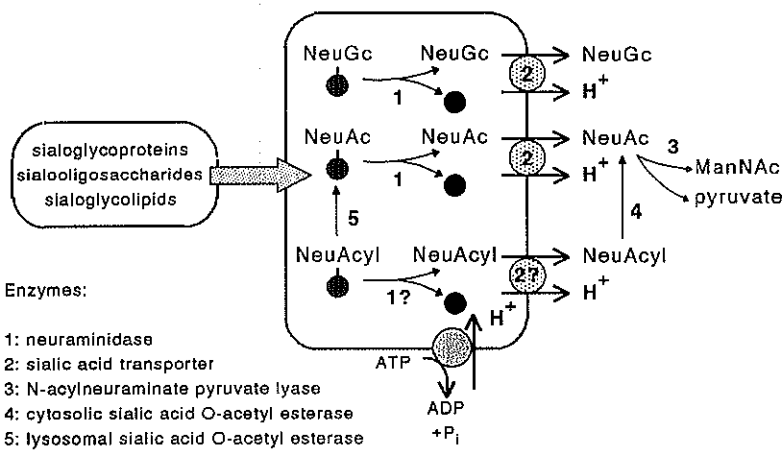

Figure 3. Sialic acid catabolism.

carrier for sialic acid, present in the lysosomal membrane (Mancini *et al.*, 1989). In the cytosol NeuAc is further degraded into pyruvate and ManNAc by the enzyme N-acyl-neuraminate pyruvate lyase (Brunetti *et al.*, 1962). Pyruvate is metabolized to acetyl CoA, oxaloacetate, lactate or alanine, and ManNAc might be reutilized to form NeuAc or other amino sugars (for a review on the synthesis of sialic acids, see Corfield and Schauer, 1982). Most reports on lysosomal neuraminidases deal with the hydrolysis of α-glycosidically linked NeuAc, the prevalent natural sialic acid. However, as described in chapter 1, also other sialic acids exist. Degradation of NeuGc containing sialoglycoconjugates appears no problem as lysosomal neuraminidase and the sialic acid transporter are able to remove and transport NeuGc (Corfield *et al.*, 1982; Mancini *et al.*, 1989). It is not clear whether the lysosomal neuraminidase and sialic acid transporter are capable of degrading and transporting O-acetylated sialic acids. If so, soluble cytosolic sialic acid O-acetyl esterases may be involved in the de-acetylation of O-acetylated sialic acids, allowing further degradation of the latter by N-acylneuraminate pyruvate-lyase (Higa *et al.*, 1987; Schauer, 1987; Schauer *et al.*, 1988, 1989). Alternatively, based on the observation that one of the membrane bound sialic acid O-acetyl esterases from rat liver demonstrated a pH optimum around 5 and a latency corresponding to that of the lysosomal enzyme ß-hexosaminidase, the existence of a lysosomal O-acetyl esterase was suggested (Higa *et al.*, 1987, 1989). In the latter model, the esterase will prepare the O-acetylated sialic acid residue for hydrolysis by the lysosomal neuraminidase. Recently, this esterase was biochemically characterized and its lysosomal origin was established (Butor et al., 1993a,b).

Lysosomal storage disorders and neuraminidase deficiency

Proper lysosomal function depends on the concerted action of the complete panel of hydrolases, activator proteins and lysosomal membrane transport proteins. The dysfunction of only one component is sufficient to cause intralysosomal accumulation of substrates or reaction products, which in time will cause cellular and organ malfunctioning. Today, over 35 human lysosomal storage diseases are known (for a complete overview see: *The Metabolic Basis of Inherited Disease*; Scriver *et al.*, 1989). Except for Fabry disease (α-galactosidase deficiency) and Hunter disease (iduronate sulfatase deficiency), which are X-linked, all lysosomal storage disorders are inherited as an autosomal recessive trait. Lysosomal storage disorders are rare ($<1:100,000$) but in isolated populations high carrier frequencies (1:20-30) may occur in certain diseases, as is the case for G_{M2}-gangliosidosis (hexosaminidase A deficiency) and Gaucher diseases (glucocerebrosidase deficiency) among the Ashkenazi-Jews and Salla disease (sialic acid transport defect) in the Northern Finnish population (Petersen *et al.*, 1983; Aula *et al.*, 1986; Matoth et al., 1987). With the exception of I-cell disease, where multiple soluble hydrolases are deficient due to defective lysosomal enzyme delivery, most lysosomal storage disorders result from mutations/deletions in a single lysosomal protein encoding gene. Classical biochemical studies on the synthesis and processing of lysosomal proteins as well as comparison of normal and patient genes or gene transcripts have revealed that these alterations may affect transcription, mRNA stability, mRNA splicing, protein targeting (i.e generation mannose-6-phosphate recognition marker, retention in the endoplasmic reticulum), protein stability, proteolytic processing, subunit association, or protein activity (for reviews see: Hoefsloot, 1991; Neufeld, 1991).

Clinically, lysosomal storage disorders are heterogeneous. Pathological effects largely depend on the residual activity and substrate specificity of the enzyme involved, as well as substrate supply and product reutilization rates in different organs. Mutations in different hydrolases may cause clinically indistinguishable disorders due to storage of the same substrate (keratan sulfate in galactosamine-6-sulfate sulfatase deficient Morquio A and ß-galactosidase deficient Morquio B syndrome), whereas different mutations in one enzyme may give rise to clinically different disorders as a result of different storage products (G_{M1}-gangliosides and keratan sulfate in ß-galactosidase deficient G_{M1}-gangliosidosis and Morquio B syndrome, respectively). Even within one disease the age of onset and clinical appearance (mild to severe) can vary, depending on the rate at which substrates accumulate, which in turn is determined by the residual enzyme activity and substrate influx (Conzelmann and Sandhoff, 1983). Since the activity of all lysosomal enzymes is expressed in cultured amniotic fluid cells and chorionic villi, prenatal diagnosis can be offered to parents at risk (Galjaard, 1980; Kleijer, 1990).

Lysosomal neuraminidase was first associated with a lysosomal storage disorder in 1977, when patients with mucolipidosis I (Spranger and Wiedemann, 1970) and cherry-red-spot-myoclonus syndrome, a mild form of mucolipidosis I, were shown to excrete large amounts of bound sialic acids in their urine as a result of a single deficiency of lysosomal neuraminidase (Cantz *et al.*, 1977). Shortly afterwards several ß-galactosidase deficient patients thought to suffer from a variant form of G_{M1}-gangliosidosis, were shown to be deficient in neuraminidase as well (Wenger *et al.*, 1978). Accordingly, disorders with an isolated neuraminidase deficiency were designated *sialidosis* whereas *galactosialidosis* is reserved for patients with the combined neuraminidase/ß-galactosidase deficiency (Durand *et al.*, 1977; Andria *et al.*, 1981).

Sialidosis. According to the clinical features and age of onset different forms of sialidosis are distinguished (reviewed by Beaudet and Thomas, 1989). *Type I (normomorphic) sialidosis* is a rather mild form of sialidosis, corresponding to the cherry-red-spot-myoclonus syndrome, where symptoms usually appear in the second decade and patients present with myoclonus and progressive impaired vision (Durand *et al.*, 1977; O'Brien, 1977; Thomas *et al.*, 1978). *Type II (dysmorphic) sialidosis* is characterized by progressively developing mental retardation, myoclonus, cherry-red spots, hepatosplenomegaly, dysostosis multiplex and coarse facial features (Cantz *et al.*, 1977; Kelly and Graetz, 1977; Spranger *et al.*, 1977). Most severe is the *congenital form* of sialidosis type II. Patients are stillborn or die shortly after birth. In the *infantile* and *juvenile form* of sialidosis type II symptoms appear within one year after birth or before adulthood, repectively. Biochemically, sialidosis is diagnosed by the finding of a deficient lysosomal neuraminidase activity in cultured fibroblasts or leucocytes (preferentially lymphocytes), and in case of prenatal diagnosis, cultured amniotic or chorionic villi cells (Galjaard, 1980; Johnson *et al.*, 1980; Mueller and Wenger, 1981). Residual activities vary from 0 to 10 percent, the lowest activities being observed in type II sialidosis (Lowden and O'Brien, 1979; O'Brien and Warner, 1980). In line with the neuraminidase deficiency as a primary defect, half normal activities are observed in obligatory heterozygotes (Thomas *et al.*, 1978). Chemical and/or NMR-spectroscopical analysis of storage products in patient urine and cultured fibroblasts demonstrated increased levels of linear and branched sialyloligosaccharides (Michalski *et al.*, 1977; Strecker *et al.*, 1977; Dorland *et al.*, 1978; van Pelt *et al.*, 1988b).

Galactosialidosis. Clinically, different forms of galactosialidosis have been observed, resembling those of sialidosis type II (reviewed by Andria *et al.*, 1981, O'Brien, 1989). Patients with the *early infantile form* suffer from severe edema, ascites, skeletal dysplasia, cherry-red macular spots and often present as fetal hydrops (Kleijer *et al.*, 1979). In the *late infantile form* dysostosis multiplex, visceromegaly, macular cherry-red spots and mental retardation become apparent 6 to 12 months after birth (Andria *et al.*, 1981). In the *late*

juvenile form, patients present between late infancy and adulthood with skeletal dysplasia, dysmorphism, myoclonus, corneal clouding, macular cherry-red spots, angiokeratoma and mental retardation (Loonen *et al.*, 1974; Okada *et al.*, 1979; Suzuki et al., 1883, 1984). Galactosialidosis is biochemically diagnosed by the finding of a combined deficiency of lysosomal ß-galactosidase and neuraminidase in cultured fibroblasts and leucocytes, whereas cultured amniotic or chorionic villi cells can be used for prenatal diagnosis (Kleijer *et al.*, 1979; Galjaard, 1980). Activities for neuraminidase are negligible whereas ß-galactosidase activity usually is 10 to 15 percent of control values. Galactosialidosis has been shown to result from a deficiency of a third protein, the so called "protective protein", which is required for neuraminidase activity and for protection of ß-galactosidase against enhanced intralysosomal degradation (d'Azzo *et al.*, 1982). Analysis of the storage products in cultured fibroblasts and urine from patients with galactosialidosis and comparison with those observed in sialidosis revealed the accumulation of essentially the same sialyloligosaccharides (van Pelt *et al.*, 1988a,b,c,1989). The sialidosis-like clinical appearance and the similarity of storage products indicates that the neuraminidase deficiency is mainly responsible for the pathogenic effects in galactosialidosis.

In addition to the lysosomal storage disorders caused by impaired degradation of sialylated compounds, two other diseases have been connected to a defect in the catabolic pathway of sialic acids. Salla disease and infantile sialic acid storage disease (ISSD), characterized by the lysosomal storage of free sialic acid, have been shown to result from impaired function of a carrier protein, responsible for the transport of sialic acids and other acidic sugars to the cytosol (Mancini *et al.*, 1991b; for review see Mancini, 1991a).

Purification of lysosomal neuraminidase

Lysosomal neuraminidase has first been described some 25 years ago by Mahadevan and coworkers (1967) and Horvath and Touster (1968). Partially purified lysosomes from rat liver were shown to contain a neuraminidase which - in isotonic sucrose buffer - exhibited the latency characteristic for lysosomal hydrolases. The enzyme showed an acidic pH optimum and was active towards sialyllactose, sialoglycoproteins and gangliosides. In the following years, as summarized in table 1, the enzyme has been demonstrated in different tissues in a variety of other mammals. In a comparative study using different organs of the mouse, highest neuraminidase activities were observed in liver and kidney (Potier *et al.*, 1979). As discussed, lysosomal neuraminidase has also been detected in cultured human fibroblasts and the observation that this enzyme is deficient in fibroblasts from patients suffering from the lysosomal storage disorders sialidosis (Cantz *et al.*, 1977; Kelly and Graetz, 1977; Thomas *et al.*, 1979; Suzuki *et al.*, 1981) and galactosialidosis (Wenger *et al.*,

Table 1 Lysosomal neuraminidase

species	source	method[a]	pur. (x)	sp.act[b] (mU/mg)	substr.[c]	pH opt[d]	reference	
rat	liver	dif. centrifugation	63	a.u.	SGpGa	4.0-4.4	Mahadevan *et al.*	1967
		dif. centrifugation	7	1.1 ^{S}	SGpGa	4.2	Horvath & Touster	1968
		dif. centrifugation	-	2.8 ^{S}	SGpGa	4.4	Tulsiani & Carubelli	1970
		dif.centr.+ion ex.	226	12.5 ^{S}	MSGp(Ga)	4.7	Miyagi & Tsuiki	1984
	mam. gland	density centrifugation	-	0.33 ^{S}	SGpGa	4.4	Tulsiani & Carubelli	1971
chick	liver	dif. centrifugation	2	0.075 ^{S}	SGpGa	3.8	Tulsiani & Carubelli	1972
mouse	liver	total homogenate	-	0.056 ^{M}	M	*4.4*	Potier *et al.*	1979
	kidney	total homogenate	-	0.131 ^{M}	M	*4.4*	Potier *et al.*	1979
	heart	total homogenate	-	0.006 ^{M}	M	*4.4*	Potier *et al.*	1979
	lung	total homogenate	-	0.011 ^{M}	M	*4.4*	Potier *et al.*	1979
	brain	total homogenate	-	0.010 ^{M}	M	*4.4*	Potier *et al.*	1979
		density centrifugation	59	17.55 ^{Ga}	Ga	4.0-4.2	Fiorilli *et al.*	1991
rabbit	spermatozoa	acros.extr.+chrom.	46	370 ^{Gp}	SGp	4.3	Srivastava *et al.*	1977
bovine	thyroid	dif.centr.+aff.chrom.	3450	a.u.	MSGa	3.5-4.5	van Dessel *et al.*	1984
	testis	copurification ß-gal.	316	114 ^{M}	M	*4.3*	Verheijen *et al.*	1982
porcine	testis	copurification ß-gal.	180	316 ^{M}	MSGp(Ga)	4.2	Yamamoto *et al.*	1987
human	fibroblasts	total homogenate	-	0.305 ^{S}	S	*4.4*	Cantz *et al.*	1977
		total homogenate	-	0.36 ^{Mpn}	Mpn	*4.2*	Kelly & Graetz	1977
		total homogenate	-	0.33 ^{S}	S	4.2-4.6	Thomas *et al.*	1979
		total homogenate	-	1.235 ^{M}	MSGa	*4.0*	Caimi *et al.* (review)	1981
		total homogenate	-	0.64 ^{M}	M	*4.3*	Suzuki *et al.*	1981
		total homogenate	-	0.101 ^{Ga}	Ga	*4.4*	Lieser *et al.*	1989
		density centrifugation	-	1.8 ^{Ga}	Ga	*4.3*	Zeigler *et al.*	1989
		total homogenate	-	3.3 ^{Ga}	Ga	*4.5*	Schneider-Jakob et al	1991
	leukocytes	total homogenate	-	0.024 ^{M}	M	4.0-4.2	Nguyen Hong *et al.*	1980
		total homogenate	-	0.021 ^{M}	M	*4.3*	Suzuki *et al.*	1981
		total homogenate	-	0.030 ^{M}	M	4.1	Verheijen *et al.*	1983
		$4.8x10^{4}g$ pel.+chrom.	40	40 ^{S}	SGp(Ga)	4.6	Schauer *et al.*	1984
	liver	density centrifugation	11	0.58 ^{S}	S	4.0	Meyer *et al.*	1981
		dif.centr.+aff.chrom.	1200	6.2 ^{S}	SGpGa	4.0-4.5	Michalski *et al.*	1982
		dif. centrifugation	14	2.2 ^{M}	MSGpGa	*4.6*	Spaltro & Alhadeff	1987
	placenta	total homogenate	-	.00017 ^{M}	MSGa	4.4	McNamara *et al.*	1982
		copurification ß-gal.	>4500	555 ^{M}	M	*4.3*	Verheijen *et al.*	1985
		copurification ß-gal.	3580	41 ^{M}	MSGpGa	4-5	Hiraiwa *et al.*	1987

[a] Abbreviations used: acros.extr.: acrosomal extract; aff.chrom.: affinity chromatography; dif.centr.: differential centrifugation; ion ex.: ion exchange chromatography.

[b] Specific activity measured with sialyllactose (^{S}), MU-NeuAc (^{M}), gangliosides (^{Ga}), glycoproteins (^{Gp}) or 2-(3-methoxyphenyl)-NeuAc (^{Mpn}); a.u.: arbitrary units.

[c] Substrates: S: sialyllactose; M: MU-NeuAc; Ga: gangliosides; Gp: glycoproteins; Mpn: 2-(3-methoxyphenyl)-NeuAc. Substrates between brackets are not or only poorly hydrolyzed.

[d] pH optimum; values in italics indicate pH used in neuraminidase assay (not empirically determined)

1978; Andria *et al.*, 1981) further emphasized the lysosomal origin of this neuraminidase.

Initial attempts to isolate lysosomal neuraminidase from rat liver, rat mammary glands, chick liver and human liver by partial purification of lysosomes resulted in enzyme preparations with only slightly enhanced specific activities. As 75 to 100 % of the activity was membrane bound, further purification required solubilization of the enzyme. Neuraminidase activity could be solubilized to some extend by repeated freeze-thawing but the soluble enzyme was shown to be extremely labile (Horvath and Touster, 1968; Tulsiani and Carubelli 1970; Meyer *et al.*, 1981). After solubilization of purified lysosomes by detergents, neuraminidase has been significantly purified from rabbit spermatozoa, human liver, bovine thyroid and human leukocytes by conventional separation procedures (gel filtration, ion exchange chromatography, affinity chromatography on Sepharose-coupled NeuAc derivatives, etc.; see table 1). Native polyacrylamide gel electrophoresis of the purified acrosomal neuraminidase from rabbit spermatozoa demonstrated only one major protein band of unspecified molecular mass (Srivastava and Abou-Issa, 1977). The human leukocyte neuraminidase preparation showed one protein with a molecular mass of 48 kDa (Schauer et al., 1984). In case of the human liver neuraminidase an enzymatically active protein band with a molecular mass of approximately 70 kDa was observed after incubation of the gel slices with substrate (Michalski et al., 1982). These purified proteins have not been further characterized.

In 1982 Verheijen and coworkers discovered that lysosomal neuraminidase from bovine testis is present in a high molecular mass complex with lysosomal ß-galactosidase and its "protective protein". After isolation of the glycoprotein fraction by Concanavalin A chromatography the neuraminidase activity containing complex could be isolated via a substrate specific affinity column for ß-galactosidase. The same procedure has later also successfully been applied for the purification of lysosomal neuraminidase from human placenta and porcine testis. Surprisingly, detergents were not required for the isolation of the complex. Either the isolated neuraminidase represents a soluble form of a membrane bound lysosomal enzyme, as is the case for acid phosphatase (Waheed *et al.*, 1988; Gottschalk *et al.*, 1989), or the use of frozen tissue is sufficient to solubilize the protein by freeze-thawing. Together with the purified acrosomal neuraminidase from rabbit spermatozoa, these complexes form the best purified active mammalian lysosomal neuraminidase preparations available thusfar. The mammalian lysosomal neuraminidase/ß-galactosidase/protective protein (carboxypeptidase) complex and the relation between the individual components from this complex will be discussed in chapter 4.

Several investigators have obtained evidence for the existence of multiple forms of lysosomal neuraminidase. Two neuraminidases with different stabilities but similar kinetic characteristics have been demonstrated in human fibroblasts (Potier *et al.*, 1979) and

leukocytes (Nguyen Hong *et al.*, 1980). Both the labile and stable enzyme are deficient in cells from patients with sialidosis and might represent different forms of the same enzyme. In contrast, Verheijen *et al.* (1983) observed two genetically different neuraminidases in human leukocytes. One form binds to Concanavalin A sepharose and is present in a complex with ß-galactosidase and the protective protein, whereas the other is not. Although both forms are of lysosomal origin, as demonstrated by density centrifugation, only the first is deficient in cells from a patient with sialidosis. In addition, Suzuki *et al.* (1981) reported the occurrence of two neuraminidases with different stabilities upon repeated freeze-thawing in human liver, fibroblasts and leukocytes. Only the freeze labile form was shown to be deficient in cells from a patient with galactosialidosis. A possible lysosomal origin of the other form was not further investigated.

Plasma membrane neuraminidase

Along with the discovery of lysosomal neuraminidases Leibovitz and Gatt (1968) demonstrated in an acetone extract from bovine brain a neuraminidase active towards ganglioside rather than glycoprotein substrates. Cell fractionation studies with bovine and rat brain homogenates showed that this neuraminidase was localized on the synaptosomal membrane (Schengrund and Rosenberg, 1970; Tettamanti *et al.*, 1972; Miyagi *et al.*, 1990a). With the aid of ganglioside substrates neuraminidase could also be observed in other tissues and species as well as in cultured fibroblasts, as summarized in table 2. Cellular fractionation of tissues and cultured cells by differential centrifugation, density centrifugation or free flow electrophoresis has shown that also in non-neuronal tissues this neuraminidase is localized on the outer cell surface.

The plasma membrane neuraminidase is strongly membrane bound. Detergent solubilization of acetone precipitates was required to obtain a 7-fold purification of the enzyme from brain homogenates. Only in few cases significant purification of the plasma membrane neuraminidase has been reported. A 3412-fold enriched preparation of a ganglioside hydrolyzing neuraminidase solubilized from rat heart by sonication, has been obtained via conventional separation procedures including gel filtration, ion exchange chromatography and isoelectric focussing (Talman and Brady, 1973). The enzyme preparation showed only one band after native polyacrylamide gel electrophoresis but was not further characterized. A preparation with similar specific activity has been purified from rat brain after solubilization of the enzyme with detergents (Miyagi *et al.*, 1990a). Chiarini *et al.* (1990) have shown that the plasma membrane neuraminidase from porcine brain could also be solubilized by the phosphatidylinositol specific phospholipase C, which indicates that the protein is linked to the membrane via a phosphatidylinositol membrane anchor.

Table 2 Plasma membrane neuraminidase

Species	Source	Method	Pur. (x)	Sp.act[b] (mU/mg)	Substr.[c]	pH opt.[d]	Reference	
bovine	brain	acetone powder extr.	7	1.01	Ga(SGp)	*4.4*	Leibovitz & Gatt	1968
		dif. centrifugation	-	a.u.	GaS	*3.9*	Schengrund *et al.*	1970
	thyroid	dif.+dens. centr.	-	a.u.	Ga(MS)	3.5-4.5	van Dessel *et al.*	1984
rat	heart	$3.4x10^4g$ pel.+chrom.	3412	13.53	GaGp(S)	5.0	Tallman & Brady	1973
	liver	dif. centrifugation	-	0.091	Ga	*4.2*	Schengrund *et al.*	1972
		dif. centrifugation	-	0.21	Ga	4.5	Miyagi & Tsuiki	1986
	brain	dif. centrifugation	10	0.62	Ga	*4.4*	Tettamanti *et al.*	1972
		$7.8x10^4g$ pel.+chrom.	450	27.75	Ga(SMGp)	*4.8*	Miyagi *et al.*	1990a
	erythrocytes	solubil.ghosts +chrom.	30	1.81	Ga(SMGp)	*4.8*	Sagawa *et al.*	1990
pig	brain	PI-PLC + Am.sulf.↓[a]	-	1.24	Ga	4.2	Chiarini *et al.*	1990
human	fibroblasts	total homogenate	-	0.050	GaS(Gp)	4.0	Caimi *et al.*	1979
		total homogenate	-	0.0129	Ga	*4.5*	Cantz & Messer	1979
		density centrifugation	-	a.u.	Ga		Zeigler & Bach	1981
		total homogenate	-	0.0256	Ga	4.7	Baumkötter & Cantz	1983
		free flow electr.	-	0.021	Ga	4.5	Lieser *et al.*	1989
		total homogenate	-	0.017	Ga	*4.5*	Schneider-Jakob *et al.*	1991
	brain	acetone powder extr.	7	2.3	GaSGp	*4.4*	Öhman *et al.*	1970

[a] phosphatidylinositol phospholipase C (PI-PLC) solubilization and ammonium sulfate fractionation
[b] Specific activity measured with gangliosides.
[c] Substrates cleaved by the enzyme: sialyllactose (S), MU-Neu5Ac (M), gangliosides (Ga), glycoproteins (Gp); substrates between brackets are not or poorly hydrolyzed.
[d] pH optimum; values in italics indicate pH used in neuraminidase assay (not empirically determined).

Despite its acidic pH optimum, resembling that of the lysosomal neuraminidase, several lines of evidence indicate that the plasma membrane neuraminidase is unrelated to its lysosomal counterpart. Apart from their different subcellular location, the lysosomal and plasma membrane neuraminidase were shown to respond differently upon stimulation of the ganglioside degrading activity by detergents (Lieser *et al.*, 1989; see also chapter 3). Moreover, normal plasma membrane neuraminidase activities were detected in fibroblasts from patients with sialidosis (Cantz and Messer, 1979; Lieser *et al.*, 1989; Schneider-Jakob and Cantz, 1991). In addition Miyagi *et al.* (1990a) have shown that the plasma membrane and lysosomal neuraminidase are immunologically unrelated as an antibody directed against the plasma membrane neuraminidase does not precipitate the lysosomal enzyme and vice versa.

At this moment the biological function of the plasma membrane neuraminidase is not clear. A possible role of the synaptosomal membrane neuraminidase in the stabilization of

neuronal circuits in brain tissue has been proposed by Veh and Sander (1981). According to their "calcium buffer hypothesis", gangliosides provide a reservoir of Ca^{2+}ions in the immediate vicinity of the presynaptosomal membrane by forming Ca^{2+}-ganglioside complexes. From the major gangliosides present in the neuronal membrane (G_{M1}, G_{D1a} and other polysialogangliosides), ganglioside G_{M1} is the most efficient Ca^{2+}buffer. After stimulation of the nerve, the presynaptosomal Ca^{2+}influx at the nerve ending causes the release of acetylcholine in the synaptic cleft. Due to the hydrolysis of acetylcholine by acetylcholine esterase the intrasynaptosomal pH will temporarily drop to a value around 4, the optimal pH for the synaptosomal neuraminidase. As repeated stimulation of the neuron will generate the ideal environment for the synaptosomal neuraminidase to convert polysialogangliosides to ganglioside G_{M1}, frequently used synapses will therefore contain the ganglioside with the best "calcium buffering" capacity. The function of the plasma membrane neuraminidase in non-neuronal tissues remains also to be resolved. It has been suggested that the enzyme might reach the endosomes where, after acidification, it would hydrolyze gangliosides (Conzelmann and Sandhoff, 1987).

Cytosolic neuraminidase

In contrast to the membrane bound lysosomal and plasma membrane neuraminidases, a soluble neuraminidase has been demonstrated in high speed supernatants of tissue homogenates (table 3). This cytosolic neuraminidase, first described by Tulsiani and Carubelli (1970), is fully active at a pH around 6 although some investigators report lower values. The enzyme readily hydrolyzes small natural and synthetic substrates like sialyllactose and 4-methylumbelliferyl-NeuAc. Glycopeptides and gangliosides also serve as substrates although the specific activity with the latter is approximately 5-fold lower.

Cytosolic neuraminidase has been purified from porcine brain, rat liver and rat skeletal muscle (see table 3). In case of the porcine brain enzyme two forms with a slightly different pH optimum and different kinetic parameters could be separated on a hydroxyapatite/cellulose gel column. As both forms have not been further investigated, it is not clear whether they represent different enzymes or variant forms of the same enzyme. The best characterized cytosolic neuraminidase is the rat skeletal muscle enzyme (Miyagi and Tsuiki, 1985). It has been purified till homogeneity and had a molecular mass of 43 kDa after SDS-polyacrylamide gel electrophoresis. Immunologically the skeletal muscle cytosolic neuraminidase appears to be different from the lysosomal and plasma membrane neuraminidases as a polyclonal antibody against the 43 kDa protein exclusively inhibits and precipitates the cytosolic neuraminidase activity. Although the cytosolic neuraminidase appears to be the best purified mammalian neuraminidase, it has not been further investigated yet and the biological function of the enzyme remains to be resolved.

Table 3 Cytosolic neuraminidase

Species	Source	Method	Pur. (x)	Sp.act[a] (mU/mg)	Substr.[b]	pH opt.[c]	Reference	
rat	liver	$1.5x10^4g$ supernatant	-	0.89 [S]	SGp(Ga)	5.8	Tulsiani & Carubelli	1970
		$1x10^5g$ supernatant	-	0.040 [S]	SGpGa	5.8	Carubelli & Tulsiani	1971
		$8x10^4g$ sup.+chrom.	83000	5132 [S]	SMGpGa	6.0-6.5	Miyagi and Tsuiki	1985
	mam. gland	$1x10^5g$ supernatant	-	0.054 [S]	SGpGa	5.8	Tulsiani & Carubelli	1971
	brain	$1x10^5g$ supernatant	-	0.112 [S]	SGpGa	5.8	Carubelli & Tulsiani	1971
	muscle	$8x10^4g$ sup.+chrom.	22700	10600 [M]	M	*6.0*	Miyagi *et al.*	1990b
porcine	brain	$1x10^5g$ sup.+chrom.	500	3220 [S]	S	4.7	Venerando *et al.*	1975
		$1x10^5g$ sup.+chrom.	400	2300 [S]	S	4.9		
chick	liver	$1x10^5g$ supernatant	-	0.055 [S]	SGpGa	4.4	Tulsiani & Carubelli	1972

[a] Specific activity measured with sialyllactose ([S]) or MU-Neu5Ac ([M]).

[b] Substrates cleaved by the enzyme: sialyllactose (S), MU-Neu5Ac (M), gangliosides (Ga), glycoproteins (Gp); substrates between brackets are not or poorly hydrolyzed.

[c] pH optimum; values in italics indicate pH used in neuraminidase assay (not empirically determined).

References

Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W.S. and Dodson, W.E. (1981) Infantile neuraminidase and ß-galactosidase deficiencies (galactosialidosis) with mild clinical courses. In Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 379-395, Edi. Ermes, Milan

Aula, P., Renlund, M., Raivio, K.O. and Koskela, S.L. (1986) Screening of inherited oligosaccharidurias among mentally retarded patients in northern Finland. *J. Ment. Def. Res.* **30**:365-368

Bainton, D.F. (1981) The discovery of lysosomes. *J. Cell Biol.* **91**:66S-77S

Beaudet, A.L. and Thomas, G.H. (1989) Disorders of glycoprotein degradation: Mannosidosis, fucosidosis, sialidosis and aspartylglycosaminuria. In: The metabolic basis of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. eds.), pp. 1603-1621, McGraw-Hill, New York

Baumkötter, J. and Cantz, M. (1983) Decreased ganglioside neuraminidase activity from fibroblasts from mucopolysaccharidosis patients. Inhibition of the activity in vitro by sulfated glycosaminoglycans and other compounds. *Biochim. Biophys. Acta* **761**:163-170

Brunetti, P., Jourdian, G.W. and Roseman, S. (1962) The sialic acids. III Distribution and properties of animal N-acetylneuraminic aldolase. *J. Biol. Chem.* **237**:2447-2453

Butor, C., Diaz, S. and Varki, A. (1993) High level of O-acetylation of sialic acids on N-linked oligosaccharides of rat liver membranes. *J. Biol. Chem.* **268**:10197-10206

Butor, C., Higa, H.H. and Varki, A. (1993) Structural, immunological, and biosynthetic studies of a sialic acid specific O-acetylesterase from rat liver. *J. Biol. Chem.* **268**:10207-10213

Caimi, L., Lombardo, A., Preti, A., Wiesmann, U. and Tettamanti, G. (1979) Optimal conditions for the assay of fibroblast neuraminidase with different natural substrates. *Biochim. Biophys. Acta* **571**:137-146

Caimi, L., Wiesmann, U.N., Lombardo, A., Preti, A., Marchesini, S. and Tettamanti, G. (1981) Sialidases in human cultured fibroblasts. In Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 169-190, Edi. Ermes, Milan

Cantz, M., Gehler, J. and Spranger, J. (1977) Mucolipidosis I: increased sialic acid content and deficiency of an α-N-acetylneuraminidase in cultured fibroblasts. *Biochem. Biophys. Res. Commun.* **74**:732-738

Cantz, M. and Messer, H. (1979) Oligosaccharide and ganglioside neuraminidase activities of mucolipidosis I (sialidosis) and mucolipidosis II (I-cell disease) fibroblasts. *Eur. J. Biochem.* **97**:113-118

Carubelli, R. and Tulsiani, D.R.P. (1971) Neuraminidase activity in brain and liver of rats during development. *Biochim. Biophys. Acta* **237**:78-87

Chiarini, A., Fiorilli, A., Siniscalco, C., Tettamanti, G. and Venerando, B. (1990) Solubilization of the membrane bound sialidase from pig brain by treatment with bacterial phosphatidylinositol phospholipase C. *J. Neurochem.* **55**:1576-1584

Conzelmann, E., and Sandhoff, K. (1983) Partial enzyme deficiencies: residual activies and the development of neurological disorders. *Dev. Neurosci.* **6**:58-71

Conzelmann, E. and Sandhoff, K. (1987) Glycolipid and glycoprotein degradation. *Adv. Enzymol.* **60**:89-216

Corfield, A.P. and Schauer, R. (1982) Metabolism of sialic acids. In: Sialic acids. Chemistry, metabolism and function (Schauer, R. ed) pp. 195-262, Springer-Verlag, New York

d'Azzo, A., Hoogeveen, A.T., Reuser, A.J., Robinson, D. and Galjaard, H. (1982) Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. USA* **79**:4535-4539

de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**:604-617

de Duve, C. (1969) The lysosome in retrospect. In: Lysosomes in biology and pathology, vol.1 (Dingle, J.T. and Fell, H.B. eds.), pp. 3-40, North Holland, Amsterdam.

de Duve, C. (1983) Lysosomes revisited. *Eur. J. Biochem.* **137**:391-397

Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Strecker, G., Michalski, J.C., Fournet, B., Spik, G. and Montreuil, J. (1978) 360 MHz^{1}H nuclear-magnetic resonance spectroscopy of sialyloligosaccharides from patients with sialidosis (mucolipidosis I and II). *Eur. J. Biochem.* **87**:323-329

Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C. and Strecker, G. (1977) Sialidosis (mucolipidosis I). *Helv. Paediat. Acta* **32**:391-400

Fiorilli, A., Siniscalco, C., Chiarini, A., Di Francesco, L., Venerando, B. and Tettamanti, G. (1991) Occurrence of sialidase activity in two distinct and highly homogeneous populations of lysosomes prepared from the brain of developing mouse. *FEBS Lett.* **282**:235-238

Fürst, W. and Sandhoff, K. (1992) Activator proteins and topology of lysosomal sphingolipid catabolism. *Biochim. Biophys. Acta* **1126**:1-16

Galjaard, H. (1980) Genetic metabolic diseases. Early diagnosis and prenatal analysis. Elsevier/North-Holland, Amsterdam

Gottschalk, S., Waheed, A., Schmidt, B., Laidler, P. and von Figura, K. (1989) Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. *EMBO J.* **8**:3215-3219

Higa, H.H., Diaz, S. and Varki, A. (1987) Biochemical and genetical evidence for distinct membrane-bound and cytosolic sialic acid O-acetyl-esterases: serine active site enzymes. *Biochem. Biophys Res. Commun.*

144:1099-1108

Higa, H.H., Manzi, A. and Varki, A. (1989) O-acetylation and de-O-acetylation of sialic acids. Purification, characterization, and properties of a glycosylated rat liver esterase specific for 9-O-acetylated sialic acids. *J. Biol. Chem.* **264**:19435-19442

Hiraiwa, M., Uda, Y., Nishizawa, M. and Miyatake, T. (1987) Human placental sialidase: partial purification and characterization. *J. Biochem.* **101**:1273-1279

Hoefsloot, L.H. (1991) Glycogenosis type II. Cloning and characterization of the human lysosomal α-glucosidase gene. Thesis, Erasmus University Rotterdam

Holzman, E. (1989) Lysosomes. Plenum Press, New York

Hoogeveen, A.T., Verheijen, F.W., d'Azzo, A. and Galjaard, H. (1980) Genetic heterogeneity in human neuraminidase deficiency. *Nature* **285**:500-502

Horvath, A. and Touster, O. (1968) On the lysosomal occurrence and the properties of the neuraminidase of rat liver and of Ehrlich ascites tumor cells. *J. Biol. Chem.* **243**:4380-4390

Johnson, W.G., Thomas, G.H., Miranda, A.F., Driscoll, J.M., Wigger, J.H., Yeh, M.N., Schwartz, R.C., Cohen, C.S., Berdon, W.E. and Königsberger, M.R. (1980) Congenital sialidosis: biochemical studies; clinical spectrum in four sibs; two successful prenatal diagnoses. *Am. J. Hum. Genet.* **32**:43A

Kelly, T.E. and Graetz, G. (1977) Isolated acid neuraminidase deficiency: A distinct lysosomal storage disorder. *Am. J. Med. Genet.* **1**:31-46

Kleijer, W.J., Hoogeveen, A.T., Verheijen, F.W., Niermeijer, M.F., Galjaard, H., O'Brien, J.S. and Warner, T.G. (1979) Prenatal diagnosis of sialidosis with combined ß-galactosidase and neuraminidase deficiency. *Clin. Genet.* **16**:60-61

Kleijer, W.J. (1990) Prenatal diagnosis. In: Inborn metabolic diseases. Diagnosis and treatment (Fernandes, J., Saudubray, J.M. and Tada, K. eds), pp. 683-695, Springer-Verlag, Berlin, Heidelberg

Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**:483-525

Leibovitz, Z. and Gatt, S. (1968) Enzymatic hydrolysis of sphingolipids. VII Hydrolysis of gangliosides by a neuraminidase from calf brain. *Biochim. Biophys. Acta* **152**:136-143

Lieser, M., Harms, E., Kern, H., Bach, G. and Cantz, M. (1989) Ganglioside G_{M3} sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucolipidosis IV. Subcellular distribution and some properties. *Biochem. J.* **260**:69-74

Loonen, M.C.B., Reuser, A.J.J., Visser, P. and Arts, W.F.M. (1984) Combined sialidase (neuraminidase) and ß-galactosidase deficiency. Clinical, morphological and enzymological observations in a patient. *Clin. Genet.* **26**:139-149

Lowden, J.A. and O'Brien, J.S. (1979) Sialidosis: A review of human neuraminidase deficiency. *Am. J. Hum. Genet.* **31**:1-18

Mahadevan, S., Nduaguba, J.C. and Tappel, A.L. (1967) Sialidase of rat liver and kidney. *J. Biol. Chem.* **242**:4409-4413

Mancini, G.M.S., de Jonge, H.R., Galjaard, H. and Verheijen, F.W. (1989) Characterization of a proton-driven carrier for sialic acid in the lysosomal membrane. *J. Biol. Chem.* **264**:15247-15254

Mancini, G.M.S. (1991a) Lysosomal membrane transport: physiological and pathological events. Thesis, Erasmus University Rotterdam

Mancini, G.M.S., Beerens, C.E.M.T., Aula, P.P. and Verheijen, F.W. (1991b) Sialic acid storage diseases. A multiple lysosomal transport defect for acidic monosaccharides. *J. Clin. Invest.* **87**:1329-1335

Matoth, Y., Chazan, S., Cnaan, A., Gelernter, I. and Klibansky, C. (1987) Frequency of carriers of chronic

(type I) Gaucher disease in Ashkenazi Jews. *Am. J. Med. Genet.* **27**:561-565

McNamara, D., Beauregard, G., Nguyen, H.V., Yan, D.L.S., Bélisle, M. and Potier, M. (1982) Characterization of human placental neuraminidases. *Biochem. J.* **205**:345-351

Meyer, D.M., Lemonnier, M. and Bourrillon, R. (1981) Human liver neuraminidase. *Biochem. Biophys. Res. Commun.* **103**:1302-1309

Michalski, J.C., Strecker, G., Fournet, B., Cantz, M. and Spranger, J. (1977) Structures of sialyl-oligosaccharides excreted in the urine of a patient with mucolipidosis I. *FEBS Lett.* **79**:101104

Michalski, J.C., Corfield, A.P. and Schauer, R. (1982) Solubilization and affinity chromatography of a sialidase from human liver. *Hoppe-Seyler's Z. Physiol. Chem.* **363**:1097-1102

Miyagi, T. and Tsuiki, S. (1984) Rat liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase. *Eur. J. Biochem.* **141**:75-81

Miyagi, T. and Tsuiki, S. (1985) Purification and characterization of cytosolic sialidase from rat liver. *J. Biol. Chem.* **260**:6710-6716

Miyagi, T. and Tsuiki, S. (1986) Evidence for sialidase hydrolyzing gangliosides GM_2 and GM_1 in rat liver plasma membrane. *FEBS Lett.* **206**:223-228

Miyagi, T., Sagawa, J., Konno, K., Handa, S. and Tsuiki, S. (1990a) Biochemical and immunological studies on two distinct ganglioside hydrolyzing sialidases from the particulate fraction of rat brain. *J. Biochem.* **107**:787-793

Miyagi, T., Sagawa, J., Konno, K. and Tsuiki, S. (1990b) Immunological discrimination of intralysosomal, cytosolic, and two membrane sialidases present in rat tissues. *J. Biochem.* **107**:794-798

Mueller, O.T. and Wenger, D.A. (1981) Mucolipidosis I: studies of sialidase activity and a prenatal diagnosis. *Clin. Chim. Acta* **109**:313-324

Neufeld, E.F. (1991) Lysosomal storage diseases. *Annu. Rev. Biochem.* **60**:257-280

Nguyen Hong, V., Beauregard, G., Potier, M., Bélisle, M., Mameli, L., Gatti, R. and Durand, P. (1980) Studies on the sialidoses. Properties of human leucocyte neuraminidases. *Biochim. Biophys. Acta* **616**:259-270

O'Brien, J.S. (1977) Neuraminidase in the cherry-red-spot-myoclonus syndrome. *Biochem. Biophys. Res. Commun.* **79**:1136-1141

O'Brien, J.S. and Warner, T.G. (1980) Sialidosis: delineation of subtypes by neuraminidase assay. *Clin. Genet.* **17**:35-38

O'Brien, J.S. (1989) ß-Galactosidase deficiency (GM1 Gangliosiosis, Galactosialidosis, and Morquio B Syndrome); Ganglioside sialidase deficiency (Mucolipidosis IV). In: The metabolic basis of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. eds.), pp. 1797-1806, McGraw-Hill, New York

Öhman, R., Rosenberg, A. and Schengrund, C.L. (1970) Human brain sialidase. *Biochemistry* **9**:3774-3782

Ohkuma, S. (1987) The lysosomal proton pump and its effects on protein breakdown. In: Lysosomes: their role in protein breakdown (Glaumann, H. and Ballard, F.J. eds.), pp. 115-148 , Academic Press, London

Okada, S., Yutaka, T., Kato, T., Ikehara, C., Yabuuchi, H., Okawa, M., Inui, M. and Chiyo, H. (1979) A case of neuraminidase deficiency associated with a partial ß-galactosidase defect. *Eur. J. Pediatr.* **130**:239-249

Petersen, G.M., Rotter, J.I., Cantor, R.M., Field, L.L., Greenwald, S., Lim, J.S.T., Roy, C., Schoenfeld, V., Lowden, J.A. and Kaback, M.M. (1983) The Tay-Sachs disease gene in North American Jewish populations: geographic variation and origin. *Am. J. Hum. Genet.* **35**:1258-1269

Pisoni, R.L. and Thoene, J.G. (1991) The transport system of mammalian lysosomes. *Biochim. Biophys. Acta*

1071:351-373

Potier, M., Lu Shun Yan, D. and Womack, J.E. (1979) Neuraminidase deficiency in the mouse. *FEBS Lett.* **108**:345-348

Sagawa, J., Miyagi, T. and Tsuiki, S. (1990) Characterization of the major sialidases of various types of rat blood cells: their comparison with rat liver sialidases. *J. Biochem.* **107**:452-456

Schauer, R., Wember, M. and Tschesche, H. (1984) Isolation and characterization of an oligosaccharide- and glycoprotein-specific sialidase from human leucocytes. *Hoppe-Seyler's Z. Physiol. Chem.* **365**:419-426

Schauer, R. (1987) Sialic acids: metabolism of O-acetyl groups. *Methods Enzymol.* **138**:611-626

Schauer, R., Reuter, G. and Stoll, S. (1988) Sialate O-acetylesterases: key enzymes in sialic acid metabolism. *Biochimie* **70**:1511-1519

Schauer, R., Reuter, G., Stoll, S. and Shukla, A.K. (1989) Partial purification and characterization of sialate O-acetylesterase from bovine brain. *J. Biochem.* **106**:143-150

Schengrund, C.L. and Rosenberg, A. (1970) Intracellular location and properties of bovine brain sialidase. *J. Biol. Chem.* **245**:6196-6200

Schengrund, C.L., Jensen, D.S. and Rosenberg, A. (1972) Localization of sialidase in the plasma membrane of rat liver cells. *J. Biol. Chem.* **247**:2742-2746

Schneider-Jakob, H.R. and Cantz, M. (1991) Lysosomal and plasma membrane ganglioside G_{M3} sialidase of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biol. Chem. Hoppe-Seyler* **372**:443-450

Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. eds. (1989) The metabolic basis of inherited disease. pp. 1565-1839, McGraw-Hill, New York

Spaltro, J. and Alhadeff, J.A. (1987) Cellular localization and substrate specificity of isoelectric forms of human liver neuraminidase activity. *Biochem. J.* **241**:137-143

Spranger, J.W. and Wiedemann, H.R. (1970) The genetic mucolipidoses: diagnosis and differential diagnosis. *Humangenetik* **9**:113-139

Spranger, J., Gehler, J. and Cantz, M. (1977) Mucolipidosis I - A sialidosis. *Am. J. Med. Genet.* **1**:21-29

Srivastava, P.N. and Abou-Issa, H. (1977) Purification and properties of rabbit spermatozoal acrosomal neuraminidase. *Biochem. J.* **161**:193-200

Strecker, G., Peers, M.C., Michalski, J.C., Hondi-Assah, T., Fournet, B., Spik, G., Montreuil, J., Farriaux, J.P., Maroteaux, P. and Durand, P. (1977) Structure of nine sialyl-oligosaccharides accumulated in urine of eleven patients with three different types of sialidosis, mucolipidosis II and two new types of mucolipidosis. *Eur. J. Biochem.* **75**:391-403

Suzuki, Y., Sakuraba, H., Potier, M., Akagi, M., Sakai, M. and Beppu, H. (1981) ß-Galactosidase neuraminidase deficiency in adults: deficiency of a freeze-labile neuraminidase in leukocytes and fibroblasts. *Hum. Genet.* **58**:387-389

Suzuki, Y., Sakuraba, H., Yamanaka, T., Ko, Y.M. and Okamura, Y. (1983) Galactosialidosis (ß-galactosidase-neuraminidase deficiency): a new heriditary metabolic disease with abnormal degradation of enzyme molecules. *Acta Paediatr. Jpn.* **25**:31-37

Suzuki, Y., Sakuraba, H., Yamanaka, T., Ko., Y.M., Limori, Y., Okamura, Y. and Hoogeveen, A.T. (1984) Galactosialidosis: a comparative study of clinical and biochemical data on 22 patients. In: The developing brain and its disorders (Arina, M., Suzuki, Y. and Yabuuchi, H. eds.), pp. 161-175, University of Tokyo Press, Tokyo

Tallmann, J.F. and Brady, R.O. (1973) The purification and properties of a mammalian neuraminidase

(sialidase). *Biochim. Biophys. Acta* **293**:434-443

Tettamanti, G., Morgan, I.G., Gombos, G., Vincendon, G. and Mandal, P. (1972) Sub-synaptosomal localization of brain particulate neuraminidase. *Brain Res.* **47**:515-518

Thomas, G.H., Tipton, R.E., Ch'ien, L.T., Reynolds, L.W. and Miller, C.S. (1978) Sialidase (α-N-acetyl neuraminidase) deficiency: the enzyme defect in an adult with macular cherry-red spots and myoclonus without dementia. *Clin. Genet.* **13**:369-379

Thomas, G.H., Reynolds, L.W. and Miller, C.S. (1979) Characterization of neuraminidase activity of cultured human fibroblasts. *Biochim. Biophys. Acta* **568**:39-48

Tulsiani, D.R.P. and Carubelli, R. (1970) Studies on the soluble and lysosomal neuraminidases of rat liver. *J. Biol. Chem.* **245**:1821-1827

Tulsiani, D.R.P. and Carubelli, R. (1971) Studies on the soluble and lysosomal neuraminidases of rat mammary glands. *Biochim. Biophys. Acta* **227**:139-153

Tulsiani, D.R.P. and Carubelli, R. (1972) Soluble and lysosomal neuraminidase in the liver of developing chicks. *Biochim. Biophys. Acta* **284**:257-267

van Dessel, G., de Wolf, M., Lagrou, A., Hilderson, H. and Dierick, W. (1984) Characterization, purification and subcellular localization of bovine thyroid sialidase. *J. Biochem.* **96**:937-947

van Pelt, J., Kamerling, J.P., Vliegenthart, J.F.G., Hoogeveen, A.T. and Galjaard, H. (1988a) A comparative study of the accumulated sialic acid-containing oligosaccharides from cultured human galactosialidosis and sialidosis fibroblasts. *Clin. Chim. Acta* **174**:325-336

van Pelt, J., Kamerling, J.P., Vliegenthart, J.F.G., Verheijen, F.W. and Galjaard, H. (1988b) Isolation and structural characterization of sialic acid-containing storage material from mucolipidosis I (sialidosis) fibroblasts. *Biochim. Biophys. Acta* **965**:36-45

van Pelt, J. (1988c) Isolation and structural analysis of sialyloligosaccharides from sialidosis and galactosialidosis fibroblasts, placenta and urine. Thesis, Rijksuniversiteit Utrecht

van Pelt, J., Hard, K., Kamerling, J.P., Vliegenthart, J.F.G., Reuser, A.J.J. and Galjaard, H. (1989) Isolation and characterization of twenty-one sialyloligosaccharides from galactosialidosis urine. *Biol. Chem. Hoppe Seyler* **370**:191-203

Veh, R.W. and Sander, M. (1981) Differentiation between ganglioside and sialyllactose sialidases in human tissues. In Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 71-109, Ermes, Milan

Venerando, B., Tettamanti, G. Cestaro, B. and Zambotti, V. (1975) Studies on brain cytosol neuraminidase. I. Isolation and partial characterization of two forms of the enzyme from pig brain. *Biochim. Biophys. Acta* **403**:461-472

Verheijen, F.W., Brossmer, R. and Galjaard, H. (1982) Purification of acid ß-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. *Biochem. Biophys. Res. Commun.* **108**:868-875

Verheijen, F.W., Janse, H.C., van Diggelen, O.P., Bakker, H.D., Loonen, M.C.B., Durand, P. and Galjaard, H. (1983) Two genetically different MU-NANA neuraminidases in human leucocytes. *Biochem. Biophys. Res. Commun.* **117**:470-478

Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985) Human placental neuraminidase. Activation, stabilization and association with ß-galactosidase and its 'protective protein'. *Eur. J. Biochem.* **149**:315-322

Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H. and

von Figura, K. (1988) Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. *EMBO J.* 7:2351-2358

Wenger, D.A., Tarby, T.J. and Wharton, C. (1978) Macular cherry-red spots and myoclonus with dementia: Co-existent neuraminidase and ß-galactosidase deficiencies. *Biochem. Biophys. Res. Commun.* **82**:589-595

Yamamoto, Y. and Nishimura, K. (1987) Copurification and separation of ß-galactosidase and sialidase from porcine testis. *Int. J. Biochem.* **19**:435-442

Zeigler, M. and Bach, G. (1981) Cellular localization of neuraminidases in cultured human fibroblasts. *Biochem. J.* **198**:505-508

Zeigler, M., Sury, V. and Bach, G. (1989) The identification of lysosomal ganglioside sialidase in human cells. *Eur. J. Biochem.* **183**:455-458

Chapter 3

SUBSTRATE SPECIFICITY AND ACTIVE SITE

Substrate specificity

Considering the pronounced diversity in sialic acids, reflected by the natural occurrence of at least 23 different neuraminic acid derivatives, and the wide range of soluble and membrane bound sialic acid bearing compounds such as oligosaccharides, polysaccharides, glycopeptides, glycoproteins and glycolipids, neuraminidases are confronted with a large assortment of potential substrates. Therefore, knowledge about the substrate specificity of a neuraminidase would contribute to a better understanding of its biological function. However, most studies deal with viral and bacterial neuraminidases as mammalian neuraminidases are hard to obtain in pure form and substrate specificity studies require rather pure enzyme preparations (reviewed by Corfield *et al.*, 1981a). The substrate specificity of neuraminidases is set by a number of features, including the chemical structure of the sialic acid residue (see chapter 1, figure 1), as well as the composition of the aglycone (= non-sugar) part of the substrate:

(a) *Anomeric conformation.* Neuraminidases specifically hydrolyze α-O-glycosidic linkages. Neither ß-glycosides nor α-N- or α-S-glycosides are cleaved by bacterial and viral neuraminidases (Kuhn and Brossmer, 1958; Meindl and Tuppy, 1965; Khorlin *et al.*, 1970).
(b) *Presence of negative charge.* The negatively charged carboxylic group of sialic acids is

required for proper function of the neuraminidase. Removal of the carboxylic group by esterification or by reduction to a primary alcohol renders substrates uncleavable by viral and bacterial neuraminidases (Gottschalk, 1962; Kuhn *et al.*,1966; Brossmer and Holmquist, 1971). Introduction of an additional anionic site in the aglycone part, close to the carboxylic group of NeuAc, has no effect on neuraminidase activity, as shown by the normal hydrolysis of the carboxymethyl α-ketoside of NeuAc the *Vibrio cholerae* enzyme (Holmquist and Brossmer, 1972).

(c) *N-substituent*. The nature of the N-substituents greatly influences the neuraminidase activity. In a comparative study Corfield *et al.* (1981b) demonstrated that viral and bacterial neuraminidases as well as partially purified liver lysosomal neuraminidase hydrolyze both NeuAc and NeuGc containing substrates with a preference for the first. Substitution of the N-acetyl group for N-formyl or N-propionyl groups strongly reduces the cleavage rate, whereas N-succinyl and N-butyryl groups abolish bacterial neuraminidase activity (Meindl and Tuppy, 1966; Brossmer and Nebelin, 1969).

(d) *C_7-C_9 side chain*. Shortening of the side chain of glycoprotein or ganglioside bound NeuAc results in a decrease in the hydrolysis rate (Suttajit and Winzler, 1971; Veh *et al.*, 1977). Introduction of a carboxyl group in the side chain renders α-benzyl-glycosides uncleavable by Vibrio cholerae neuraminidase (Brossmer *et al.*, 1974). It is not clear whether this is the result of the altered size of the side chain or the introduction of a second anionic group in the sialic acid molecule. Introduction of a bulky aryl azide group at position C_9 of 2-deoxy-2,3-didehydro-N-acetyl neuraminic acid (NeuAc2en) does not alter the K_i of this strong competitive neuraminidase inhibitor (chapter 5: publications 2 and 3).

(e) *O-acetylation*. Depending on the position and amount of O-acetyl groups, the hydrolysis rate of O-acetylated sialic acids by bacterial neuraminidases is reduced, even when O-acetylation results in a higher affinity of the substrate for the enzyme. Most resistant against bacterial and viral neuraminidase action are 4-O-acetylated sialic acids (Schauer and Faillard, 1968; Shukla and Schauer, 1986; Kleineidam *et al.*, 1990).

(f) *Orientation of hydroxyl groups*. Bacterial neuraminidase and partially purified bovine testis lysosomal neuraminidase hydrolyze the benzyl α-glycoside of N-acetyl-4-epi-neuraminic acid at strongly reduced rates (Gross *et al.*, 1988). Together with the results obtained with 4-O-acetylated NeuAc, this indicates that proper enzyme-substrate interaction requires an unsubstituted, equatorially oriented hydroxyl group at C-atom 4.

(g) *Glycosidic linkage*. Depending on the type of linkage, α(2-3), α(2-6) or α(2-8), different hydrolysis rates are observed. In general, bacterial, viral and mammalian neuraminidases hydrolyze α(2-3) linkages at higher rates than α(2-6) linkages, which in turn are faster degraded than α(2-8) bonds, although with bacterial neuraminidases the difference between α(2-3) and α(2-6) linkages is less pronounced (Corfield *et al.*, 1981a).

(h) *Position of sialic acid.* Also the position of the sialic acid residue in sialoglycoconjugates influences the cleavage rate obtained with bacterial, viral and mammalian neuraminidases. This is illustrated by the resistance of the α(2-3) linked terminal side chain sialic acid residue in ganglioside G_{M1} and G_{M2} towards the action of neuraminidases, probably as a result of steric hindrance by the acetyl group of GalNAc (Kuhn and Wiegandt, 1963; Ledeen and Salsman, 1965).

(i) *Aglycone part.* Small synthetic substrates, like 4-methylumbelliferyl-NeuAc are readily cleaved by most neuraminidases with K_m values ranging from 0.1 to 1 mM. In contrast, hydrolysis of natural substrates by neuraminidases is strongly influenced by the nature and size of the aglycone part (Corfield *et al.*, 1981a). Least selective are the microbial neuraminidases, but in case of the various mammalian neuraminidases a narrow substrate specificity can be observed, as illustrated by the plasma membrane neuraminidase which predominantly hydrolyzes gangliosides (see chapter 2, table 2). The influence of the size of the aglycone part is best demonstrated by the rat liver lysosomal neuraminidase, purified by Miyagi and Tsuiki (1984). The enzyme failed to hydrolyze intact mucin but readily hydrolyzed the glycopeptides obtained after pronase digestion of mucin.

Apart from the intrinsic properties of substrate molecules, neuraminidase activity is influenced by a variety of other compounds, like divalent cations, detergents etc. Although many detailed and sometimes conflicting reports exist on the stimulating or inhibiting influence of ions on neuraminidase activity (for review see Drzeniek, 1973; Corfield *et al.*, 1982) it is difficult to obtain general rules as the effect is dependent on the source and purity of the neuraminidase, the substrate and pH used in the enzyme reaction, as well as the ionic concentration. Competitive inhibition of microbial as well as mammalian neuraminidases is observed with NeuAc, the product of the neuraminidase reaction (Drzeniek, 1973). The best competitive inhibitor of neuraminidases known thusfar is 2-deoxy-2,3-didehydro-N-acetyl neuraminic acid (NeuAc2en)(Meindl and Tuppy, 1969). Non-competitive inhibition of viral and bacterial neuraminidases has been observed with polyanionic compounds like nucleic acids and heparin (Drzeniek, 1973).

Substrates for lysosomal neuraminidase

Characterization of the storage products in urine and cultured fibroblasts from patients with sialidosis revealed that sialylated oligosaccharides represent a major natural substrate for the lysosomal neuraminidase (Michalski *et al.*, 1977; Strecker *et al.*, 1977, Dorland *et al.*, 1978; van Pelt *et al.*, 1988a,b). Also sialoglycopeptides and sialoglycoproteins serve as substrates for the enzyme although the hydrolytic rate may be lower (Tulsiani and Carubelli,

1970, 1971; Miyagi and Tsuiki, 1984). The involvement of lysosomal neuraminidase in the degradation of water-insoluble sialoglycolipids has been a matter of debate. *In vitro*, homogenates from cultured human fibroblasts were shown to hydrolyze gangliosides in the presence of detergents like Triton X-100. The responsible neuraminidase could be localized at the plasma membrane and was normally active in fibroblasts from a patient with sialidosis, suggesting the existence of a lysosomal "oligosaccharide neuraminidase" and a genetically distinct plasma membrane "ganglioside neuraminidase" (Cantz and Messer, 1979; Caimi *et al.*, 1979; Zeigler and Bach, 1981). Meanwhile, analysis of the ganglioside degrading activity of partially purified lysosomal neuraminidase preparations yielded conflicting results, probably because of differences in assay conditions or contamination with plasma membrane neuraminidase, leaving the participation of the lysosomal "oligosaccharide neuraminidase" in the catabolism of gangliosides an open question (for references see chapter 2, table 1).

The observation that cultured fibroblasts from patients with sialidosis or galacto-sialidosis accumulate ganglioside G_{M3} after feeding with radiolabeled ganglioside G_{M1}, strongly favoured the idea that the lysosomal neuraminidase must be involved in the lysosomal degradation of sialoglycolipids (Mancini *et al.*, 1986). In fact, in the presence of sodium cholate, highly purified lysosomal neuraminidase from human placenta, as present in a complex with ß-galactosidase and its protective protein, was shown to hydrolyze gangliosides G_{M3}, G_{D1a} and G_{D1b}, and to some extent gangliosides G_{M1} and G_{M2}. Triton X-100, known to stimulate the plasma membrane neuraminidase, had no effect on or even inhibited the lysosomal neuraminidase (Hiraiwa *et al.*, 1987, 1988). After inactivation of the plasma membrane neuraminidase with Cu^{2+}, a cholate dependent lysosomal neuraminidase could also be detected in cultured human fibroblasts. The absence of this cholate dependent lysosomal neuraminidase rather than the Triton dependent plasma membrane neuraminidase in fibroblasts from sialidosis patients further demonstrated that the lysosomal neuraminidase accomodates both oligosaccharide and ganglioside degrading activities (Lieser *et al.*, 1989; Schneider-Jakob and Cantz,1991).

In line with the proposed role of lysosomal neuraminidase in the catabolism of sialoglycolipids, autopsy material from a patient with sialidosis revealed an 8-fold increase in lipid bound sialic acids (mainly gangliosides G_{M3} and G_{D3}) in liver, spleen and kidney, with normal amounts in brain tissue (Ulrich-Bott *et al.*, 1987). Storage of gangliosides could also not be detected in a total brain homogenate from a patient with galactosialidosis (Sakuraba *et al.*, 1983). However, after dissection of the nervous system of another galactosialidosis patient, accumulation of ganglioside G_{M3} was demonstated in the thalamus, cerebellar cortex and sympathetic and spinal ganglia (Yoshino *et al.*, 1990).

Although detergents are handy tools for the detection of the glycolipid degrading capacity of neuraminidase and other lysosomal hydrolases, the reaction conditions do not

Table 4 Spingolipid Activator Proteins

genes	: G_{M2}-activator	prosaposin			
proteins	: G_{M2}-activator	*sap*-A	*sap*-B	*sap*-C	*sap*-D
other names:	SAP-3	saposin A	saposin B SAP-1 G_{M1}-activator sulfatide act.	saposin C SAP-2 co-ß-glucosidase	saposin D
enzymes involved	: hexosaminidase A	ß-glucosyl-ceramidase ß-galactosyl-ceramidase	arylsulfatase A α-galactosidase ß-galactosidase sphingomyelinase	ß-glucosyl ceramidase ß-galactosyl-ceramidase	sphingomyelinase
mechanism	: extracts substrate and presents it to the hydrolase		extracts substrate and presents it to the hydrolase	associates with hydrolase and alters K_m/V_{max}	
deficient in	: AB-variant G_{M2}-gangliosidosis		MLD-like storage diseases	Gaucher-like storage diseases	

note: SAP: sphingolipid activator protein
sap : SAP from saposin protein family

adapted from Fürst and Sandhoff (1992)

reflect the physiological situation occurring *in vivo*. Instead, living cells possess small heat-stable glycoproteins, known as sphingolipid activator proteins (SAP), that assist lysosomal hydrolases in the degradation of water-insoluble substrates (Mehl and Jatzkewitz, 1964; Conzelmann and Sandhoff, 1979; O'Brien *et al.*, 1988). Activator proteins (a) extract sphingolipids from the membrane and present them as a 1:1 protein-lipid complex to the enzyme in solution, (b) lift sphingolipids just far enough out of the membrane to allow a lawn-mower-like removal of the terminal sugar residue by the enzyme, or (c) bind to membrane associated enzymes hereby increasing the V_{max} and reducing the K_m of the enzyme for glycolipid substrates (Fürst and Sandhoff, 1992). In addition to their role in the hydrolysis of glycolipids, activator proteins have been shown to transfer sphingolipids from donor to acceptor liposomes or membranes (Conzelmann *et al.*, 1982; Vogel *et al.*, 1991; Hiraiwa *et al.* 1992). The occurrence of lysosomal storage disorders resulting from an activator protein deficiency illustrates the biological significance of this class of proteins. The molecular biology, structure, function and pathology of the activator proteins have recently been reviewed and will not be further discussed (Conzelmann and Sandhoff, 1987b; O'Brien and Kishimoto, 1991; Fürst and Sandhoff, 1992). The major features of the 5 known activator proteins - the G_{M2}-activator and *sap*-A to D, four homologous proteins derived from

a common precursor (prosaposin) - are summarized in table 4.

Recently, as will be discussed in detail in the next chapter, we have shown that the desialylation of gangliosides by purified lysosomal neuraminidase in absence of detergents is stimulated by *sap*-B (chapter 5: publication 5).

The active site

The active site represents the most vital part of an enzyme: it forms the catalytic centre that determines substrate specificity and the type of reaction performed. Considering the overall size of a protein, the active site only makes up a small portion of the total volume, the vast majority of amino acids not coming into contact with the substrate; a few specific amino acids are precisely arranged into a three-dimensional structure complementary to that of the substrate. The active site of neuraminidases has been studied for a number of reasons. In case of viral and bacterial neuraminidases knowledge about the structure of the active site and the reaction mechanism would facilitate the design of specific inhibitors that may control the spread of neuraminidase containing pathogens. In addition, the construction of active site directed, neuraminidase specific probes would allow identification of neuraminidases at the molecular level. For lysosomal neuraminidase biochemical determination of active site residues might help to mark point mutations affecting kinetic properties of the enzyme in patients with sialidosis, once cDNA's encoding the gene have been cloned. This section describes the approaches used to explore the active site of neuraminidases and to unravel the reaction mechanism.

Affinity labeling

Photoaffinity labeling has been proven to be a powerful tool for the identification of specific receptors and enzymes in complex protein mixtures or the identification of ligand or substrate binding domains within such proteins (for review see Bayley, 1983). A photoreactive probe is a radioactively or fluorescently labeled, chemically inert biological compound (ligand, substrate, inhibitor, etc.) which, upon radiation with visible or near ultraviolet light, turns into a short-living but highly reactive intermediate. This intermediate will mainly form covalent cross-links with the binding site or active site of the protein for which it has been designed as a result of the the high 'local' concentration. To minimize non-specific labeling it is essential that the probe has a high affinity for the protein of interest (K_m, K_i or K_d in the μmolar range), and thus can be used at sufficiently low concentrations.

The first photoreactive probes designed for the specific labeling of neuraminidases and other sialic acid binding proteins had important drawbacks. An aryl azido thioglycoside of

NeuAc with the photoreactive group in the non-cleavable aglycone part demonstrated insufficient affinity ($K_i \approx 1.5$ mM) towards neuraminidases (Warner and Lee, 1988; Warner and Loftin 1989), whereas 9-S-(4-azido-2-nitrophenyl)NeuAc2en (9-PANP-NeuAc2en), a high affinity NeuAc2en derivative containing a thio-linked aryl azide group at carbon atom C_9 ($K_i \approx 10$ μM) could not be labeled to high specific activity (Warner, 1987; Warner and Loftin, 1989). In 1990 we have synthesized ASA-NeuAc2en (chapter 5: publication 2), a photoreactive derivative of NeuAc2en with a N-linked aryl azide group at C_9 and high affinity for neuraminidase ($K_i \approx 15$ μM). In contrast to 9-PANP-NeuAc2en, ASA-NeuAc2en could easily be radioiodinated to high specific activity. Using this probe we were successful in labeling the *Clostridium perfringens* neuraminidase specifically at the active site (publication 2). After CNBr cleavage of the protein two radiolabeled peptides were observed, demonstrating that photoaffinity labeling combined with peptide mapping is a valuable tool to localize within the neuraminidase protein regions containing active site residues. Recently, by using an identical radioiodinated probe, Warner *et al.* (1992) performed a similar experiment for recombinant *Salmonella typhimurium* LT2 neuraminidase overproduced in *E.coli*. In this case the identification of photoaffinity labeled peptides facilitates the design of mutagenesis experiments with the cloned gene.

Photoaffinity labeling does not require previous knowledge about the protein structure or sequence and as such can be helpful for the identification of neuraminidases in complex protein structures. We have used radioiodinated ASA-NeuAc2en to identify the neuraminidase protein in the lysosomal ß-galactosidase/neuraminidase/protective protein complex from bovine testis (chapter 5: publication 3).

A photoreactive analogue of ganglioside G_{M1} has been prepared to study proteins involved in the uptake of this ganglioside by cultured human fibroblasts (Sonnino *et al.*, 1989). In addition to the photoreactive NeuAc2en derivatives, photoreactive gangliosides might be valuable for the study of neuraminidases.

Recently, Driguez *et al.* (1992) reported the synthesis of ortho-(difluoromethyl)-phenyl-α-ketoside of NeuAc, a mechanism based inhibitor of *Clostridium perfringens* neuraminidase. In contrast to photoreactive inhibitors, which are activated by irradiation with UV-light, this inhibitor is activated by the neuraminidase itself: hydrolysis of the compound generates a reactive intermediate that binds irreversible with a nucleophilic amino acid residue in the reactive center. Once prepared in a radioactive form this suicide substrate will be of great value for identification of neuraminidases and active site domains.

Conserved sequence motifs

Sequence comparison of proteins may reveal conserved amino acid sequences which

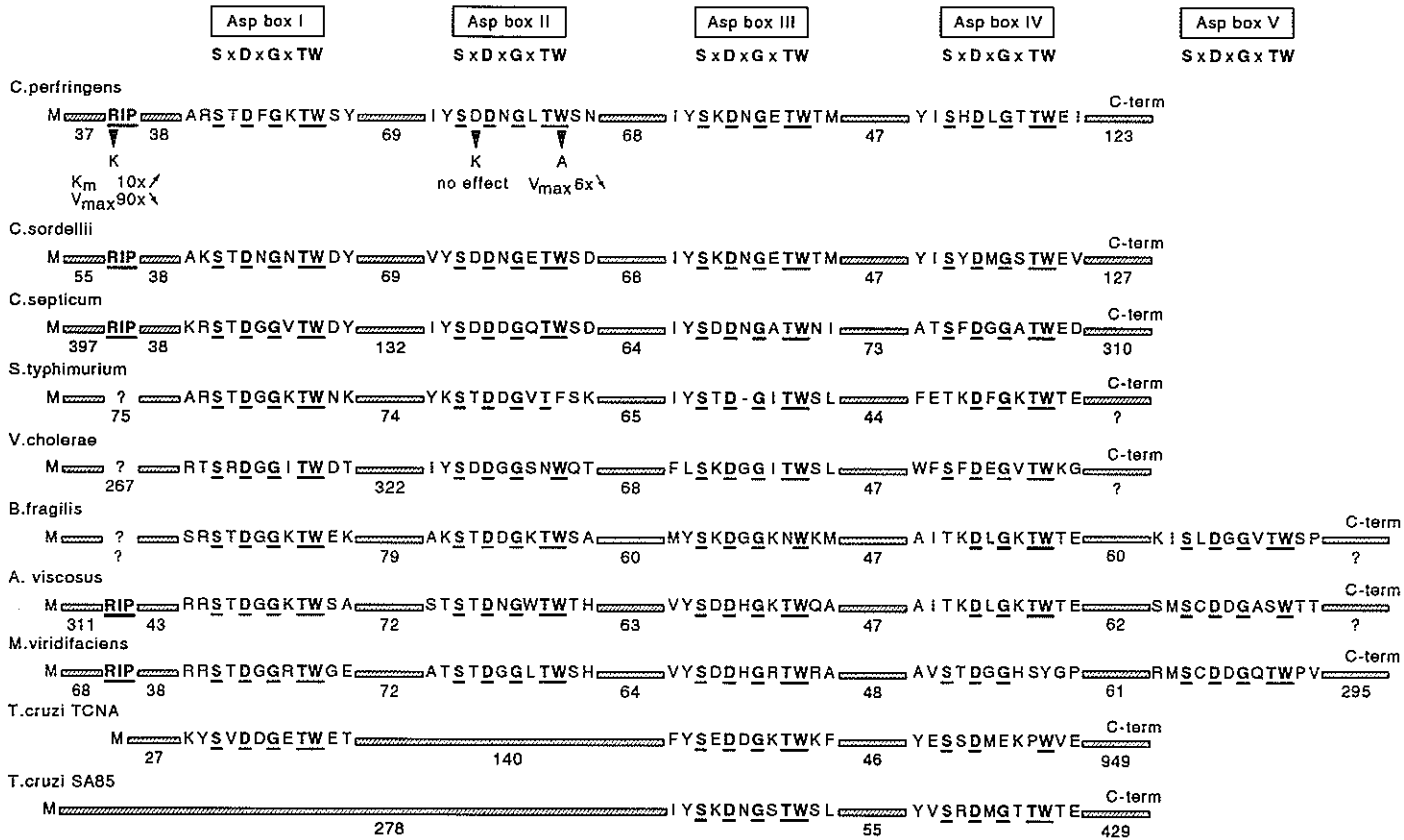

Figure 4. Comparison of conserved, repeated sequences in neuraminidases from bacteria and *Trypanosoma cruzi* and effect of amino acid substitutions in the in vitro mutagenized Clostridium perfringens neuraminidase. Numbers indicate the number of residues between the starting methionine, the arginine in the conserved tripeptide structure, the central asparagine residues in the Asp-boxes, and the C-terminus of the protein. References are given in the text.

are particularly important for biological function. At this moment this approach is only applicable to microbial and viral neuraminidases as these microorganisms form the only source from which genes encoding neuraminidases have been cloned.

During the last 5 years genes encoding bacterial neuraminidases have been isolated from *Clostridium perfringens* (Roggentin *et al.*, 1988), *Vibrio cholerae* (Vimr *et al.*, 1988), *Clostridium sordellii* (Rothe *et al.*, 1989), *Salmonella typhimurium* (Roggentin *et al.*, 1989; Hoyer *et al.*, 1992), *Bacteroides fragilis* (Russo *et al.*, 1990), *Clostridium septicum* (Rothe *et al.*, 1991) *Actinomyces viscosus* (Henningsen *et al.*, 1991) and *Micromonospora viridifaciens* (Sakurada *et al.*, 1992). Notwithstanding the significant mutual homology observed between Clostridial neuraminidases, sequence alignment of bacterial neuraminidases from different genera reveals only poor homology (<35 %) except for a short 8 amino acid sequence motif (Roggentin *et al.*, 1989; Rothe *et al.*, 1991). This motif, containining the sequence Ser-X-Asp-X-Gly-X-Thr-Trp, has been designated *Asp-box* due to the 100 % conservation of the aspartic acid residue (see figure 4). Asp-boxes occur 4 or 5 times in each bacterial neuraminidase, in most cases separated by 40 to 80 amino acids. Asp-boxes have also been observed in the neuraminidases from Trypanosoma cruzi: 2 completely and 1 partially conserved Asp-boxes in the TCNA (Pereira *et al.*, 1991) and 2 completely conserved Asp-boxes in members of the SA85 family (Kahn *et al.*, 1991). Conserved repeated Asp-boxes have not been observed in viral neuraminidases.

The role of Asp-boxes in bacterial and Trypanosomal neuraminidase activity remains to be resolved. However, the absence of such boxes in a photoaffinity labeled peptide of *Salmonella typhimurium* LT2 neuraminidase (Warner *et al.*, 1992) and the occurrence of repeated Asp(like)-boxes in non-neuraminidase proteins like the galactose recognizing *Myxococcus xanthus* hemagglutinin (Romeo *et al.*, 1986; Rothe *et al.*, 1991), argue against the involvement of the Asp-boxes in neuraminidase activity. As the only common feature of these proteins and neuraminidases is their ability to bind sugars, it has been suggested that the Asp-boxes are involved in substrate binding (Rothe *et al.*, 1991). Studies on the three-dimensional protein structure, recently started for a bacterial neuraminidase (Taylor *et al.*, 1992) in combination with site directed mutagenesis experiments with cloned genes should reveal whether Asp-box amino acid residues are located within the active site and required for enzymatic activity. Initial experiments with the *Clostridium perfringens* neuraminidase by Roggentin *et al.* (1992) revealed that substitution of the non-conserved Asp143 (→Lys) in the second Asp-box did not affect the kinetic parameters. Substitution of Trp149 (→Ala) in the same box leads to a 6 fold decrease in the V_{max} for the methylumbelliferyl substrate. In contrast, substitution of Arg37 (→Lys) in a short conserved motif upstream of the first box resulted in a 10-fold increased K_m and a 90-fold decreased V_{max}. Using chimeric protein constructs of an active and inactive *Trypanosoma cruzi* neuraminidase (TCNA), two potential

active site residues were also shown to reside outside an Asp-box (Uemura *et al.*, 1992).

Active site residues and reaction mechanism

Viral neuraminidases and in particular the influenza virus neuraminidase are best understood, probably as a result of their pathophysiological importance and the fact that the protein and its gene are relatively easily isolated in comparison to the mammalian neuraminidases. Nature itself has largely facilitated the identification of active site residues via a phenomenon called antigenic variation (Webster and Laver, 1975; Webster *et al.*, 1982, 1983). Genetic reassortment of the neuraminidase (and hemagglutinin) genes causes large alterations in the structure of the neuraminidase protein, resulting in the occurrence of influenza A and B virus neuraminidases (antigenic shift), whereas point mutations in the viral genome cause only minor changes, giving rise to antigenically different subtypes of influenza A virus as well as influenza B virus neuraminidase (antigenic drift). Amino acid sequence

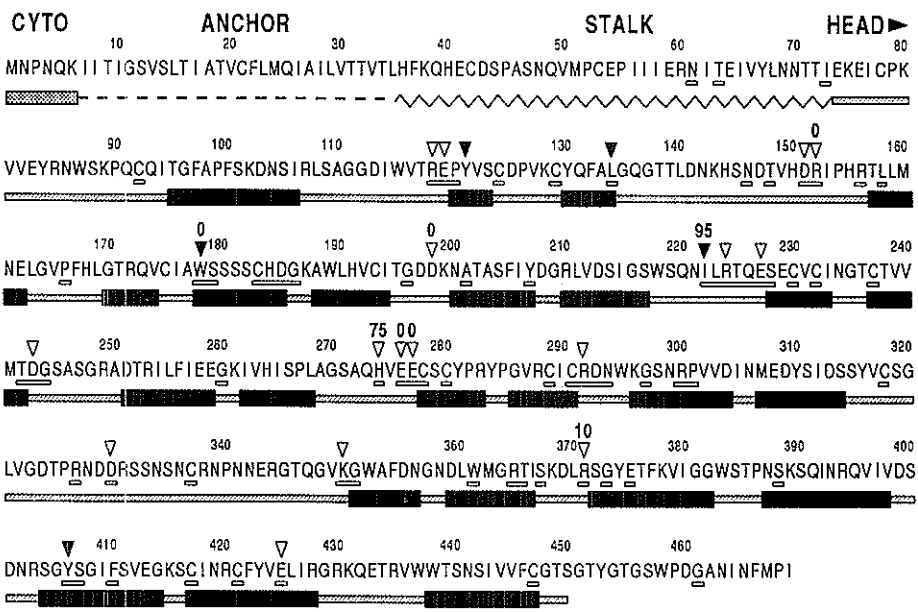

Figure 5. Analysis of the influenza A neuraminidase. Deduced amino acid sequence of influenza A neuraminidase subtype N2 (Lentz et al., 1984) with ß-sheet (▬) and loop (═) structure of the head region as deduced from crystallization studies (Varghese et al., 1983). Amino acids conserved in influenza neuraminidase subtypes N1,2,5,7,8 and 9 (Harley et al., 1989) are underlined. Conserved charged and hydrophobic amino acids near the active site are indicated with ▿ and ▾ respectively (Colman et al., 1983). Residual activity (in %) after in vitro mutagenesis of conserved amino acids (Lentz et al., 1987) is given as $\frac{0}{\nabla}$.

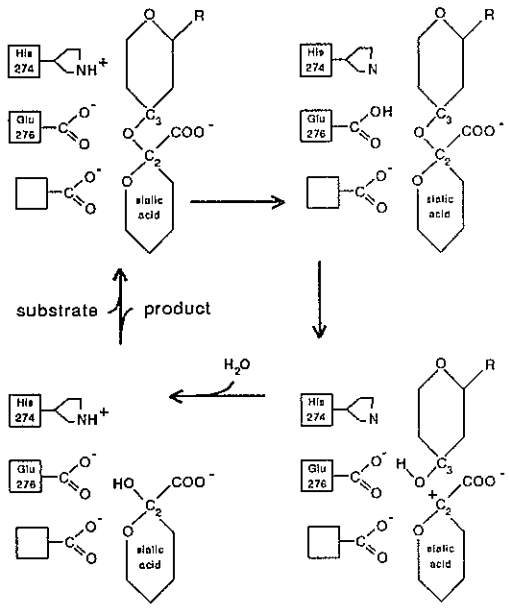

Figure 6. Proposed catalytic mechanism for influenza virus neuraminidase (adapted from Lentz et al., 1987).

homologies within and between the different subtypes are approximately 90 and 50 %, respectively. Assuming that amino acids required for special functions are not subject to antigenic variation, sequence comparison of serologically different subtypes of influenza A virus neuraminidase (resulting from antigenic drift), as well as influenza A and B neuraminidase (arisen by antigenic shift) must reveal amino acids involved in neuraminidase catalytic function. Since 1981, a great number of sequence reports concerning influenza A neuraminidase subtypes and influenza neuraminidase B has appeared (for references see: Shaw *et al.*, 1982; Varghese *et al.*, 1983; Harley *et al.*, 1989). As shown in figure 5, approximately 15 % of the amino acids is conserved in the different influenza A and B neuraminidases.

Crystallization studies have revealed the three dimensional structure of the N2 subtype neuraminidase (Varghese *et al.*, 1983, Colman *et al.*, 1983). The protein is composed of four identical subunits. Each subunit contains 6 four-stranded ß-sheets and, viewed from the top, resembles a six bladed propeller with the active site cleft in the middle. An unusually large number of charged residues, conserved amongst the different influenza strains (see figure 5), is localized in the active site and around its rim. In addition the active site contains four hydrophobic residues Tyr121 (Phe in N1 and B strains), Leu134, Trp178 and Tyr406.

Based on *in vitro* mutagenesis experiments Lentz *et al.* (1987) proposed a catalytic mechanism for the influenza neuraminidase (figure 6): His274 donates its proton to Glu276, which in turn uses this proton to open the glycosidic bond between sialic acid and the adjacent sugar residue. The ionized sialic acid intermediate, containing a carbonium ion, is stabilized by the ionized carboxyl group of a nearby acidic amino acid residue (possibly Glu277). Finally a water molecule is used to donate a hydroxyl group to the sialic acid intermediate and to reprotonate His274. During release from the active site, the α-anomer of NeuAc mutarotates to give the ß-anomer (Friebolin *et al.*, 1980). In this mechanism the

function of the His residue is to elevate the pKa of Glu276, thus allowing it to act as proton donor. This is supported by the observation that mutations in His274 only result in decreased enzymatic activity at a lower pH-optimum. In the absence of the histidine residue, a lower pH is required to obtain a protonated Glu276. The other amino acid residues required for activity, Arg152, Trp178, Asp198 and Arg371, are thought to be involved in the binding of substrate.

Recently, von Itzstein and coworkers (1993) have demonstrated that crystallographic studies of the influenza virus neuraminidase, in addition to providing information about the three dimensional structure of the active site, enable the computer-assisted design of potential drugs for therapeutic or prophylactic treatment of influenza infections. 4-Guanidino-NeuAc2en not only appeared to be a powerful inhibitor of the viral enzyme (K_i 75 nM) but also inhibited the replication of the virus in cell culture and animal models.

As discussed, after the identification of conserved amino acid sequences in bacterial and *Trypanosoma cruzi* neuraminidases, a start has been made with the unraveling of the structure and function of the microbial enzymes by in vitro mutagenesis and protein crystalization studies (Roggentin *et al.*, 1992; Taylor *et al.*, 1992; Uemura *et al.*, 1992). Analogously, cloning of cDNA's encoding the mammalian neuraminidases will enable a detailed structure/function analysis for this class of enzymes. In case of the lysosomal neuraminidase, naturally occurring mutations in sialidosis patients may be of help in delineating functional domains.

References

Bayley, H. (1983) Photogenerated reagents in biochemistry and molecular biology. Laboratory techniques in biochemistry and molecular biology, Vol. 12 (Work, T.S. and Burdon, R.H. eds.), Elsevier, Amsterdam

Brossmer, R. and Nebelin, E (1969) Synthesis of N-formyl- and N-succinyl-D-neuraminic acid. *FEBS Lett.* 4:335-336

Brossmer, R. and Holmquist, L. (1971) On the specificity of neuraminidase. *Hoppe-Seyler's Z. Physiol. Chem.* **352**:1715-1719

Brossmer, R., Bürk, G., Eschenfelder, V., Holmquist, L., Jäckh, R., Neumann, B. and Rose, U. (1974) Recent aspects of the chemistry of N-acetyl-D-neuraminic acid. *Behring Inst. Mitt.* **55**:119-123

Caimi, L., Lombardo, A., Preti, A., Wiesmann, U. and Tettamanti, G. (1979) Optimal conditions for the assay of fibroblast neuraminidase with different natural substrates. *Biochim. Biophys. Acta* **571**:137-146

Cantz, M. and Messer, H. (1979) Oligosaccharide and ganglioside neuraminidase activities of mucolipidosis I (sialidosis) and mucolipidosis II (I-cell disease) fibroblasts. *Eur. J. Biochem.* **97**:113-118

Colman, P.M., Varghese, J.N. and Laver, W.G. (1983) Structure of the catalytic site and antigenic sites in

influenza virus neuraminidase. *Nature* **303**:41-44

Conzelmann, E., and Sandhof, K. (1979) Purification and characterization of an activator protein for the degradation of glycolipids GM2 and GA2 by hexosaminidase A. *Hoppe-Seyler's Z. Physiol. Chem.* **360**:1837-1849

Conzelmann, E., Burg, J., Stephan, G. and Sandhoff, K. (1982) Complexing of glycolipids and their transfer between membranes by the activator protein for degradation of ganglioside G_{M2}. *Eur. J. Biochem.* **123**:455-464

Conzelmann, E. and Sandhoff, K. (1987a) Glycolipid and glycoprotein degradation. *Adv. Enzymol.* **60**:89-216

Conzelmann, E. and Sandhoff, K. (1987b) Activator proteins for lysosomal glycolipid hydrolysis. *Meth. Enzymol.* **138**:792-815

Corfield, A.P., Michalski, J.C. and Schauer, R. (1981a) The substrate specificity of sialidases from microorganisms and mammals. In Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 3-70, Ermes, Milan

Corfield, A.P., Veh, R.W., Wember, M., Michalski, J.C. and Schauer, R. (1981b) The release of N-acetyl- and N-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases. *Biochem. J.* **197**:293-299

Corfield, A.P. and Schauer. R. (1982) Metabolism of sialic acids. In: Sialic acids. Chemistry, metabolism and function (Schauer, R. ed) pp. 195-261, Springer-Verlag, New York

Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Strecker, G., Michalski, J.C., Fournet. B., Spik, G. and Montreuil, J. (1978) 360 MHz ^{1}H nuclear-magnetic resonance spectroscopy of sialyloligosaccharides from patients with sialidosis (mucolipidosis I and II). *Eur. J. Biochem.* **87**:323-329

Driguez, P.A., Barrere, B., Chantegrel, B., Deshayes, C., Doutheau, A. and Quash, G. (1992) Synthesis of soudium salt of ortho-(difluoromethyl)phenyl-α-ketoside of N-acetylneuraminic acid: a mechanism-based inhibitor of Clostridium perfringens neuraminidase. *Bioorg. Med. Chem Lett.* **2**:1361-1366

Drzeniek, R. (1973) Substrate specificity of neuraminidases. *Histochem. J.* **5**:271-290

Friebolin, H., Brossmer, R., Keilich, G., Ziegler, D. and Supp, M. (1980) ^{1}H-NMR-spektroskopischer nachweis der N-acetyl-α-D-neuraminsäure als primäres spaltprodukt der neuraminidases. *Hoppe-Seyler's Z. Physiol. Chem.* **361**:697-702

Fürst, W. and Sandhoff, K. (1992) Activator proteins and topology of lysosomal sphingolipid catabolism. *Biochim. Biophys. Acta* **1126**:1-16

Gottschalk, A. (1962) The relation between structure and function in some glycoproteins. *Perspectives Biol. Med.* **5**:327-337

Gross, H.J., Kovac, A., Rose, U., Watzlawick, H. and Brossmer, R. (1988) Interaction of N-acetyl-4-epi-D-neuraminic acid with key enzymes in sialic acid metabolism. *Biochemistry* **27**:4279-4283

Harley, V.R., Ward, C.W. and Hudson, P.J. (1989) Molecular cloning and analysis of the N5 neuraminidase subtype from an avian influenza virus. *Virology* **169**:239-243

Henningsen, M., Roggentin, P. and Schauer, R. (1991) Cloning, sequencing and expression of the sialidase gene from Actinomyces viscosus DSM 43798. *Biol. Chem. Hoppe-Seyler* **372**:1065-1072

Hiraiwa, M., Uda, Y., Nishizawa, M. and Miyatake, T. (1987) Human placental sialidase: partial purification and characterization. *J. Biochem.* **101**:1273-1279

Hiraiwa, M., Nishizawa, M., Uda, Y., Nakajima, T. and Miyatake, T. (1988) Human placental sialidase: further purification and characterization. *J. Biochem.* **103**:86-90

Hiraiwa, M., Soeda, S., Kishimoto, Y. and O'Brien, J.S. (1992) Binding and transport of gangliosides by

prosaposin. *Proc. Natl. Acad. Sci. USA* **89**:11254-11258

Holmquist, L. and Brossmer, R. (1972) On the specificity of neuraminidase. The carboxymethyl α-ketoside of N-acetyl-D-neuraminic acid, a Vibrio cholerae neuraminidase substrate having two anionic sites. *FEBS Lett.* **22**:46-48

Kahn, S., Colbert, T.G., Wallace, J.C., Hoagland, N.A. and Eisen, H. (1991) The major 85-kDa surface antigen of the mammalian-stage forms of Trypanosoma cruzi is a family of sialidases. *Proc. Natl. Acad. Sci. USA* **88**:4481-4485

Khorlin, A., Privalova, I.M., Zakstelskaya, L., Molibog, E.V. and Evstigneeva, N.A. (1970) Synthetic inhibitors of Vibrio cholerae neuraminidase and neuraminidases of some influenza virus strains. *FEBS Lett.* **8**:17-19

Kleineidam, R.G., Furuhata, K., Ogura, H. and Schauer, R. (1990) 4-Methylumbelliferyl-α-glycosides of partially O-acetylated N-acetylneuraminic acids as substrates of bacterial and viral sialidases. *Biol. Chem. Hoppe-Seyler* **371**:715-719

Kuhn, R. and Brossmer, R. (1958) Die Konstitution der Lactaminsäurelactose, α-Ketosidase Wirkung von Viren der Influenza-Gruppe. *Angew. Chem.* **70**:25

Kuhn, R. and Wiegandt, H. (1963) Die Konstitution der Ganglio-N-tetraose und des Gangliosids G_1. *Chem. Ber.* **96**:866-880

Kuhn, R., Lutz, P. and MacDonald, D.L. (1966) Synthese anomerer Sialinsäure-methylketoside. *Chem. Ber.* **99**:611-617

Ledeen, R. and Salsman, K. (1965) Structure of the Tay-Sachs' ganglioside I. *Biochemistry* **4**:2225-2232

Lentz, M.R., Air, G.M., Laver, W.G. and Webster, R.G. (1984) Sequence of the neuraminidase genes of A/Tokyo/67 influenza virus and of variants selected with monoclonal antibodies. *Virology* **135**:257-265

Lentz, M.R., Webster, R.G. and Air, G.M. (1987) Site-directed mutagenesis of the active site of influenza neuraminidase and implications for the catalytic mechanism. *Biochemistry* **26**:5351-5358

Lieser, M., Harms, E., Kern, H., Bach, G. and Cantz, M. (1989) Ganglioside G_{M3} sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucolipidosis IV. Subcellular distribution and some properties. *Biochem. J.* **260**:69-74

Mancini, G.M.S., Hoogeveen, A.T., Galjaard, H., Månsson, J.E. and Svennerholm, L. (1986) Ganglioside GM_1 metabolism in living human fibroblasts with ß-galactosidase deficiency. *Hum. Genet.* **73**:35-38

Mehl, E. and Jatzkewitz, H. (1964) Eine Cerebrosidsulfatase aus Schweineniere. *Hoppe-Seyler's Z. Physiol. Chem.* **339**:260-276

Meindl, P. and Tuppy, H. (1965) Uber synthetische Ketoside der N-Acetyl-D-neuraminsäure. 1. Mitt.: Darstellung einer Reihe durch Neuraminidase spaltbarer Ketoside. *Monatsh. Chem.* **96**:802-815

Meindl, P. and Tuppy, H. (1966) Darstellung und enzymatische Spaltbarkeit von α-Ketosiden der N-Propionyl, N-Butyryl- und N-Benzoyl-D-neuraminsäure. *Monatsh. Chem.* **97**:1628-1647

Meindl, P. and Tuppy, H. (1969) Uber 2-Desoxy, 2,3,dehydrosialinsäuren. II. Kompetitive Hemmung der Vibrio cholerae Neuraminidases durch 2-Desoxy, 2,3,dehydro-N-acylneuraminsäuren. *Hoppe-Seyler's Z. Physiol. Chem.* **350**:1088-1092

Michalski, J.C., Strecker, G., Fournet, B., Cantz, M. and Spranger, J. (1977) Structures of sialyl-oligosaccharides excreted in the urine of a patient with mucolipidosis I. *FEBS Lett.* **79**:101104

Miyagi, T. and Tsuiki, S. (1984) Rat liver lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase. *Eur. J. Biochem.* **141**:75-81

O'Brien, J.S., Kretz, K.A., Dewji, N., Wenger, D.A., Esch, F. and Fluharty, A.L. (1988) Coding of two

sphingolipid activator proteins (SAP-1 and SAP-2) by same genetic locus. *Science* **241**:1098-1101

Pereira, M.E.A., Mejia, J.S., Ortega-Barria, E., Matzilevitch, D. and Prioli, R.P. (1991) The Trypanosoma cruzi neuraminidase contains sequences similar to bacterial neuraminidases, YWTD repeats of the low density lipoprotein receptor, and type III modules of fibronectin. *J. Exp. Med.* **174**:179-191

Roggentin, P., Rothe, B., Lottspeich, F. and Schauer, R. (1988) Cloning and sequencing of a Clostridium perfringens sialidase gene. *FEBS Lett.* **238**:31-34

Roggentin, P., Rothe, B., Kaper, J.B., Galen, J., Lawrisuk, L., Vimr, E. and Schauer, R. (1989) Conserved sequences in bacterial and viral sialidaes. *Glycoconjugate J.* **6**:349-353

Roggentin, T., Kleineidam, R.G., Schauer, R. and Roggentin, P. (1992) Effects of site specific mutations on the enzymatic properties of a sialidase from Clostridium perfringens. *Glycoconjugate J.* **9**:235-240

Romeo, J.M., Esmon, B. and Zusman, D.R. (1986) Nucleotide sequence of the myxobacterial hemagglutinin gene contains four homologous domains. *Proc. Natl. Acd. Sci. USA* **83**:6332-6336

Rothe, B., Roggentin, P., Frank, R., Blöcker, H. and Schauer, R. (1989) Cloning, sequencing and expression of a sialidase gene from Clostridium sordellii G12. *J. Gen. Microbiol.* **135**:3087-3096

Rothe, B., Rothe, B., Roggentin, P. and Schauer, R. (1991) The sialidase gene from Clostridium septicum: cloning sequencing, expression in Escherichia coli and identification of conserved sequences in sialidases and other proteins. *Mol. Gen. Genet.* **226**:190-197

Russo, T.A., Thompson, J.S., Godoy, V.G. and Malamy, M.H. (1990) Cloning and expression of the Bacteroides fragilis TAL2480 neuraminidase gene, nanH, in Escherichia coli. *J. Bacteriol.* **172**:2594-2600

Sakuraba, H., Suzuki, Y., Akagi, M., Sakai, M. and Amano, N. (1983) ß-Galactosidase-neuraminidase deficiency (galactosialidosis): clinical, pathological, and enzymatic studies in a postmortem case. *Ann. Neurol.* **13**:497-503

Sakurada, K., Ohta, T. and Hasegawa, M. (1992) Cloning, expression, and characterization of the Micromonospora viridifaciens neuraminidase gene in Streptomyces lividans. *J.Bacteriol.* **174**:6896-6903

Schauer, R. and Faillard, H. (1968) Zur Wirkungsspezifität der Neuraminidase. Das Verhalten isomerer N.O.-Diacetyl-neuraminsäureglykoside im Submaxillarismucin von Pferd und Rind bei Einwirkung bakterieller Neuraminidase. *Hoppe-Seyler's Z. Physiol. Chem.* **349**:961-968

Schneider-Jakob, H.R. and Cantz, M. (1991) Lysosomal and plasma membrane ganglioside G_{M3} sialidase of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biol. Chem. Hoppe-Seyler* **372**:443-450

Shaw, M.W., Lamb, R.A., Erickson, B.W., Briedis, D.J. and Choppin, P.W. (1982) Complete nucleotide sequence of the neuraminidase gene of influenza B virus. *Proc. Natl. Acad. Sci. USA* **79**:6817-6821

Shukla, A.K. and Schauer, R. (1986) Analysis of sialidase and N-acetylneuraminate pyruvate-lyase substrate specificity by high-performance liquid chromatography. *Anal. Biochem.* **158**:158-164

Sonnino, S., Chigorno, V., Acquotti, D., Pitto, M., Kirschner, G. and Tettamanti, G. (1989) A photoreactive derivative of radiolabeled GM1 ganglioside: preparation and use to establish the involvement of specific proteins in GM1 uptake by human fibroblasts in culture. *Biochemistry* **28**:77-84

Strecker, G., Peers, M.C., Michalski, J.C., Hondi-Assah, T., Fournet, B., Spik, G., Montreuil, J., Farriaux, J.P., Maroteaux, P. and Durand, P. (1977) Structure of nine sialyl-oligosaccharides accumulated in urine of eleven patients with three different types of sialidosis, mucolipidosis II and two new types of mucolipidosis. *Eur. J. Biochem.* **75**:391-403

Suttajit, M. and Winzler, R.J. (1971) Effect of modification of N-acetylneuraminic acid on the binding of glycoproteins to influenza virus and on susceptibility to cleavage by neuraminidase. *J. Biol. Chem.*

246:3398-3401

Taylor, G., Vimr, E., Garman, E. and Laver, G. (1992) Purification, crystallization and preliminary crystallographic study of neuraminidase from Vibrio cholerae and Salmonella Typhimurium LT2. *J. Mol. Biol.* **226**:1287-1290.

Tulsiani, D.R.P. and Carubelli, R. (1970) Studies on the soluble and lysosomal neuraminidases of rat liver. *J. Biol. Chem.* **245**:1821-1827

Tulsiani, D.R.P. and Carubelli, R. (1971) Studies on the soluble and lysosomal neuraminidases of rat mammary glands. *Biochim. Biophys. Acta* **227**:139-153

Uemura, H., Schenkman, S., Nussenzweig, V. and Eichinger, D. (1992) Only some members of a gene family in Trypanosoma cruzi encode proteins that express both trans-sialidase and neuraminidase activities. *EMBO J.* **11**:3837-3844

Ulrich-Bott, B., Klem, B., Kaiser, R., Spranger, J. and Cantz, M. (1987) Lysosomal sialidase deficiency: increased ganglioside content in autopsy tissues of a sialidosis patient. *Enzyme* **38**:262-266

van Pelt, J., Kamerling, J.P., Vliegenthart, J.F.G., Verheijen, F.W. and Galjaard, H. (1988a) Isolation and structural characterization of sialic acid-containing storage material from mucolipidosis I (sialidosis) fibroblasts. *Biochim. Biophys. Acta* **965**:36-45

van Pelt, J. (1988b) Isolation and structural analysis of sialyloligosaccharides from sialidosis and galactosialidosis fibroblasts, placenta and urine. Thesis, Rijksuniversiteit Utrecht

Varghese, J.N., Laver, W.G. and Colman, P.M. (1983) Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature* **303**:35-40

Veh, R.W., Corfield, A.P., Sander, M. and Schauer, R. (1977) Neuraminic acid-specific modification and tritium labeling of gangliosides. *Biochim. Biophys. Acta* **486**:145-160

Vimr, E.R., Lawrisuk, L., Galen, J. and Kaper, J.B. (1988) Cloning and expression of the Vibrio cholerae neuraminidase gene nanH in Escherichia coli. *J. Bacteriol.* **170**:1495-1504

Vogel, A., Schwarzmann, G. and Sandhoff, K. (1991) Glycosphingolipid specificity of the human sulfatide activator protein. *Eur. J. Biochem.* **200**:591-597

von Itzstein, M., Wu, W.Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Phan, T.V., Smythe, M.L., White, H.F., Oliver, S.W., Colman, P.M., Varghese, J.N., Ryan, D.M., Woods, J.M., Bethell, R.C., Hotham, V.J., Cameron, J.M. and Penn, C.R. (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**:418-423

Warner, T.G. (1987) A photoreactive competitive inhibitor of the human lysosomal neuraminidase in cultured skin fibroblasts. *Biochem. Biophys. Res. Commun.* **148**:1323-1329

Warner, T.G. and Lee, L.A. (1988) An azidoaryl thioglycoside of sialic acid. A potential photoaffinity probe of sialidases and sialic acid binding proteins. *Carbohydr. Res.* **176**:211-218

Warner, T.G. and Loftin, S.K. (1989) Photolysis of the lysosomal neuraminidase in cultured human skin fibroblasts in the presence of a photoreactive competitive inhibitor. *Enzyme* **42**:103-109

Warner, T.G., Harris, R., McDowell, R. and Vimr, E.R. (1992) Photolabelling of Salmonella typhimurium LT2 sialidase. *Biochem. J.* **285**:957-964

Webster, R.G. and Laver, W.G. (1975) Antigenic variation of influenza viruses. In: The influenza viruses and influenza (Kilbourne, E.D. ed.), pp. 269-314, Academic, New York

Webster, R.G., Laver, W.G., Air, G.M. and Schild, G.C. (1982) Molecular mechanism of variation in influenza viruses. *Nature* **296**:115-121

Webster, R.G., Laver, W.G. and Air, G.M. (1983) Antigenic variation among type A influenza viruses. In:

Genetics of influenza viruses (Palese, P. and Kingsbury, D.W. eds.), pp. 127-168, Springer-Verlag, Vienna

Yoshino, H., Miyashita, K., Miyatani, N., Ariga, T., Hashimoto, Y., Tsuji, S., Oyanagi, K., Ohama, E., Ikuta, F., Suzuki, A. and Miyatake, T. (1990) Abnormal glycosphingolipid metabolism in the nervous system of galactosialidosis. *J. Neurol. Sci.* **97**:53-65

Zeigler, M. and Bach, G. (1981) Cellular localization of neuraminidases in cultured human fibroblasts. *Biochem. J.* **198**:505-508

Chapter 4

EXPERIMENTAL WORK

Introduction to the ß-galactosidase/neuraminidase/protective protein complex

Most lysosomal storage disorders result from a single enzyme or transport protein deficiency. However, with the discovery of galactosialidosis, caused by a combined deficiency of lysosomal neuraminidase and ß-galactosidase, the question was raised whether both hydrolases require an additional protein for their proper functioning. Genetic complementation studies (Galjaard *et al.*, 1975; Hoogeveen *et al.*, 1980) with fibroblasts from patients with sialidosis (neur$^-$), G_{M1} gangliosidosis (ß-gal$^-$) and galactosialidosis (ß-gal$^-$ neur$^-$) indicated that different genes/proteins were involved in these disorders and initiated a series of studies leading to the discovery of the ß-galactosidase/neuraminidase/protective protein complex (for reviews see Verheijen, 1986; Hoogeveen, 1987; Galjart, 1991; Morreau, 1992).

ß-Galactosidase and its "protective protein"

Complementation studies not only revealed increased neuraminidase and ß-galactosidase activities in hybrid sialidosis/galactosialidosis fibroblasts, but also in galactosialidosis fibroblasts which had been cocultivated with sialidosis cells. The responsible "corrective factor", also secreted by normal and G_{M1}-gangliosidosis fibroblasts but absent in

the medium of galactosialidosis and I-cell fibroblasts, appeared to be glycoprotein, internalized by galactosialidosis fibroblasts via mannose-6-phosphate receptor mediated endocytosis (Hoogeveen *et al.*, 1980, 1981). Turnover studies with a suicide substrate for ß-galactosidase revealed an enhanced intralysosomal degradation of endogenous and ingested normal ß-galactosidase by galactosialidosis fibroblasts, suggesting that the "corrective factor" protects ß-galactosidase from being degraded and that the primary defect in galactosialidosis is a deficiency of this "protective" factor (van Diggelen *et al.*, 1981, 1982). The nature of the molecular defect in galactosialidosis was elucidated by d'Azzo *et al.* (1982). In normal fibroblasts, ß-galactosidase was shown to be synthesized as a 85 kDa precursor which was subsequently modified into a 64 kDa mature form. In fibroblasts from a patient with galactosialidosis the mature form rapidly disappeared. The enhanced intralysosomal degradation of the mature ß-galactosidase in galactosialidosis fibroblasts could be partially prevented by administration of the protease inhibitor leupeptine. The primary protein defect in galactosialidosis appeared to be a deficiency of a 32 kDa protein and its 54 kDa precursor, the latter being identical to the previously described "corrective factor". In control fibroblasts, ß-galactosidase exists as a high molecular mass multimer, whereas in galactosialidosis fibroblasts the enzyme is present in monomeric form (Hoeksema *et al.* 1979). Addition of the 54 kDa corrective factor to the medium of galactosialidosis fibroblasts restored the formation of ß-galactosidase multimers. From this fact and the presence of the 32 and 20 kDa processing products of the corrective factor in these multimers, it was concluded that the protection of ß-galactosidase against enhanced proteolytic degradation is based on complex formation between ß-galactosidase and the "protective protein" (Hoogeveen *et al.*, 1983).

During the last few years cDNA's and genes encoding mammalian protective protein and ß-galactosidase have been cloned (Galjart *et al.*, 1988, 1990; Oshima *et al.*, 1988; Morreau *et al.*, 1989, 1991; Nanba and Suzuki, 1990; 1991). Analysis of deduced amino acid sequences has revealed unexpected features. The protective protein demonstrated a marked homology with yeast and plant serine carboxypeptidases and labeling of the mature 32/20 kDa heterodimeric protective protein with a serine protease specific affinity probe further emphasized a possible function as protease/esterase (Galjart *et al.*, 1988, 1990). Enzymatically, the protective protein was shown to exhibit cathepsin A-like carboxypeptidase activity at acidic pH and deamidase/esterase at neutral pH, all activities being deficient in galactosialidosis fibroblasts (Kase *et al.*, 1990; Tranchemontagne *et al.*, 1990; Galjart *et al.*, 1991). In addition, a peptidase/deamidase released by human platelets and involved in the degradation of biologically active peptides appears to be identical to the protective protein (Jackman *et al.*, 1990, 1992). Recently, by employing the baculovirus expression system, recombinant protective protein has been synthesized on a large scale for elucidation of the

three dimensional structure of the protein (d'Azzo et al., 1993). Cloning of ß-galactosidase cDNA's revealed that the precursor mRNA is alternatively spliced into an abundant 2.5 kb transcript and a minor 2.0 kb species. The major transcript directs the synthesis of normal, enzymatically active ß-galactosidase whereas the minor transcript gives rise to a ß-galactosidase related protein devoid of enzymatic activity (Morreau et al., 1989). Recently, Hinek *et al.* (1993) demonstrated sequence homology as well as immunological and functional similarity between the frame shift generated sequence in the alternatively spliced ß-galactosidase and the ligand binding motif of the 67 kDa elastin/laminin binding protein (EBP).

The biosynthesis of ß-galactosidase and protective protein has been studied in human control and mutant fibroblasts. In particular expression studies with cloned cDNA's encoding these proteins, have provided detailed information about the intracellular transport and maturation of normal and mutated proteins. In control fibroblasts, the protective protein and ß-galactosidase are synthesized as glycosylated precursors with a molecular mass of 54 kDa and 85 kDa respectively and transported to the lysosome via the mannose-6-phosphate dependent pathway (d'Azzo *et al.*, 1982; Palmeri *et al.*, 1986). Expression studies in COS-1 cells suggest that exit from the endoplasmic reticulum requires dimerization of the protective protein precursor (Zhou *et al.*, 1991). In the late endosomal/lysosomal compartment the protective protein precursor is processed into the mature heterodimeric form consisting of 32 and 20 kDa polypeptides, which results in the release of carboxypeptidase activity (Galjart *et al.*, 1988, 1990). ß-Galactosidase is processed into the mature 64 kDa form and associates in the late endosomal/lysosomal compartment with the protective protein to prevent rapid intralysosomal degradation of the former (d'Azzo et al., 1982; Hoogeveen *et al.*, 1983; Willemsen *et al.*, 1986). Coexpression of ß-galactosidase and the protective protein in COS-1 cells showed that their precursors may already associate in the endoplasmic reticulum at neutral pH (Morreau *et al.*, 1992).

By employing panels of human-mouse hybrids, the genes encoding human ß-galactosidase and the protective protein have been shown to reside on chromosome 3 and 20, respectively (Shows *et al.*, 1979; Mueller *et al.*, 1986). Hybridization studies with a partial cDNA fragment further mapped the ß-galactosidase gene on chromosome 3p21-3pter (Yamamoto *et al.*, 1990), whereas in situ hybridization experiments placed the gene encoding the protective protein on chromosome 20q13.1 (Wiegant *et al.*, 1991).

Neuraminidase and the complex

Purified acid ß-galactosidase from a variety of mammalian organs exists as a monomeric, dimeric as well as multimeric form and consists of a protein with a molecular

mass of approximately 64 kDa. The multimeric form was shown to contain two additional proteins with a molecular mass of 32 and 20 kDa, which are now known to represent the protective protein (Norden *et al.*, 1974; Cheetham and Dance 1976; Frost *et al.*, 1978). During purification of lysomal ß-galactosidase via a ß-galactosidase specific substrate affinity chromatography step, lysosomal neuraminidase from bovine testis and human placenta was shown to copurify with the enzyme at high specific activity (Verheijen *et al.*, 1982, 1985). Sucrose density centrifugation revealed two multimeric forms of ß-galactosidase with different densities. Neuraminidase activity cosedimented with the high density form and could be precipitated with monospecific antibodies against ß-galactosidase or protective protein, demonstrating the existence of a complex containing neuraminidase, ß-galactosidase and protective protein. These results have been confirmed by others (Hiraiwa *et al.*, 1987, 1988; Potier *et al.*, 1990b) and extended to other tissues such as human lymphocytes (Verheijen *et al.*, 1983), porcine testis (Yamamoto and Nishimura, 1987) or rat liver (Scheibe *et al.*, 1990).

Especially the purification of the ß-galactosidase/neuraminidase/protective protein complex from human placenta has contributed to a better understanding of the relation between neuraminidase and the protective protein. In contrast to other tissues, neuraminidase activity is barely detectable in the placental homogenate. However, large amounts of neuraminidase activity can be generated by concentration of the glycoprotein fraction, as shown by the increase in specific activity (figure 7a). This activity is very labile and readily lost upon dilution, unless it is stabilized by incubation of the concentrated glycoprotein fraction at 37 °C. Depending on the pH, ionic strength and protein concentration the different forms of ß-galactosidase are known to interconvert (Cheetham and Dance, 1976; Yamamoto *et al.*, 1982; Yamamoto and Nishimura, 1980, 1986; Taguchi *et al.* 1981). Sucrose density gradient centrifugation experiments revealed that generation of neuraminidase activity during concentration is linked to the formation of the high density complex (Verheijen *et al.*, 1985). Reversely, partial dissociation of the human placental ß-galactosidase/neuraminidase/protective protein complex with the chaotropic agent KSCN results in the specific loss of neuraminidase activity and conversion of the high density into the low density multimeric form (Verheijen *et al.*, 1987). It was proposed that association of an unknown (inactive) neuraminidase polypeptide with the protective protein is essential for catalytic activity of lysosomal neuraminidase (Verheijen *et al.*, 1985). In addition to its protective function towards ß-galactosidase, the protective protein would act as a subunit for neuraminidase, explaining the neuraminidase deficiency in galactosialidosis. It is not clear whether the protective protein also protects the neuraminidase polypeptide from being degraded as data concerning the turnover of the neuraminidase protein are lacking.

With the aid of human-mouse hybrids, the gene encoding the human neuraminidase

polypeptide has been localized on chromosome 10pter→q23 (Mueller *et al.*, 1986). The normal neuraminidase activity in a patient with a terminal deletion of chromosome 10 implies that the neuraminidase gene is not located to 10pter→p15.1 (Takano *et al.*, 1993). In contrast, on basis of a family study of a patient with a combined neuraminidase/21-hydroxylase deficiency suffering from sialidosis and congenital adrenal hyperplasia, the neuraminidase gene has been reported to segregate with the HLA locus on chromosome 6 (Oohira *et al.*, 1985). The unequivocal chromosomal assignment of the neuraminidase locus probably will have to await the cloning of the neuraminidase cDNA or gene.

Identification of the neuraminidase polypeptide

To gain insight in the structure and function of the lysosomal neuraminidase in normal individuals and in patients with sialidosis and galactosialidosis, identification and further purification of the protein, finally leading to the cloning of the corresponding gene, is a prerequisite. Partial dissociation of the purified human placental complex and subsequent removal of the remaining ß-galactosidase/protective protein complex via ß-galactosidase specific affinity chromatography rendered a preparation containing the inactive neuraminidase polypeptide and consisting of polypeptides with molecular masses of 66 and 76 kDa. A polyclonal antibody against this fraction specifically precipitated neuraminidase activity as present in the complex, but a direct link between a specific protein and neuraminidase activity could not be made (Verheijen *et al.*, 1987). **Publication 1** describes the application of this anti-neuraminidase antibody for the identification of the lysosomal neuraminidase polypeptide from human placenta. Immunoblotting experiments with the purified ß-galactosidase/neuraminidase/protective protein complex revealed a single protein with a molecular mass of 66 kDa, which coprecipitated with neuraminidase activity in an immunetitration experiment using a human placental glycoprotein preparation. The 66 kDa protein was only detectable when electrophoresis was performed under non-reducing conditions, indicating that the polyclonal antibody is mainly directed against one or more conformation determinants. Moreover, specific removal of the 66 kDa protein from an unconcentrated glycoprotein preparation, containing all components of the complex in an unassociated form, prevented the generation of neuraminidase activity during concentration, demonstrating that this protein is indeed the human placental neuraminidase polypeptide (figure 7b).

The availability of active site specific affinity probes would greatly facilitate research on neuraminidases. Without requiring previous knowledge about the protein structure, they would allow direct molecular identification of neuraminidases by virtue of their enzymatic activity (see also chapter 3). As such probes were not available, we have developed a

photoreactive radioiodinatable affinity probe. As described in **publication 2**, an aryl azide group was introduced at C-atom 9 of the potent competitive neuraminidase inhibitor NeuAc2en. The resulting compound, ASA-NeuAc2en (5-N-acetyl-9-(4-azidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid), maintained the inhibitory characteristics of NeuAc2en, could be radioiodinated at high specific activity and was photoactivated upon irradiation with UV-light. When tested on commercially available preparations of *Clostridium perfringens* neuraminidase, radioiodinated ASA-NeuAc2en was shown to label a 72 kDa protein. The incorporation of label was proportional to the amount of neuraminidase activity used and decreased in the presence of the competitive inhibitor NeuAc2en. This demonstrates that labeling occurs specifically at the active site. After CNBr cleavage of the 72 kDa protein, only two labeled peptides were observed. Consequently, in addition to its use for the identification of neuraminidases, ASA-NeuAc2en is a powerful tool for the specific mapping of active site sequences within neuraminidase proteins. Recently, ASA-NeuAc2en has also been used for the labeling of *Salmonella typhimurium* LT2 neuraminidase (Warner et al., 1992).

After the successful labeling of a bacterial neuraminidase, we have applied the photoaffinity labeling technique to mammalian lysosomal neuraminidases. **Publication 3** describes the identification of a 55 kDa protein as the neuraminidase polypeptide in the bovine testis ß-galactosidase/neuraminidase/protective protein complex. The reduced molecular mass of the bovine testis neuraminidase polypeptide in comparison to its human placental counterpart might be due to tissue or species differences. Using the human placental complex, we were not able to obtain a specifically labeled protein, probably as a result of the low stability of the enzyme. In contrast, Warner *et al.* (1990) reported the specific labeling of a 61 kDa protein in the human placental complex. Although this protein could be identical to the 66 kDa neuraminidase polypeptide described in **publication 1**, it is not clear whether in the former report labeling is specific as all proteins display reduced incorporation of label in presence of the competitor NeuAc2en.

Several other proteins have been described as candidate neuraminidase polypeptides. The 46 kDa protein in the purified human placental complex reported by Hiraiwa and coworkers appeared to be N-acetyl-α-galactosaminidase (Hiraiwa *et al.*, 1988, 1991). In another report, the neuraminidase polypeptide was presumed to be a 60 kDa processing product of prosaposin (Potier *et al.*, 1990a). This protein was later identified as an IgG heavy chain protein (Hiraiwa *et al.*, 1991). Moreover, prosaposin deficient fibroblasts were shown to contain normal neuraminidase activity towards the methylumbelliferyl substrate, which definitely excluded a possible neuraminidase function for the prosaposin precursor (Paton *et al.*, 1992).

Proteins required for neuraminidase function

Formation of neuraminidase activity in human placental glycoprotein preparations is a two step procedure, requiring association of an inactive neuraminidase polypeptide with the protective protein into a high density ß-galactosidase complex (activation) and stabilization of the generated neuraminidase activity to prevent dissociation of the active enzyme upon dilution (Verheijen *et al.*, 1985, 1987). The mechanism of activation and stabilization of neuraminidase is further investigated in **publication 1**. Analogous to the removal of the 66 kDa neuraminidase polypeptide, as discussed in the former section, partial reduction of the protective protein with ß-mercaptoethanol or removal of ß-galactosidase precluded the formation of neuraminidase activity in human placental glycoprotein preparations during concentration (figure 7c and d). The three modified glycoprotein preparations complemented each other for generation of neuraminidase activity, demonstrating that *in vitro* all three

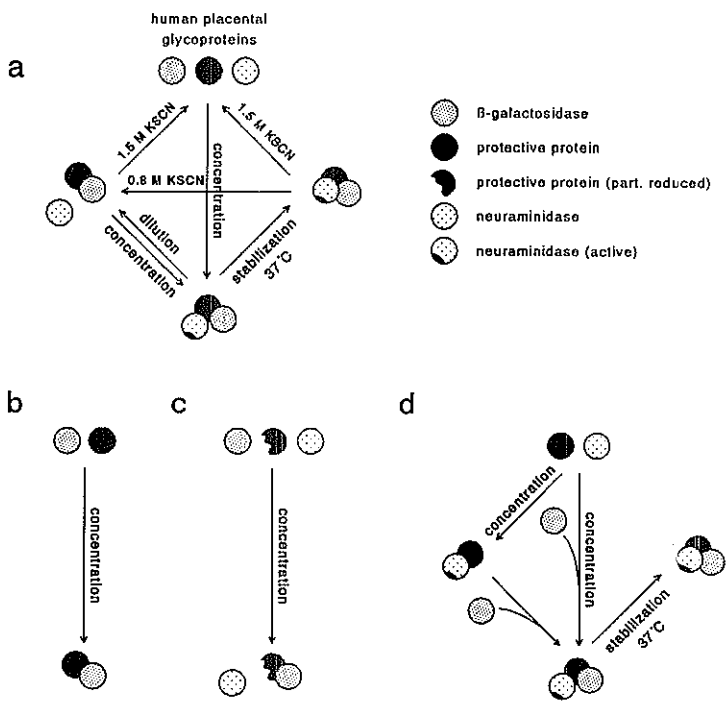

Figure 7. Association and dissociation of components of the ß-galactosidase/neuraminidase/protective protein complex from human placenta during *in vitro* manipulation of the complex. For simplicity, the stoichiometry of the components in the complex is ignored. Shown are the possible protein interactions in the presence of all three components (panel a), after immunoprecipitation of the neuraminidase polypeptide (panel b), after partial reduction of the protective protein (panel c) and after removal and addition of ß-galactosidase (panel d).

components of the complex are involved in neuraminidase function. The neuraminidase polypeptide and protective protein are indispensable components for the generation of neuraminidase activity. In the absence of ß-galactosidase however, near normal amounts of neuraminidase activity were detected after concentration of the glycoprotein preparation, but this activity could not be stabilized by subsequent incubation at 37 °C. This led us to conclude that *in vitro* ß-galactosidase is required for the stabilization rather than the activation of neuraminidase activity.

In contrast to the human placental ß-galactosidase/neuraminidase/protective protein complex, lysosomal neuraminidase in the bovine testis complex is rather stable and does not require generation of activity during purification (Verheijen *et al.*, 1982). In **publication 4** the bovine testis neuraminidase polypeptide is further investigated after partial disruption of the complex at increased pH (figure 8a). The dissociated protein fraction, containing the 55 kDa protein identified as the bovine testis neuraminidase polypeptide by means of photoaffinity labeling (**publication 3**), is able to restore the activation of neuraminidase activity in a human placental glycoprotein preparation lacking the 66 kDa neuraminidase polypeptide (figure 8b). Similarly, reconstitution with bovine testis glycoprotein or preparations containing the purified ß-galactosidase/protective protein multimer, rendered active neuraminidase (figure 8c). Activity in both the hybrid and bovine testis complexes could be stabilized by incubation at 37 °C, demonstrating that after dissociation from the complex, the bovine testis and human placenta neuraminidase polypeptide display a similar

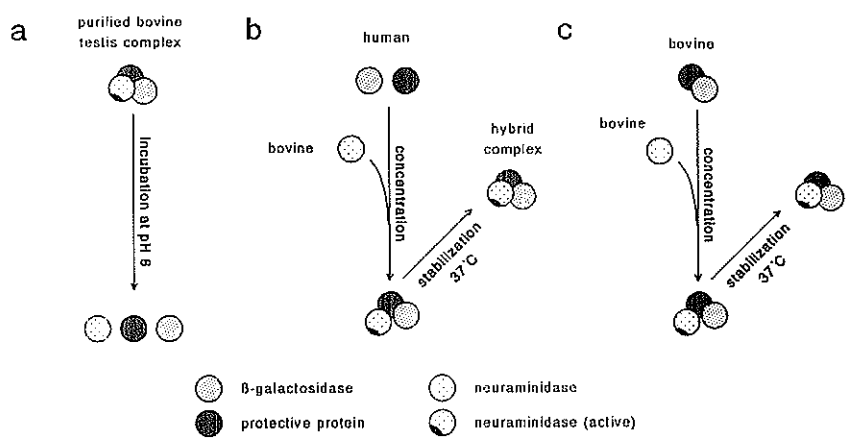

Figure 8. Dissociation of components of the ß-galactosidase/neuraminidase/protective protein complex from bovine testis (panel a) and *in vitro* reconstitution of neuraminidase activity in bovine/human hybrid (panel b) and bovine complex (panel c). For simplicity, the stoichiometry of the components in the complex is ignored.

behaviour with respect to activation and stabilization of neuraminidase activity.

In view of its protease activity, the protective protein has been suggested to act as a processing enzyme for ß-galactosidase and neuraminidase (Galjart *et al.*, 1988). However, *in vitro* mutagenized protective protein lacking the active site serine, is still able to correct the neuraminidase and ß-galactosidase deficiency in galactosialidosis fibroblasts, demonstrating that the protease function is distinct from the protective and subunit function towards ß-galactosidase and neuraminidase, respectively (Galjart *et al.*, 1991). Interestingly, the carboxypeptidase activity of human protective protein isolated from stably transformed chinese hamster ovary cells is stabilized by *sap*-proteins and in particular *sap*-B (Itoh *et al.*, 1993).

Information about the stoichiometry of the components in the complex is limited. In a rather speculative report the purified human placental ß-galactosidase/neuraminidase/ protective protein complex is calculated to consist of a core hexamer of ß-galactosidase and neuraminidase polypeptides, surrounded by five protective protein molecules (Potier *et al.*, 1990a). Recently, the same group was able to reconstitute purified ß-galactosidase and protective protein into a multimeric form containing the proteins in a 1:2 molar ratio (Pshezhetsky and Potier, 1993). As purified ß-galactosidase and protective protein were shown to form tetramers and dimers respectively, the native ß-galactosidase/protective protein complex is proposed to consist of four protective protein dimers, associated to one ß-galactosidase tetramer. The presence of protective protein dimers in the reconstituted as well as in the native complex was confirmed with cross-linking experiments.

Irrespective the exact composition of the complex, it appears that formation as well as stabilization of neuraminidase activity does not require a strict order of association events. The ß-galactosidase/neuraminidase/protective protein complex, bearing active neuraminidase, not only results from association of the separate components, but is also formed by binding of ß-galactosidase to neuraminidase polypeptide/protective protein units or binding of the neuraminidase polypeptide to ß-galactosidase/protective protein units (figures 7d and 8c; **publications 1 and 4**). These experiments also point to the presence of different binding domains for ß-galactosidase and the neuraminidase polypeptide on the protective protein. The binding site for the neuraminidase polypeptide can be destroyed by partial reduction of the protective protein.

The exact appearance and size of membrane associated active neuraminidase in the intact cell remains to be solved. Given the normal neuraminidase activity in ß-galactosidase mRNA$^-$ G_{M1}-gangliosidosis fibroblasts, association with ß-galactosidase is not essential for neuraminidase functioning *in vivo*. Nevertheless, the copurification of solubilized active neuraminidase from cultured fibroblasts and lymphocytes with ß-galactosidase, and lower stability of neuraminidase activity in fibroblast homogenates from patients with G_{M1}-

gangliosidosis argue in favour of the presence of ß-galactosidase in the catalytically active neuraminidase/protective protein multimer under normal conditions (Verheijen et al., 1983; Verheijen, van der Horst, unpublished results).

Purification and sequencing of the neuraminidase polypeptide

Cloning of the cDNA encoding the lysosomal neuraminidase polypeptide would make a contribution to the unraveling of the structure and function of the enzyme in the normal and disease state. Initial attempts to obtain NH_2-terminal amino acid sequences of the immuno-purified human placental lysosomal neuraminidase polypeptide or its 55 kDa bovine testis counterpart as present in the purified complex, were hampered by the presence of impurities and, as appeared later, the fact that the neuraminidase polypeptide is blocked. **Publication 4** describes the further purification and sequencing of the bovine testis neuraminidase polypeptide, after partial dissociation of the ß-galactosidase/neuraminidase/protective protein complex at increased pH. By employing a reconstitution assay for screening of the inactive lysosomal neuraminidase polypeptide, we have demonstrated that the protein resides in the unretained fraction of the ß-galactosidase specific affinity column, used to remove the ß-galactosidase and protective protein. In line with the photoaffinity labeling experiments described in **publication 3**, the unretained protein fraction was shown to contain increased amounts of a 55 kDa protein. Since the proteins in this fraction could not efficiently be further separated by any chromatographic procedure, the NH_2-terminal amino acid sequence of each protein was determined. Except for the 55 kDa protein, which appeared to be blocked, all proteins could be identified as known lysosomal proteins. On the basis of this result and the outcome of the photoaffinity labeling experiments, the 55 kDa protein appears to be the only candidate neuraminidase polypeptide left. Amino acid sequences of tryptic peptides of this protein did not show any homology to known proteins. On the basis of these amino acid sequences it is now possible to design oligonucleotide probes for the cloning of cDNA encoding the bovine lysosomal neuraminidase polypeptide, which in turn can be used for the cloning of the human cDNA.

Once the cDNA encoding the lysosomal neuraminidase polypeptide has been cloned, the active site of the enzyme can be explored in detail. To facilitate the design of *in vitro* mutagenesis experiments for the identification of active site residues, we have investigated the role of certain amino acids in the catalytic mechanism of neuraminidase with amino acid modifying agents. In **publication 4** the active site of lysosomal neuraminidase is shown to harbour histidine as well as acidic amino acid residues, suggesting a similar catalytic mechanism as observed for the influenza virus neuraminidase (see chapter 3). This finding may also help the identification of point mutations affecting the kinetic parameters of the

enzyme in patients with sialidosis.

Degradation of gangliosides by lysosomal neuraminidase

With the establishment of the ganglioside degrading capacity of lysosomal neuraminidase in the presence of detergents (Hiraiwa *et al.*, 1987, 1988; Lieser *et al.*, 1989), an important question was how those substrates would be degraded *in vivo*. From the results described in **publication 5** it is clear that sphingolipid activator protein B (*sap*-B, sulfatide activator, see also chapter 3) mediates the degradation of gangliosides by lysosomal neuraminidase. In the absence of detergents, purified human liver *sap*-B stimulates the degradation of gangliosides G_{M3}, G_{T1b} and G_{D1a} by the purified human placental ß-galactosidase/neuraminidase/protective protein complex, whereas ganglioside G_{D1b} is hardly cleaved. From the resistance of the α(2-8) disialo linkage towards the action of lysosomal neuraminidase, it appears that *in vivo* the conversion of ganglioside G_{D3} to G_{M3} is the bottle-neck in the degradation of the b-series gangliosides. Since ganglioside G_{D1b} is readily cleaved by lysosomal neuraminidase when the activator protein is substituted for taurodeoxycholate, *sap*-B is proposed to influence the substrate specificity of the enzyme towards gangliosides. In view of the observed stabilization of purified protective protein/carboxypeptidase by *sap* proteins, and in particular *sap*-B (Itoh *et al.*, 1993), it may well be possible that degradation of gangliosides by lysosomal neuraminidase involves the binding of the activator/lipid complex to the protective protein subunit.

Occasionally, activator protein mediated stimulation of sphingolipid degradation by lysosomal enzymes *in vitro* have not been indicative for the situation *in vivo*. For example, *sap*-B has clearly been shown to facilitate the *in vitro* degradation of ganglioside G_{M1} by ß-galactosidase, but the absence of ganglioside G_{M1} storage in patients with a *sap*-B or prosaposin deficiency as well as the normal turnover of ganglioside G_{M1} in activator protein deficient fibroblasts throw doubts upon a similar role *in vivo* (Li and Li, 1976; Vogel *et al.*, 1987; Wenger *et al.*, 1989; Schmid *et al.*, 1992). However, accumulation of ganglioside G_{M3}, was clearly observed in *sap*-B and prosaposin deficient fibroblasts loaded with ganglioside G_{M1}, whereas, amongst other sphingolipids, increased amounts of ganglioside G_{M3} have been reported in the liver of a fetus affected with a prosaposin deficiency (Smíd *et al.*, 1991; Schmid *et al.*, 1992). Taken together, these data demonstrate that *in vitro* and *in vivo*, *sap*-B is required for the degradation of gangliosides by the lysosomal neuraminidase, at least in non-neuronal tissues. In brain tissue, despite the high concentration and turnover of gangliosides, patients with sialidosis and galactosialidosis accumulate relatively small amounts of ganglioside (Sakuraba *et al.*, 1983; Ulrich-Bott *et al.*, 1987; Yoshino *et al.*, 1990). As hypothesized in **publication 5**, small amounts of endocytosed plasma membrane

neuraminidase, having an acidic pH optimum and abundantly present in the brain, may compensate for the missing lysosomal neuraminidase and prevent or slow down ganglioside storage in this tissue.

Future perspectives

With the identification of the lysosomal neuraminidase polypeptide and determination of amino acid sequences, the cloning of the cDNA encoding this protein is within reach. Once available, the predicted amino acid sequence of the lysosomal neuraminidase polypeptide will disclose the structural features of the protein, whereas comparison of sequences might reveal sequence similarities with other neuraminidases or lysosomal proteins. A possible occurrence of one ore more transmembrane domains will answer the question whether the neuraminidase polypeptide is an integral membrane protein.

The experiments described in this thesis deal with a soluble form of neuraminidase activity, present in the purified human placental and bovine testicular ß-galactosidase/ neuraminidase/protective protein complex. Considering the membrane bound state of the neuraminidase in the living cell, the cloning of its cDNA will enable the biochemical characterization of normal as well as *in vitro* mutagenized lysosomal neuraminidase under conditions, mimicking the in vivo situation. After overexpression of the cDNA in stably transformed CHO cells, reconstitution experiments may be performed with purified lysosomes or lysosomal membranes, containing the lysosomal neuraminidase polypeptide in its natural environment, and purified protective protein and ß-galactosidase. Moreover, this system may serve to study the mode of action of *sap*-B in determining the substrate specificity of lysosomal neuraminidase towards ganglioside substrates.

Cloning of the cDNA will also allow the examination of the biosynthetic pathway of the neuraminidase polypeptide. Analogous to the situation for acid phosphatase, a soluble lysosomal hydrolase that is synthesized and transported to the lysosome as an integral membrane protein (Waheed et al., 1988; Gottschalk et al., 1989), unequivocal and detailed knowledge about the synthesis, lysosomal routing and processing of the lysosomal neuraminidase polypeptide might only be obtained via expression studies in COS cells. The same system can be used to study the interaction between the neuraminidase polypeptide and the protective protein and ß-galactosidase *in vivo*. This approach will also address the question whether, in addition to its subunit function for neuraminidase, the protective protein prevents the intralysosomal degradation of the neuraminidase polypeptide. Finally, cloning of the cDNA encoding the lysosomal neuraminidase polypeptide will allow the molecular analysis of mutations leading to the different clinical forms of sialidosis.

References

Cheetham, P.S.J. and Dance, N.E. (1976) The separation and characterization of the methylumbelliferyl ß-galactosidases of human liver. *Biochem. J.* **157**:189-195

d'Azzo, A., Hoogeveen, A.T., Reuser, A.J., Robinson, D. and Galjaard, H. (1982) Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. USA* **79**:4535-4539

d'Azzo, A., Bonten, E., Willemsen, R. and Rudenko, G. (1993) Biogenesis of human protective protein/cathepsin A in insect cells: large scale production for 3D structure analysis. Abstractbook of the Second International Duodecim Symposium "Molecular biology of lysosomal diseases", Helsinki, Finland, p. 31

Frost, R.G., Holmes, E.W., Norden, A.G.W. and O'Brien, J.S. (1978) Characterization of human liver acid ß-D-galactosidases A2 and A3. *Biochem. J.* **175**:181-188

Galjaard, H., Hoogeveen, A.T., Keijzer, W., de Wit-Verbeek, H.A., Reuser, A.J.J., Ho, M.W. and Robinson, D. (1975) Genetic heterogeneity in GM1-gangliosidosis. *Nature* **257**:60-62

Galjart, N.J., Gillemans, N., Harris, A., van der Horst, G.T.J., Verheijen, F.W., Galjaard, H. and d'Azzo, A. (1988) Expression of cDNA encoding the human "protective protein" associated with lysosomal ß-galactosidase and neuraminidase: homology to yeast proteases. *Cell* **54**:755-764

Galjart, N.J., Gillemans, N., Meijer, D. and d'Azzo, A. (1990) Mouse protective protein. cDNA cloning, sequence comparison and expression. *J. Biol. Chem.* **265**:4678-4684

Galjart, N.J. (1991) The protective protein: a multifunctional lysosomal enzyme. Doctoral thesis, Erasmus University Rotterdam

Galjart, N.J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E.J. and d'Azzo, A. (1991) Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. *J. Biol. Chem.* **266**:14754-14762

Gottschalk, S., Waheed, A., Schmidt, B., Laidler, P. and von Figura, K. (1989) Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. *EMBO J.* **8**:3215-3219

Hinek, A., Rabinovitch, M., Keeley, F., Okamura-Oho, Y. and Callahan, J. (1993) The 67 kDa elastin/laminin binding protein is related to an enzymatically inactive, alternatively spliced form of ß-galactosidase. *J. Clin. Invest.* **91**:1198-1205

Hiraiwa, M., Uda, Y., Nishizawa, M. and Miyatake, T. (1987) Human placental sialidase: partial purification and characterization. *J. Biochem.* **101**:1273-1279

Hiraiwa, M., Nishizawa, M., Uda, Y., Nakajima, T. and Miyatake, T. (1988) Human placental sialidase: further purification and characterization. *J. Biochem.* **103**:86-90

Hiraiwa, M., Uda. Y., Tsuji, S., Miyatake, T., Martin, B.M., Tayama, M., O'Brien, J.S. and Kishimoto, Y. (1991) Human placental sialidase complex. Characterization of the 60 kDa protein that cross-reacts with anti-saposin antibodies. *Biochem. Biophys. Res. Commun.* **177**:1211-1216

Hiraiwa, M., Soeda, S., Kishimoto, Y. and O'Brien, J.S. (1992) Binding and transport of gangliosides by prosaposin. *Proc. Natl. Acad. Sci. USA* **89**:11254-11258

Hoeksema, J., van Diggelen, O.P. and Galjaard, H. (1979) Intergenic complementation after fusion of fibroblasts from different patients with ß-galactosidase deficiency. *Biochim. Biophys. Acta* **566**:72-79

Hoogeveen, A.T., Verheijen, F.W., d'Azzo, A. and Galjaard, H. (1980) Genetic heterogeneity in human neuraminidase deficiency. *Nature* **285**:500-502

Hoogeveen, A., d'Azzo, A., Brossmer, R. and Galjaard, H. (1981) Correction of combined ß-galactosidase/

neuraminidase deficiency in human fibroblasts. *Biochem. Biophys. Res. Commun.* **103**:292-300

Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1983) The relation between human lysosomal ß-galactosidase and its protective protein. *J. Biol. Chem.* **258**:12143-12146

Hoogeveen, A.T. (1987) ß-Galactosidase in normal and mutant human cells. Doctoral thesis, Erasmus University Rotterdam

Itoh, K., Takiyama, N., Kondoh, K., Sano, A., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) Purification and characterization of human lysosomal protective protein expressed in stably transformed chinese hamster ovary cells. *J. Biol. Chem.* **268**:1180-1186

Jackman, H.L., Tan, F., Tamei, H., Beurling-Harbury, C., Li, X.Y., Skidgel, R.A. and Erdös, E.G. (1990) A peptidase in human platelets that deamidates tachykinins. Probable identity with the lysosomal "protective protein". *J. Biol. Chem.* **265**:11265-11272

Jackman, H.L., Morris, P.W., Deddish, P.A., Skidgel, R.A. and Erdös, E.G. (1992) Inactivation of endothelin I by deamidase (Lysosomal protective protein). *J. Biol. Chem.* **267**:2872-2875

Kase, R., Itoh, K., Takiyama, N., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) Galactosialidosis: simultaneous deficiency of esterase, carboxy-terminal deamidase and acidic carboxypeptidase activity. *Biochem. Biophys. Res. Commun.* **172**:1175-1179

Li, S.C. and Li, Y.T. (1976) An activator stimulating the enzymic hydrolysis of sphingoglycolipids. *J. Biol. Chem.* **251**:1159-1163

Morreau, H., Galjart, N.J., Gillemans, N., Willemsen, R., van der Horst, G.T.J. and d'Azzo, A. (1989) Alternative splicing of ß-galactosidase mRNA generates the classic lysosomal enzyme and a ß-galactosidase related protein. *J. Biol. Chem.* **264**:20655-20663

Morreau, H., Bonten, E., Zhou, X.Y. and d'Azzo, A. (1991) Organization of the gene encoding human lysosomal ß-galactosidase. *DNA Cell Biol.* **10**:495-504

Morreau, H. (1992) Structural and functional analysis of lysosomal ß-galactosidase and its relation to the protective protein. Doctoral thesis, Erasmus University Rotterdam

Morreau, H., Galjart, N.J., Willemsen, R., Gillemans, N., Zhou, X.Y. and d'Azzo, A. (1992) Human lysosomal protective protein: Glycosylation, intracellular transport and association with ß-galactosidase in the endoplasmic reticulum. *J. Biol. Chem.* **267**:17949-17956

Mueller, O.T., Henrey, W.M., Haley, L.L., Byers, M.G., Eddy, R.L. and Shows, T.B. (1986) Sialidosis and galactosialidosis: chromosomal assignment of two genes associated with neuraminidase-deficiency disorders. *Proc. Natl. Acad. Sci. USA* **83**:1817-1821

Nanba, E. and Suzuki, K. (1990) Molecular cloning of mouse acid ß-galactosidase cDNA: sequence, expression of catalytic activity and comparison with the human enzyme. *Biochem. Biophys. Res. Commun.* **173**:141-148

Nanba, E. and Suzuki, K. (1991) Organization of the mouse acid ß-galactosidase gene. *Biochem. Biophys. Res. Commun.* **178**:158-164

Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974) Ganglioside G_{M1} ß-galactosidase A: purification and studies of the enzyme from human liver. *J. Biol. Chem.* **249**:7969-7976

Oohira, T., Nagata, N., Akaboshi, I., Matsuda, I. and Naito, S. (1985) The infantile form of sialidosis type II associated with congenital adrenal hyperplasia: possible linkage between HLA and the neuraminidase deficiency gene. *Hum. Genet.* **70**:341-343

Oshima, A., Tsuji, A., Nagao, Y., Sakuraba, H. and Suzuki, Y. (1988) Cloning, sequencing and expression of cDNA for human ß-galactosidase. *Biochem. Biophys. Res. Commun.* **157**:238-244

Palmeri, S., Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1986) Galactosialidosis: molecular heterogeneity among different clinical phemotypes. *Am. J. Hum. Genet.* **38**:137-148

Paton, B.C., Schmid, B., Kustermann-Kuhn, B., Poulos, A. and Harzer, K. (1992) Additional biochemical findings in a patient and fetal sibling with a genetic defect in the sphingolipid activator protein (SAP) precursor, prosaposin. *Biochem. J.* **285**:481-488

Potier, M. (1988) Structure of the lysosomal sphingolipid activator protein 1 by homology with influenza virus neuraminidase. *Biochem. Biophys. Res. Commun.* **155**:32-37

Potier, M., Lamontagne, S., Michaud, L. and Tranchemontagne, J. (1990a) Human neuraminidase is a 60 kDa processing product of prosaposin. *Biochem. Biophys. Res. Commun.* **173**:449-456

Potier, M., Michaud, L., Tranchemontagne, J. and Thauvette, L. (1990b) Structure of the lysosomal neuraminidase-ß-galactosidase-carboxypeptidase multienzymic complex. *Biochem. J.* **267**:197-202

Pshezhetsy, A.V. and Potier, M. (1993) In vitro reconstitution of human lysosomal carboxypeptidase and ß-galactosidase complex. Abstract Second International Duodecim Symposium "Molecular biology of lysosomal diseases" (Helsinki, Finland), p. 87

Sakuraba, H., Suzuki, Y., Akagi, M., Sakai, M. and Amano, N. (1983) ß-Galactosidase-neuraminidase deficiency (galactosialidosis): clinical, pathological, and enzymatic studies in a postmortem case. *Ann. Neurol.* **13**:497-503

Scheibe, R., Hein, K. and Wenzel, K.W. (1990) Lysosomal ß-galactosidase from rat liver: purification, molecular forms and association with neuraminidase. *Biomed. Biochim. Acta* **49**:547-556

Schmid, B., Paton, B.C., Sandhoff, K. and Harzer, K. (1992) Metabolism of GM1 ganglioside in cultured skin fibroblasts: anomalies in gangliosidoses, sialidoses, and sphingolipid activator protein (SAP, saposin) 1 and prosaposin deficient disorders. *Hum. Genet.* **89**:513-518

Shows, T.B., Scrafford-Wolff, L.R., Brown, J.A. and Meisler, M. (1979) GM1 Gangliosidosis: Chromosome 3 assignment of the ß-galactosidase A gene (Beta-GAL A). *Somat. Cell. Genet.* **5**:147-158

Smíd, F., Bradová, V. Cantz, M. and Harzer, K. (1991) Combined sphingolipidosis including lactosylceramidosis in sphingolipid activator protein (SAP) deficiency. Results of lipid analysis of affected fetus. Abstract 8th ESGLD Workshop (Annecy, France), p. 72

Taguchi, S., Kouyama, H. and Yago, N. (1981) Reversible aggregation and stability of lysosomal acid ß-galactosidase from porcine adrenal cortex. *J. Biochem.* **89**:483-489

Takano, T., Horigome, H. and Shibata, S. (1993) Exclusion map of the gene for neuraminidase from 10(pter→p15.1). *Clin. Genet.* **43**:166-167

Tranchemontagne, J., Michaud, L. and Potier, M. (1990) Deficient lysosomal carboxypeptidase activity in galactosialidosis. *Biochem. Biophys. Res. Commun.* **168**:22-29

Ulrich-Bott, B., Klem, B., Kaiser, R., Spranger, J. and Cantz, M. (1987) Lysosomal sialidase deficiency: increased ganglioside content in autopsy tissues of a sialidosis patient. *Enzyme* **38**:262-266

van Diggelen, O.P., Schram, A.W., Sinnott, M.L., Smith, P.J., Robinson, D. and Galjaard, H. (1981) Turnover of ß-galactosidase in fibroblasts from patients with genetically different types of ß-galactosidase deficiency. *Biochem. J.* **200**:143-151

van Diggelen, O.P., Hoogeveen, A.T., Smith, P.J., Reuser, A.J. and Galjaard, H. (1982) Enhanced proteolytic degradation of normal ß-galactosidase in the lysosomal storage disease with combined ß-galactosidase and neuraminidase deficiency. *Biochim. Biophys. Acta* **703**:69-76

Verheijen, F.W., Brossmer, R. and Galjaard, H. (1982) Purification of acid ß-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. *Biochem. Biophys. Res. Commun.* **108**:868-875

Verheijen, F.W., Janse, H.C., van Diggelen, O.P., Bakker, H.D., Loonen, M.C.B., Durand, P. and Galjaard, H. (1983) Two genetically different MU-NANA neuraminidases in human leucocytes. *Biochem. Biophys.*

Res. Commun. **117**:470-478

Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985) Human placental neuraminidase. Activation, stabilization and association with ß-galactosidase and its 'protective protein'. *Eur. J. Biochem.* **149**:315-322

Verheijen, F.W. (1986) Lysosomal neuraminidase in human genetic disorders. Doctoral thesis, Erasmus University Rotterdam

Verheijen, F.W., Palmeri, S. and Galjaard, H. (1987) Purification and partial characterization of lysosomal neuraminidase from human placenta. *Eur. J. Biochem.* **162**:63-67

Vogel, A., Fürst, W., Abo-Hashish, M.A., Lee-Vaupel, M., Conzelmann, E. and Sandhoff, K. (1987) Identity of the activator proteins for the enzymatic hydrolysis of sulfatide, ganglioside G_{M1}, and globotriaosylceramide. *Arch. Biochem. Biophys.* **259**:627-638

Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H. and von Figura, K. (1988) Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. *EMBO J.* **7**:2351-2358

Warner, T.G., Louie, A. and Potier, M. (1990) Photolabeling of the α-neuraminidase/ß-galactosidase complex from human placenta with a photoreactive neuraminidase inhibitor. *Biochem. Biophys. Res. Commun.* **173**:13-19

Warner, T.G., Harris, R., McDowell, R. and Vimr, E.R. (1992) Photolabelling of Salmonella typhimurium LT2 sialidase. *Biochem. J.* **285**:957-964

Wenger, D.A., DeGala, G., Williams, C., Taylor, H.A., Stevenson, R.E., Pruitt, J.R., Miller, J., Garen, P.D. and Balentine, J.D. (1989) Clinical, pathological and biochemical studies on an infantile case of sulfatide/GM1 activator protein deficiency. *Am. J. Med. Genet.* **33**:255-265

Wiegant, J., Galjart, N.J., Raap, A.K. and d'Azzo, A. (1991) The gene encoding human protective protein (PPGB) is on chromosome 20. *Genomics* **10**:345-349

Willemsen, R., Hoogeveen, A.T., Sips, H.J., van Dongen, J.M. and Galjaard, H. (1986) Immunoelectron microscopical localization of lysosomal ß-galactosidase and its precursor forms in normal and mutant human fibroblasts. *Eur. J. Cell Biol.* **40**:9-15

Yamamoto, Y. and Nishimura, K. (1980) pH-Dependent association-dissociation of GM1-ß-galactosidase purified from porcine spleen. *J. Biochem.* **88**:705-713

Yamamoto, Y., Fujie, M. and Nishimura, K. (1982) The interrelation between high- and low-molecular-weight forms of GM1-ß-galactosidase purified from porcine spleen. *J. Biochem.* **92**:13-21

Yamamoto, Y. and Nishimura, K. (1986) Aggregation-dissociation and stability of acid ß-galactosidase purified from porcine spleen. *Int. J. Biochem.* **18**:327-335

Yamamoto, Y. and Nishimura, K. (1987) Copurification and separation of ß-galactosidase and sialidase from porcine testis. *Int. J. Biochem.* **19**:435-442

Yamamoto, Y., Hake, C.A., Martin, B.M., Kretz, K.A., Ahern-Rindell, A.J., Naylor, S.L., Mudd, M. and O'Brien, J.S. (1990) Isolation, characterization and mapping of the human acid ß-galactosidase cDNA. *DNA Cell Biol.* **9**:119-127

Yoshino, H., Miyashita, K., Miyatani, N., Ariga, T., Hashimoto, Y., Tsuji, S., Oyanagi, K., Ohama, E., Ikuta, F., Suzuki, A. and Miyatake, T. (1990) Abnormal glycosphingolipid metabolism in the nervous system of galactosialidosis. *J. Neurol. Sci.* **97**:53-65

Zhou, X.Y., Galjart, N.J., Willemsen, R., Gillemans, N., Galjaard, H. and d'Azzo, A. (1991) A mutation in a mild form of galactosialidosis impairs dimerization of the protective protein and renders it unstable. *EMBO J.* **10**:4041-4048

Chapter 5

PUBLICATIONS

Publication 1

Identification and *in vitro* reconstitution of
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Identification and *in Vitro* Reconstitution of Lysosomal Neuraminidase from Human Placenta*

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Lysosomal neuraminidase from human placenta has been obtained in its active form by association of an inactive neuraminidase polypeptide with β-galactosidase and the protective protein. Using a specific antiserum, we have now identified a 66-kDa protein as the inactive neuraminidase polypeptide. It is specifically recognized on immunoblots only in its nonreduced state, and it coprecipitates with neuraminidase activity. The 66-kDa polypeptide is substantially glycosylated (38-kDa protein core with 7–14 *N*-linked oligosaccharide chains), a feature characteristic of lysosomal integral membrane proteins. Specific removal of the 66-kDa neuraminidase polypeptide from glycoprotein preparations prevents the generation of neuraminidase activity. Removal of β-galactosidase or destruction of the protective protein also hinders the formation of active neuraminidase. Reconstitution of neuraminidase activity is observed after mixing glycoprotein preparations, depleted in different components of the β-galactosidase-neuraminidase-protective protein complex, indicating that all three components of the complex are required for neuramindase activity. Association of the neuraminidase polypeptide and the protective protein generates unstable neuraminidase activity, whereas association with β-galactosidase is required for stability.

Lysosomal neuraminidase (sialidase, EC 3.2.1.18) is an acid hydrolase that catalyzes the removal of terminal α-ketosidically linked neuraminic acid from oligosaccharides, glycolipids, and glycoproteins (1). In man, the enzyme is deficient in a number of inherited metabolic diseases. In sialidosis, a single neuraminidase deficiency exists (2–4). At the molecular level, the mutation is still unknown. In galactosialidosis, both neuraminidase and β-galactosidase are deficient (5, 6). The defect responsible for this disease is a deficiency of the "protective protein" (7). This protein promotes the multimerization of β-galactosidase, thereby preventing the rapid intralysosomal degradation of the latter (8). It is also required for the activity of neuraminidase as demonstrated by the ability of endocytosed normal protective protein to restore neuraminidase and β-galactosidase activity in galactosialidosis fibroblasts (9). Recently, the cDNA coding for the protective protein has been cloned, and sequencing data revealed a putative role for this protein as a processing enzyme (10).

In contrast to viral and bacterial neuraminidases, purification of the enzyme from mammalian sources has been hampered by the membrane-bound, labile character of this hydrolase (11–14). However, in bovine testis, a soluble, stable form of lysosomal neuraminidase is found (15). The active enzyme is present in a high molecular weight complex with β-galactosidase and the protective protein and can be isolated as such using a substrate affinity chromatography column for β-galactosidase (15). Similarly, neuraminidase can be isolated from human placenta, although in this case, neuraminidase activity has to be generated by concentration of a glycoprotein preparation. During concentration, a complex of unknown inactive neuraminidase polypeptide(s) with the protective protein and β-galactosidase is formed (16). Partial disruption of the human placental β-galactosidase-neuraminidase-protective protein complex with KSCN results in the specific loss of neuraminidase activity and is accompanied by the dissociation of 66- and 76-kDa polypeptides from the complex (17). At least one of these polypeptides represents inactive neuraminidase since antibodies raised against the dissociated protein fraction precipitate the active enzyme present in the complex (17).

In this study, we have used these antibodies to identify and characterize the inactive neuraminidase polypeptide. In addition, we describe a method to activate and stabilize placental neuraminidase on a small scale. With this method, reconstitution experiments have been performed to investigate the role of all components of the β-galactosidase-neuraminidase-protective protein complex with respect to neuraminidase activity.

EXPERIMENTAL PROCEDURES

Purification Procedures—Human placental glycoproteins were isolated by concanavalin A-Sepharose (Pharmacia LKB Biotechnology Inc.) chromatography and concentrated to 5 mg of protein/ml (16). This protein preparation is referred to as unconcentrated glycoproteins. To obtain active, stable neuraminidase, unconcentrated glycoproteins were concentrated to 60 mg of protein/ml and incubated for 90 min at 37 °C as described earlier (16).

The β-galactosidase-neuraminidase-protective protein complex was purified by *p*-aminophenylthiogalactoside-CH-Sepharose chromatography of concentrated glycoproteins containing active, stable neuraminidase as described (16).

β-Galactosidase was purified by *p*-aminophenylthiogalactoside-CH-Sepharose chromatography of unconcentrated glycoproteins containing unassociated β-galactosidase (18).

SDS[1]-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed on 10% separating gels with a 5% stacking gel according to Laemmli (19). Protein samples were applied either directly or after reduction with 25 mM dithiothreitol. After electrophoresis, proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose as described by Towbin *et al.* (20).

Immunological Procedures—Immunological detection of proteins

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[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

on immunoblots was performed as follows. After incubation for 16 h in phosphate-buffered saline, 0.05% Tween 20 (PBST buffer) containing 2% bovine serum albumin, filters were incubated for 6 h with 1000-fold diluted antibodies against the inactive neuraminidase polypeptide (17), β-galactosidase, or the 32-kDa polypeptide of the protective protein (10). Filters were washed with PBST buffer and incubated with 1 μCi of ^{125}I-protein A (Amersham Corp.) for 2 h. All steps were carried out at room temperature. Proteins were visualized by autoradiography.

Immunotitration of active neuraminidase in a human placental glycoprotein preparation was performed as described (16) except that protein A-Sepharose was replaced by immunoprecipitin (Bethesda Research Laboratories).

For *in vitro* reconstitution experiments, the same method was applied to create glycoprotein preparations specifically depleted in one of the components of the β-galactosidase-neuraminidase-protective protein complex. Unconcentrated glycoproteins (200–1000 μl, 5 mg of protein/ml) were incubated for 90 min at 20 °C with excess amounts of IgG specific for either inactive neuraminidase polypeptide or native β-galactosicase. Immune complexes were removed with excess immunoprecipitin.

Microactivation of Neuraminidase—Microactivation of neuraminidase was performed in 20 mM sodium acetate, 100 mM NaCl (pH 5.2). When glycoprotein preparations were not buffered as such, buffer was first exchanged by rapid gel filtration through a Sephadex G-50 medium spin column (21) equilibrated with 20 mM sodium acetate, 100 mM NaCl (pH 5.2).

Unconcentrated glycoproteins (500 μg in 100–300 μl), untreated or depleted in a specific component of the β-galactosidase-neuraminidase-protective protein complex, were concentrated by ammonium sulfate precipitation (55% saturation). Pellets were dissolved in 20 mM sodium acetate, 100 mM NaCl (pH 5.2) in a final volume of 20 μl. Remaining traces of ammonium sulfate were removed by rapid gel filtration through a 350-μl Sephadex G-50 medium spin column (21) equilibrated with 20 mM sodium acetate, 100 mM NaCl (pH 5.2). After stabilization of neuraminidase activity for 90 min at 37 °C, neuraminidase and β-galactosidase activities were measured.

Reconstitution of Neuraminidase Activity—Different unconcentrated glycoprotein preparations, previously shown to be deficient in neuraminidase activity after microactivation, were mixed (1:1). After microactivation and stabilization of 500 μg of the mixtures, neuraminidase, and β-galactosidase activities were measured.

Analogously, purified β-galactosidase was added to β-galactosidase-depleted, unconcentrated glycoprotein preparations prior to microactivation of neuraminidase.

Sucrose Density Gradient Centrifugation—Sucrose density gradient centrifugation was performed using linear 20–40% (w/v) sucrose gradients in 20 mM sodium acetate, 100 mM NaCl (pH 5.2) as described (16).

Miscellaneous—Neuraminidase and β-galactosidase activities were measured with the corresponding 4-methylumbelliferyl substrates (Koch-Light, Haverhill, United Kingdom) as described (22). Activities are expressed in milliunits. One unit of activity is defined as the amount of enzyme releasing 1 μmol of 4-methylumbelliferone/min at 37 °C. Protein concentrations were determined according to Lowry *et al.* (23). The purified β-galactosidase-neuraminidase-protective protein complex was deglycosylated with glycopeptidase F as described by the manufacturer (Boehringer Mannheim).

RESULTS

Identification of Inactive Neuraminidase Polypeptide—The purified β-galactosidase-neuraminidase-protective protein complex was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 1*A*, *left*). Protein staining reveals a 76-kDa protein, the 64-kDa β-galactosidase protein, and the 32- and 20-kDa polypeptides of the protective protein. After immunoblotting, none of these proteins is recognized by antibodies raised against the inactive neuraminidase polypeptide used in a 1000-fold dilution. In contrast, after electrophoresis under nonreducing conditions, only the 64-kDa β-galactosidase and the 54-kDa protective protein are detected after protein staining, whereas the same antibodies now recognize a protein of 66 kDa (Fig. 1*A*, *right*). To demonstrate that indeed two different proteins co-migrate at a position around 64 kDa, the complex was deglycosylated with glycopeptidase F and electrophoresed under reducing and nonreducing conditions. On immunoblots, neuraminidase antibodies recognize a 38-kDa protein in nonreduced complex, whereas in reduced complex, this protein is no longer observed (Fig. 1*B*, *left*). In contrast, β-galactosidase antibodies recognize a 53-kDa protein in nonreduced complex; and under reducing conditions, a 57-kDa protein is visible (Fig. 1*B*, *right*). From these data, we conclude that neuraminidase antibodies specifically recognize a heavily glycosylated 66-kDa protein different from β-galactosidase. This protein must be the 66-kDa protein present in the inactive neuraminidase polypeptide preparation used to raise antibodies (17). It is only recognized by the antiserum in its nonreduced state.

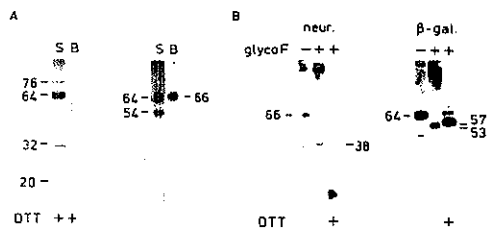

FIG. 1. **Immunoblot analysis of human placental neuraminidase.** *A*, 10 μg of purified complex was separated on a 10% SDS-polyacrylamide gel with or without reduction by dithiothreitol (*DTT*). Proteins were either stained with Coomassie Blue (*lanes S*) or blotted to nitrocellulose and treated with 1000-fold diluted neuraminidase-specific antibodies (*lanes B*). *B*, 10 μg of purified complex, with or without prior treatment with glycopeptidase F (*glycoF*), was separated on a 10% SDS-polyacrylamide gel under reducing (*DTT+*) or nonreducing (*DTT−*) conditions. Neuraminidase (*neur.*) and β-galactosidase (*β-gal.*) were visualized on immunoblots using antibodies specific for these proteins. Molecular masses are given in kilodaltons.

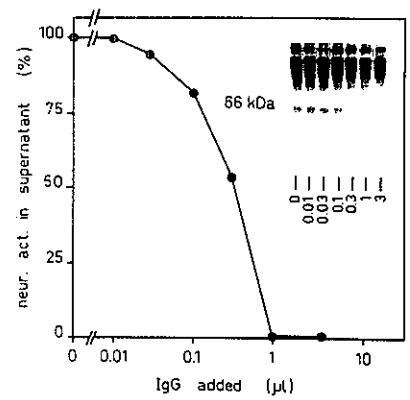

FIG. 2. **Immunotitration of activated and stabilized human placental neuraminidase.** Activated, stabilized neuraminidase was immunoprecipitated from a human glycoprotein preparation with increasing amounts of an IgG preparation prepared from neuraminidase-specific antibodies. Neuraminidase activity was measured in the supernatants. *Inset*, immunoblot analysis of supernatants using neuraminidase-specific antibodies.

Since neuraminidase-specific antibodies precipitate active neuraminidase present in the complex (17), we have performed a combined immunotitration/immunoblotting experiment to investigate whether the 66-kDa protein coprecipitates with activity. As shown in Fig. 2, the loss of neuraminidase activity from the supernatant coincides with the

disappearance of this 66-kDa protein. This indicates that neuraminidase activity is precipitated via the 66-kDa protein.

To demonstrate that the 66-kDa protein is directly involved in the generation of neuraminidase activity, we have investigated whether specific removal of this protein from unconcentrated glycoproteins affects activation of neuraminidase during concentration. In the unconcentrated glycoprotein preparation, all components of the β-galactosidase-neuraminidase-protective protein complex are present in a nonassociated form as demonstrated by the absence of multimeric β-galactosidase after sucrose density gradient centrifugation (data not shown). From this preparation (referred to as GP), the free 66-kDa protein was completely removed with neuraminidase antibodies (GP^{-n}) as demonstrated by immunoblot analysis of nonreduced proteins (Fig. 3*A*). Consistent with the data presented in Fig. 1, no differences are observed on immunoblots of reduced GP and GP^{-n}. Since the limited availability of antibodies does not allow the use of large quantities of glycoproteins, we have developed a microactivation system for the concentration of small volumes (100–300 μl). Ammonium sulfate precipitation was a convenient substitute for the ultrafiltration method used thus far (16, 17). After microactivation and stabilization of neuraminidase, enzyme activities were measured. In absence of the 66-kDa protein, neuraminidase activity is no longer generated, whereas β-galactosidase activity remains unaltered (Fig. 3*B*). From these data, we conclude that the 66-kDa protein is indispensable for the generation of neuraminidase activity and thus represents the inactive neuraminidase polypeptide.

In Vitro Reconstitution of Neuraminidase Activity—Microactivation of pretreated glycoprotein preparations was used to study the role of all the proteins of the β-galactosidase-neuraminidase-protective protein complex in neuraminidase activity (Fig. 4). The procedure is based on the principle that in the absence of an essential component, neuraminidase activity is not generated. Reconstitution of neuraminidase activity can be performed by mixing glycoproteins deficient in different components of the complex prior to microactivation.

Unconcentrated glycoprotein preparations deficient in either neuraminidase (GP^{-n}) or β-galactosidase (GP^{-g}) were obtained by immunoprecipitation with specific antibodies.

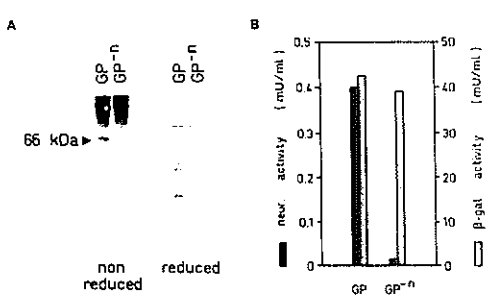

FIG. 3. **Removal of inactive neuraminidase polypeptide from glycoproteins and effect on generation of neuraminidase activity.** The inactive neuraminidase polypeptide was precipitated from an unconcentrated human placental glycoprotein preparation using the neuraminidase-specific antiserum as described under "Experimental Procedures." *A*, immunoblot analysis of normal (*lanes GP*) and neuraminidase polypeptide-depleted (*lanes* GP^{-n}) glycoprotein preparations using neuraminidase-specific antibodies. Electrophoresis was performed under reducing and nonreducing conditions. *B*, neuraminidase and β-galactosidase activities in GP and GP^{-n} after microactivation and stabilization of neuraminidase.

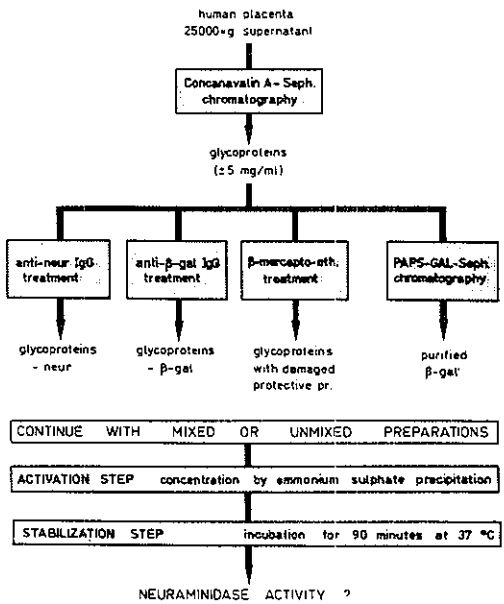

FIG. 4. **Experimental outline of reconstitution experiments.** *PAPS-GAL-Seph.*, *p*-aminophenylthiogalactoside-CH-Sepharose.

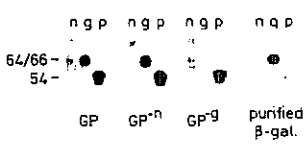

FIG. 5. **Immunoblot analysis of preparations used for reconstitution experiments.** Unconcentrated human placental glycoproteins, either untreated (*GP*) or depleted in the neuraminidase polypeptide (GP^{-n}) or β-galactosidase (GP^{-g}), and purified β-galactosidase were analyzed on immunoblots using antibodies specific for neuraminidase (*lanes n*), β-galactosidase (*lanes g*), and the protective protein (*lanes p*). Molecular masses are given in kilodaltons.

Immunoblot analysis using a panel of antibodies specific for each component of the complex clearly demonstrates the removal of the 66-kDa neuraminidase polypeptide and the 64-kDa β-galactosidase in GP^{-n} and GP^{-g}, respectively (Fig. 5). Unfortunately, the antibody approach could not be used to deplete glycoproteins from the protective protein since none of the available antibodies recognizes the mature native form of the protein. However, we have observed that even in the absence of antibodies, incubation conditions used for immunoprecipitation of the *in vivo* labeled 32-kDa polypeptide of the protective protein lead to inactivation of a component essential for neuraminidase activity. This procedure requires incubation of unconcentrated glycoproteins for 60 min at 20 °C with 100 mM β-mercaptoethanol at pH 6.5. At lower temperature or lower pH, no effect is observed (data not shown). Glycoproteins incubated in such a way will be referred to as GP^{in}. On immunoblots, GP^{in} and GP are indistinguishable (data not shown).

As shown in Fig. 6, after microactivation and stabilization of GP^{-n} and GP^{in}, markedly reduced neuraminidase activity is observed in comparison with GP, whereas β-galactosidase

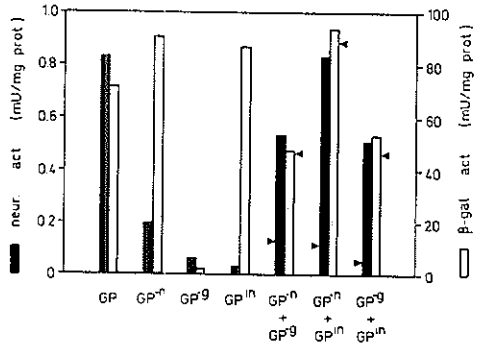

FIG. 6. **Reconstitution of neuraminidase activity using glycoprotein preparations deficient in components of β-galactosidase-neuraminidase-protective protein complex.** Unconcentrated human placental glycoproteins (500 μg), untreated (*GP*), depleted in the neuraminidase polypeptide (*GP^{-n}*) or β-galactosidase (*GP^{-g}*), or treated for 60 min at 20 °C with 100 mM β-mercaptoethanol at pH 6.5 (*GP^{-s}*), were subjected to microactivation and stabilization either directly or mixed (1:1). *Triangles* indicate calculated enzymatic activities in mixed glycoprotein preparations as expected in absence of reconstitution (average of separate components).

activities are normal. After microactivation and stabilization of GP^{-s}, β-galactosidase and neuraminidase are absent. Next, neuraminidase and β-galactosidase activities were measured after microactivation and stabilization of GP^{-n}, GP^{-s}, and GP^{in} paired in all possible combinations. In each case, β-galactosidase equals the theoretical activity, as expected in the absence of reconstitution. In GP^{-n}/GP^{-s}, neuraminidase activity is strongly increased above the hypothetical activity. This demonstrates that both the 66-kDa neuraminidase polypeptide and the 64-kDa β-galactosidase are essential components for neuraminidase activity. In the combinations GP^{-n}/GP^{in} and GP^{-s}/GP^{in}, neuraminidase activity is also restored. This indicates that in GP^{in}, the neuraminidase and β-galactosidase polypeptides are intact, but the third component of the complex, the protective protein, might be damaged.

To check whether the inactivation procedure has indeed damaged the protective protein, we have compared activated, stabilized GP and GP^{in} on sucrose density gradients (Fig. 7). In GP, the normal pattern of free β-galactosidase (peak I), β-galactosidase associated with protective protein (peak II), and the β-galactosidase-neuraminidase-protective protein complex (peak III) is observed (16). In GP^{in}, however, peak III is no longer present, indicating that the protective protein has lost its ability to associate with neuraminidase. In conclusion, these data demonstrate that generation of neuraminidase activity not only requires the association of the 54-kDa protective protein and the 66-kDa neuraminidase polypeptide, but also depends on the presence of β-galactosidase.

Function of β-Galactosidase in Neuraminidase Activity—To study in detail the role of β-galactosidase in neuraminidase activity, we have added purified β-galactosidase to GP^{-s} prior to microactivation. Fig. 5 shows the purity of the β-galactosidase preparation used. After microactivation and stabilization, neuraminidase activity is strongly elevated in the presence of increasing amounts of β-galactosidase (Fig. 8*A*). As expected, β-galactosidase activity is proportional to the amount of enzyme added (Fig. 8*B*). When neuraminidase activity is measured before stabilization, only a limited increase in activity is observed. In the absence of β-galactosidase, stabilization has no effect on neuraminidase activity (Fig.

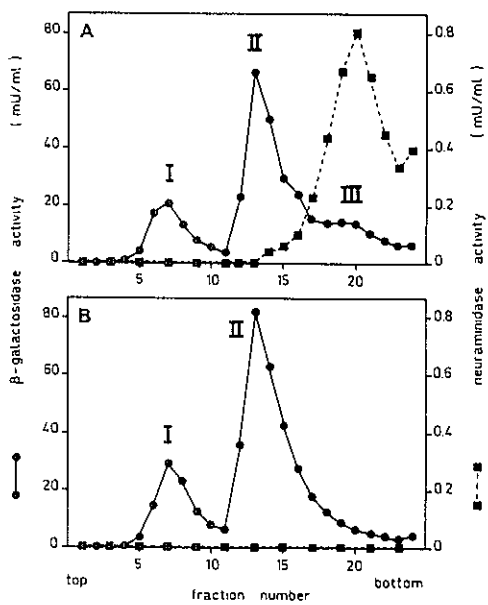

FIG. 7. **Association properties of protective protein after reduction with β-mercaptoethanol.** Sucrose density gradient centrifugation pattern of activated, stabilized glycoproteins either untreated (*A*) or after pretreatment for 60 min at 20 °C with 100 mM β-mercaptoethanol at pH 6.5 (*B*).

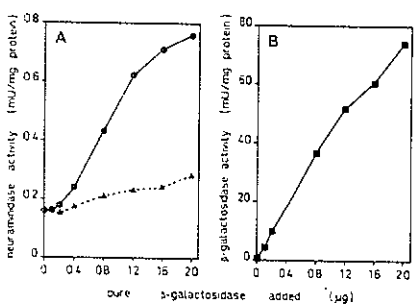

FIG. 8. **Effect of β-galactosidase on neuraminidase activity.** Increasing amounts of purified β-galactosidase were added to an unconcentrated human placental glycoprotein preparation depleted in β-galactosidase prior to microactivation and stabilization. *A*, neuraminidase activity measured before (▲) and after (●) stabilization; *B*, β-galactosidase activity (■) measured after stabilization.

8*A*). Similar results were obtained when β-galactosidase was added after microactivation, but before stabilization of GP^{-s} (data not shown). These data suggest a role for β-galactosidase in the stabilization of neuraminidase activity.

Neuraminidase activity is rapidly lost upon dilution unless the enzyme is stabilized by incubation at 37 °C for 90 min (16). To investigate the involvement of β-galactosidase in this stabilization process, we have followed neuraminidase activities in time during the stabilization of GP and GP^{-s} (Fig. 9, *upper*). In GP, an apparent increase in the specific activity of neuraminidase is observed upon prolonged incubation since the enzyme is no longer sensitive to dilution caused by the

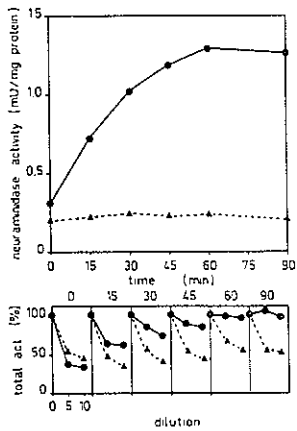

FIG. 9. **Effect of β-galactosidase on neuraminidase stability.** Unconcentrated human placental glycoproteins, untreated (●) or immunologically depleted in β-galactosidase (▲), were used to generate neuraminidase activity by microactivation. During subsequent stabilization, samples were taken at 15-min intervals, and neuraminidase activity was measured (*upper*). In addition, for each time point, total neuraminidase activity was measured in 5- and 10-fold diluted samples and expressed as percentage of the activity measured in the undiluted sample (*lower*).

enzyme assay. No such increase is observed during stabilization of GP^{-s}. Since resistance to dilution is indicative for neuraminidase stability, we have measured at each time point the activity in diluted samples (Fig. 9, *lower*). During incubation of GP, neuraminidase activity gradually becomes insensitive to dilution, demonstrating stabilization of the enzyme. In contrast, in GP^{-s}, neuraminidase activity remains labile, even after 90 min of incubation. From these data, we conclude that β-galactosidase is required for the stability of human placental neuraminidase.

DISCUSSION

Recently, we have described the purification of an inactive neuraminidase polypeptide from the partially disrupted β-galactosidase-neuraminidase-protective protein complex from human placenta (17). The preparation contained two proteins with molecular masses of 66 and 76 kDa. Since antibodies raised against this protein preparation specifically recognize neuraminidase activity, both proteins were potential candidates for being the inactive neuraminidase polypeptide.

In this study, immunoblotting experiments show that under nonreducing conditions, the 66-kDa protein is specifically recognized by highly diluted neuraminidase antibodies. In a combined immunotitration/immunoblotting experiment, this protein coprecipitates with neuraminidase activity. Moreover, the 66-kDa protein is absolutely required for neuraminidase activity since specific removal of this protein from glycoproteins, prior to formation of the complex, prevents generation of neuraminidase activity. From these data, we conclude that the 66-kDa protein is the inactive neuraminidase polypeptide.

Glycopeptidase F treatment of the complex has revealed substantial glycosylation of the 66-kDa neuraminidase polypeptide. Assuming a contribution of 2–4 kDa for each *N*-linked oligosaccharide chain to the total molecular mass, one can calculate the presence of 7–14 carbohydrate chains in the 66-kDa protein. Thus far, several lysosomal integral membrane proteins have been identified, and all have been shown to be heavily glycosylated (24–27). Since neuraminidase is a membrane-bound lysosomal hydrolase, we propose that *in vivo*, the 66-kDa protein is anchored in the lysosomal membrane. Biosynthetic labeling studies are in progress to characterize the enzyme in its natural environment.

Recently, we have suggested that the 76-kDa protein is the inactive neuraminidase polypeptide (17). Visualization of this protein on immunoblots required 10–20 fold dilutions of neuraminidase antibody (17). In this study, we show that the same antibody, when 1000-fold diluted, recognizes the 66-kDa protein, but, only in its nonreduced state. Apparently, the neuraminidase antibodies recognize a conformational determinant containing a disulfide bond. This explains why, in the past, under reducing conditions, this protein was not observed on immunoblots.

NH_2-terminal sequencing of the 76-kDa protein has revealed almost 100% homology with the NH_2-terminal end of the IgM heavy chain. The 76-kDa protein is recognized by IgM-specific antibodies on immunoblots; and likewise, antibodies raised against the electroeluted 76-kDa protein recognize the IgM heavy chain.[2] Apparently, IgM copurifies with the complex due to aspecific binding.

Active neuraminidase is present in a complex with β-galactosidase and the protective protein (16). In the absence of either one of these components, generation of stable neuraminidase activity is prevented. Restoration of neuraminidase activity is obtained after microactivation of mixtures of glycoproteins depleted in different components of the complex prior to microactivation. This proves that all three components of the complex are required for neuraminidase function.

The first component essential for neuraminidase activity is the 66-kDa neuraminidase polypeptide. Although it is inactive in its nonassociated state, most likely the active site of neuraminidase is localized on this polypeptide. We are currently investigating this by photoaffinity labeling. As discussed, it is probably also the component by which neuraminidase is integrated in the lysosomal membrane in the *in vivo* situation.

The second component essential for neuraminidase activity is the protective protein. Structural alteration of the native protein by partial reduction with β-mercaptoethanol prevents its aggregation with the 66-kDa polypeptide and thus generation of neuraminidase activity. Under these conditions, association of the protective protein and β-galactosidase is not prevented. An explanation for this behavior can be deduced from the primary structure of the protective protein as revealed from its cDNA sequence (10). The mature native protein consists of a heterodimer of 32- and 20-kDa polypeptides held together by disulfide linkages. The damaging effect of β-mercaptoethanol on the protective protein is temperature- and pH-dependent, implying that the disulfide bonds are only susceptible to reduction after unfolding of the protein (28). However, under the mild conditions used, unfolding is only partial since the 32- and 20-kDa polypeptides still form a heterodimer, and the binding site for β-galactosidase is intact. The specific loss of the binding site for the neuraminidase polypeptide suggests the use of separate domains in the protective protein for the binding of β-galactosidase and neuraminidase.

The protective protein in the complex has a different function for β-galactosidase and neuraminidase. Monomeric β-galactosidase is normally active, but it is rapidly degraded in the lysosome in the absence of the protective protein (8). In contrast, the neuraminidase polypeptide is inactive by itself and requires aggregation with the protective protein to gain

[2] G. T. J. van der Horst, N. J. Galjart, A. d'Azzo, H. Galjaard, and F. W. Verheijen, unpublished results.

activity (16). Recently, cDNA sequencing data revealed a striking homology between the protective protein and carboxypeptidase Y and the KEX1 gene product from yeast (10). This led to the hypothesis that the protective protein might act as a processing enzyme for activation of β-galactosidase and neuraminidase. The reversibility of the association process suggests that proteolytic processing is not required during the formation of the complex. Nevertheless, one can speculate that processing of the neuraminidase and β-galactosidase polypeptides is a prerequisite for association.

The third component essential for neuraminidase activity is β-galactosidase. Without β-galactosidase, neuraminidase activity remains labile upon dilution. This indicates that it is required for stabilization, but is not strictly essential for activity, at least in the *in vitro* situation. *In vivo*, neuraminidase activity can exist without β-galactosidase, as shown in the inherited metabolic disorder G_{M1} gangliosidosis, in which the latter enzyme is absent (29). However, in cellular extracts from G_{M1} gangliosidosis patients, often a lower stability of neuraminidase activity is observed.[2] Apparently, our *in vitro* observations reflect the *in vivo* situation.

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REFERENCES

1. Corfield, A. P., Michalski, J. C., and Schauer, R. (1981) in *Sialidases and Sialidoses: Perspectives in Inherited Metabolic Diseases* (Tettamanti, G., Durand, P., and Di Donato, S., eds) Vol. 4, pp. 1–70, Ermes, Milan, Italy
2. Cantz, M., Gehler, J., and Spranger, J. (1977) *Biochem. Biophys. Res. Commun.* **74**, 732–738
3. Lowden, J. A., and O'Brien, J. S. (1979) *Am. J. Hum. Genet.* **31**, 1–18
4. O'Brien, J. S. (1982) in *Genetic Errors of Glycoprotein Metabolism* (Durand, P., and O'Brien, J. S., eds) pp. 33–48, Springer-Verlag
5. Wenger, D. A., Turby, T. J., and Wharton, C. (1978) *Biochem. Biophys. Res. Commun.* **82**, 589–595
6. Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W. S., and Dodson, W. E. (1981) in *Sialidases and Sialidoses: Perspectives in Inherited Metabolic Diseases* (Tettamanti, G., Durand, P., and Di Donato, S., eds) Vol. 4, pp. 379–395, Ermes, Milan, Italy
7. D'Azzo, A., Hoogeveen, A. T., Reuser, A. J. J., Robinson, D., and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 4535–4539
8. Hoogeveen, A. T., Verheijen, F. W., and Galjaard, H. (1983) *J. Biol. Chem.* **258**, 12143–12146
9. Hoogeveen, A., D'Azzo, A., Brossmer, R., and Galjaard, H. (1981) *Biochem. Biophys. Res. Commun.* **103**, 292–300
10. Galjart, N. J., Gillemans, N., Harris, A., van der Horst, G. T. J., Verheijen, F. W., Galjaard, H., and D'Azzo, A. (1988) *Cell* **54**, 755–764
11. Nguyen, H. V., Beauregard, G., Potier, M., Belisle, M., Mameli, L., Gatti, R., and Durand, P. (1980) *Biochim. Biophys. Acta* **616**, 259–270
12. McNamara, D., Beauregard, G., Nguyen, H. V., Yan, D. L. S., Belisle, M., and Potier, M. (1982) *Biochem. J.* **205**, 345–351
13. Spaltro, J., and Aldaheff, J. A. (1984) *Biochim. Biophys. Acta* **800**, 159–165
14. Miyagi, T., and Tsuiki, S. (1984) *Eur. J. Biochem.* **141**, 75–81
15. Verheijen, F. W., Brossmer, R., and Galjaard, H. (1982) *Biochem. Biophys. Res. Commun.* **108**, 868–875
16. Verheijen, F. W., Palmeri, S., Hoogeveen, A. T., and Galjaard, H. (1985) *Eur. J. Biochem.* **149**, 315–321
17. Verheijen, F. W., Palmeri, S., and Galjaard, H. (1987) *Eur. J. Biochem.* **162**, 63–67
18. Van Diggelen, O. P., Hoogeveen, A. T., Smith, P. J., Reuser, A. J. J., and Galjaard, H. (1982) *Biochim. Biophys. Acta* **703**, 69–76
19. Laemmli, U. K. (1970) *Nature* **227**, 680–685
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
21. Peneťsky, H. S. (1977) *J. Biol. Chem.* **252**, 2891–2899
22. Galjaard, H. (1980) *Genetic Metabolic Diseases: Early Diagnosis and Prenatal Analysis*, Elsevier/North-Holland, Biomedical Press, Amsterdam
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
24. Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I., and August, J. T. (1985) *J. Cell Biol.* **101**, 85–95
25. Lippincott-Schwartz, J., and Fambrough, D. M. (1986) *J. Cell Biol.* **102**, 1593–1605
26. Barriocanal, J. G., Bonifacino, J. S., Yuan, L., and Sandoval, I. V. (1986) *J. Biol. Chem.* **261**, 16755–16763
27. Green, S. A., Zimmer, K. P., Griffiths, G., and Mellman, I. (1987) *J. Cell Biol.* **105**, 1227–1240
28. Creighton, T. E. (1984) *Methods Enzymol.* **107**, 305–329
29. Hoogeveen, A. T., Graham-Kawashima, H., d'Azzo, A., and Galjaard, H. (1984) *J. Biol. Chem.* **259**, 1974–1977

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Photoaffinity Labeling of a Bacterial Sialidase with an Aryl Azide Derivative of Sialic Acid*

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A photoreactive radioiodinatable derivative of 2-deoxy-2,3-didehydro-5-*N*-acetylneuraminic acid (NeuAc2en), 5-*N*-acetyl-9-(4-azidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid (ASA-NeuAc2-en) has been synthesized and used to label the active site of *Clostridium perfringens* sialidase. Like Neu-Ac2en, its aryl azide derivative is a strong competitive inhibitor of sialidase ($K_i \approx 15$ μM). The absorbance spectrum of ASA-NeuAc2en shows a characteristic aryl azide peak, which disappears upon photolysis with UV light. When its radioiodinated counterpart 5-*N*-acetyl-9-(4-iodoazidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid ([^{125}I]IASA-NeuAc2en) was photolyzed in the presence of *C. perfringens* sialidase a 72-kDa protein was labeled. Labeling occurred specifically in the active site since it was inhibited in the presence of NeuAc2en. Chemical cleavage of the photoaffinity-labeled 72-kDa protein demonstrates that specifically labeled peptides involved in the formation of the active site can easily be determined. ASA-Neu-Ac2en is a valuable new tool for the identification and structural/functional analysis of sialidases and other proteins, recognizing this sialic acid derivative.

Sialidases (neuraminidases EC 3.2.1.18) catalyze the removal of neuraminic acid from a variety of substrates. The enzyme is widespread in nature and has been reported in viruses, bacteria, protozoa, fungi, as well as in vertebrates (1). Sialidases are of medical importance since their occurrence in many pathogenic microorganisms is thought to be involved in the process of infection (2). Furthermore, in humans a deficiency of lysosomal sialidase is observed in two inherited metabolic diseases, sialidosis and galactosialidosis (3, 4). Despite their wide occurrence and pathophysiological importance, knowledge about sialidases is restricted to a small number of well studied enzymes from bacteria and viruses (1). Relatively little is known about the mammalian sialidases because of their labile membrane-bound character (5). Recently we have characterized the lysosomal sialidase from human placenta at the molecular level (6).

To facilitate (*a*) research on the active site of known sialidases and (*b*) molecular characterization of yet unidentified sialidases, we have prepared the photoreactive radioiodinatable affinity probe ASA-NeuAc2en[1] for labeling of the active site of this class of hydrolases. Our procedure was adapted from the method described by Shanahan *et al.* (7) for the synthesis of a photoreactive glucose derivative. We show the successful application of ASA-NeuAc2en for the specific labeling of *Clostridium perfringens* sialidase and localization of its active site at the peptide level.

EXPERIMENTAL PROCEDURES

Materials—NHS-ASA was obtained from Pierce Chemical Co. and later on prepared in our laboratory (8). The synthesis of 9-amino-NeuAc2en will be described in detail elsewhere.[2] Silica TLC Alu plates were from Merck. Na^{125}I was ordered from Amersham Corp. *C. perfringens* sialidase type VI and type X (specific activities 1.69 and 190 units/mg, respectively), NeuAc2en, 4-MU-NeuAc, and CNBr were purchased from Sigma.

Synthesis of ASA-NeuAc2en—All steps in the following procedure were performed away from direct light. 9-Amino-NeuAc2en was dissolved in water (60 mg in 1.5 ml). After the addition of 0.6 ml of 0.5 M $NaHCO_3$ and 80 mg of NHS-ASA, dissolved in 2 ml of methanol/ethyl acetate (1:1, v/v), the mixture was permitted to stand at room temperature. After 1 h, an additional 0.5 ml of 0.5 M $NaHCO_3$ and 80 mg of NHS-ASA in 2 ml of methanol/ethyl acetate (1:1, v/v) were added. After 2 more h the mixture was concentrated to a small volume under vacuum. After the addition of 5 ml of H_2O the mixture was centrifuged and the supernatant solution was passed over a DEAE-Sephadex A-25 column (10 × 4 cm). ASA-NeuAc2en was eluted from the column with 0.5 M NH_4CO_3, dried and redissolved in H_2O/diethyl ether. The aqueous phase was deionized by passage over a column of IR 120 resin (H^+ form) and lyophilized, yielding 41.7 mg of ASA-NeuAc2en (45% of the theoretical yield).

The following physical constants were obtained for the pure product: $[\alpha]_D^{20} = +24.6°$ ($c = 0.3$, CH_3OH); R_F values on silica gel thin layer chromatography with propanol-1/water (5:1, v/v) = 0.44 and with butanol-1/acetic acid/water (5:2:3, v/v) = 0.51; ^{1}H NMR data (CD_3OD) (Bruker WM-300, ^{1}H 300 MHz): δ 7.84 (d, 1 H, $J_{5',6'}$ 8.5 Hz, H-6'), δ 6.81 (dd, 1 H, $J_{3',5'}$ 2.2 Hz, H-5'), δ 6.55 (d, 1 H, $J_{3',5'}$ 2.2 Hz, H-3'). δ 5.81 (d, 1 H, $J_{3,4}$ 2.3 Hz, H-3), δ 4.40 (dd, 1 H, $J_{3,4}$ 2.3 Hz, $J_{4,5}$ 8.7 Hz, H-4), δ 1.99 (s, 3 H, AcN-5).

Photosensitivity of ASA-NeuAc2en was checked by comparing absorbance spectra of a 100 μM aqueous solution before and after irradiation with 254-nm UV light for 2 min at a distance of 1 cm (Universal UV Lamp, Camag, Muttenz, Switzerland). The half-life of ASA-NeuAc2en during UV exposure was determined by measuring A_{270} of a 100 μM aqueous solution after a 5-s interval of irradiation.

Synthesis of [^{125}I]IASA-NeuAc2en—Radioiodination of ASA-NeuAc2en was performed according to the method of Hunter and Greenwood (10). 10 μl of Na^{125}I (0.5 mCi, specific activity 2200 Ci/mmol) was mixed with 10 μl of 0.1 M NaOH. After the sequential addition of 10 μl of 0.1 M HCl, 20 μl of 5 mM ASA-NeuAc2en in 0.5 M sodium phosphate buffer (pH 7.4), and 20 μl of chloramine T (1.14 mg/ml in 0.5 M sodium phosphate buffer, pH 7.4), the reaction was allowed to proceed for 1 min. The reaction was terminated by the addition of 50 μl of 5% sodium metabisulfite. [^{125}I]IASA-NeuAc2en

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[1] The abbreviations used are: ASA-NeuAc2en, 5-*N*-acetyl-9-(4-azidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; NeuAc2en, 2-deoxy-2,3-didehydro-5-*N*-acetylneuraminic acid; IASA-NeuAc2en, 5-*N*-acetyl-9-(4-iodoazidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid; 9-amino-NeuAc2en, 9-amino-2-deoxy-2,3-didehydro-5-*N*-acetylneuraminic acid; 4-MU-NeuAc, 2-α-(4-methylumbelliferyl)-5-*N*-acetylneuraminic acid; SDS, sodium dodecyl sulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[2] R. Brossmer and U. Rose, manuscript in preparation.

was purified by preparative thin layer chromatography on a silica TLC Alu plate, developed with butanol/acetic acid/water (2:1:1, v/v). After detection by autoradiography (2-min exposure), the major radioactive band, which comigrated with ASA-NeuAc2en, was excised from the plate and eluted twice with 2 ml of methanol/water (19:1, v/v). The eluate was filtered through a Pasteur pipette with a cotton plug to remove silica particles and dried under N_2. The dried material was dissolved in 2 ml of water containing 2% ethanol (118 μCi/ml). In parallel experiments with unlabeled NaI approximately 50% of the starting material could be recovered. Therefore the [^{125}I]IASA-NeuAc2en concentration is estimated at 25 μM (specific activity 4.72 Ci/mmol).

Photoaffinity Labeling—20 μl of [^{125}I]IASA-NeuAc2en was dried under N_2 in the absence or presence of 2 μl of 0.1, 1, or 100 mM NeuAc2en. Dried material was dissolved in 20 μl of 40 mM sodium acetate, 200 mM NaCl, pH 5.2, containing *C. perfringens* sialidase type VI (0.1 unit, 60 μg) or type X (0.25 unit, 1.3 μg) and allowed to stand for 5 min at room temperature (final concentrations, 25 μM [^{125}I]IASA-NeuAc2en and 0, 0.01, 0.1, or 10 mM NeuAc2en). After cooling on ice for 30 s, samples were exposed to 254-nm UV light for 1 min at a distance of 1 cm. Under these conditions, no detectable UV-induced protein-protein cross-linking or ASA-NeuAc2en-specific inactivation of the enzyme was observed.

After labeling, protein was recovered from the reaction mixture by precipitation with 1/9 volume of 100% trichloroacetic acid. After 30 min of incubation on ice, proteins were pelleted by centrifugation for 10 min at 10,000 × *g* (4 °C). Pellets were washed once with ice-cold ethanol/ether (1:1, v/v) and dissolved either in 30 μl of electrophoresis sample application buffer or, when CNBr cleavage was performed, in 30 μl of 50 mM sodium phosphate, pH 7, containing 0.2% SDS.

CNBr Cleavage—Proteins were chemically cleaved at methionine residues by the addition of 70 μl of formic acid and 3 μl of 1 M CNBr to 30-μl protein samples (11). Alternatively, bands excised from fixed dried SDS-polyacrylamide gels were swollen in 70% formic acid containing 30 mM CNBr. After incubation for 24 h at 25 °C, samples were dried by vacuum centrifugation (Savant Speedvac).

SDS-Polyacrylamide Gel Electrophoresis—Electrophoresis was performed on 10% SDS-polyacrylamide gels according to Laemmli (12). CNBr-cleaved proteins were separated on a 16.5% gel (16.5% T, 3% C) using the Tricine-SDS polyacrylamide electrophoresis system described by Schägger and Von Jagow (13). In case excised bands were used, dried gel strips were placed in the sample well and swollen in prewarmed sample application buffer. After electrophoresis gels were fixed in 40% methanol, 10% acetic acid and either stained with Coomassie Blue or dried. Photoaffinity-labeled proteins were visualized by autoradiography at −80 °C using Kodak XAR-5 films and an intensifying screen.

Enzymatic Assays—Sialidase activity was measured with the 4-MU-NeuAc substrate as described, except that the pH was 5.2 (14). Kinetic experiments were performed with sialidase type VI preparations (3 milliunits/ml in 40 mM sodium acetate, 200 mM NaCl and 0.1% bovine serum albumin) and 4-MU-NeuAc substrate (concentrations ranging from 0.1 to 1.0 mM) in the absence or presence of ASA-NeuAc2en or NeuAc2en. K_m and K_p values (apparent K_m, measured in the presence of an inhibitor) were determined with the Enzfit computer program (Biosoft, Elsevier), and K_i values were calculated using the following formula.

$$K_i = \frac{[I]}{(K_p/K_m) - 1}$$

Protein concentrations were measured according to Lowry *et al.* (15).

RESULTS

Synthesis and Characterization of ASA-NeuAc2en—ASA-NeuAc2en was synthesized by chemical coupling of the heterobifunctional photoactivatable cross-linker NHS-ASA to the NH_2 group of 9-amino-NeuAc2en. Fig. 1 shows a schematic representation of the synthesis and molecular structures of the compounds involved. The absorbance spectrum of an aqueous solution of ASA-NeuAc2en is shown in Fig. 2. The peak at 270 nm with a shoulder at 305 nm is characteristic for the photoreactive aryl azide group. After irradiation of the compound for 2 min with 254-nm UV light, the aryl azide peak is no longer observed. This demonstrates the photosensitivity of ASA-NeuAc2en. During irradiation the aryl azide group is shown to have a half-life of approximately 8 s (Fig. 2, *inset*).

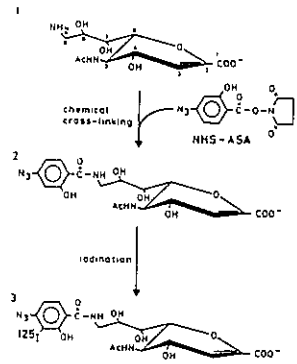

FIG. 1. **Synthesis of ASA-NeuAc2en and [^{125}I]IASA-NeuAc2en.** Molecular structures shown: compound *1*, 9-amino-NeuAc2en; compound *2*, ASA-NeuAc2en; compound *3*, [^{125}I]IASA-NeuAc2en.

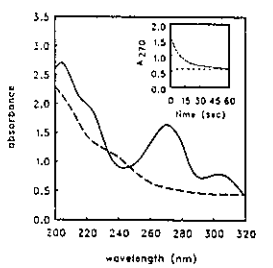

FIG. 2. **Absorbance characteristics of ASA-NeuAc2en.** Absorbance spectrum of a 100 μM aqueous solution of ASA-NeuAc2en before (——) and after (– – –) irradiation of the cuvette with 254-nm UV light for 2 min at a distance of 1 cm. *Inset*, photodecomposition of ASA-NeuAc2en in time. A 100 μM aqueous solution of ASA-NeuAc2en was repeatedly irradiated for 5-s time periods with 254-nm UV light at a distance of 1 cm, followed by measurement of A_{270}. The *dotted line* represents A_{270} of completely photolyzed ASA-NeuAc2en.

We investigated whether ASA-NeuAc2en has the same high affinity for sialidases as NeuAc2en by comparing the inhibitory effect of these compounds on the activity of *C. perfringens* sialidase. Fig. 3 shows a double-reciprocal plot of the enzyme activity as a function of the substrate concentration. K_i values calculated for ASA-NeuAc2en and NeuAc2en were 16 and 13 μM, respectively. This indicates that addition of the photoreactive aryl azide group to carbon atom C_9 has not influenced the affinity of this compound for the enzyme.

Active Site Labeling of C. perfringens Sialidase—*C. perfringens* sialidase type VI was labeled with [^{125}I]IASA-NeuAc2en as described under "Experimental Procedures." SDS-polyacrylamide gel electrophoresis of the labeled enzyme preparation revealed a single band with a molecular mass of approximately 72 kDa (Fig. 4*A*, *lane 2*). This band comigrates with the major band, visible after protein staining (Fig. 4*A*, *lane 1*). The label migrating at the front of the gel represents free photoaffinity label which is noncovalently associated with protein and released upon electrophoresis. To distinguish

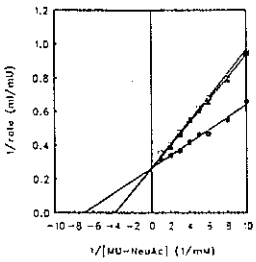

FIG. 3. **Inhibition kinetics of *C. perfringens* sialidase with NeuAc2en and ASA-NeuAc2en.** *C. perfringens* sialidase (type VI, 3 milliunits/ml) was incubated with 4-MU-NeuAc at concentrations ranging from 0.1 to 1.0 mM in the absence (●) or presence of 30 μM NeuAc2en (□) or ASA-NeuAc2en (▲). The measured activity is plotted double reciprocal against the substrate concentration.

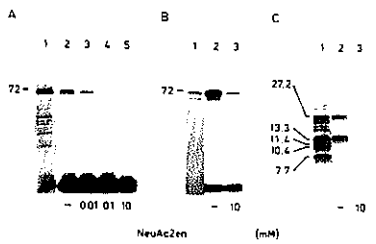

FIG. 4. **Photoaffinity labeling of *C. perfringens* sialidase.** *C. perfringens* sialidase type VI was photoaffinity-labeled with [^{125}I]IASA-NeuAc2en in the absence or presence of NeuAc2en (*panel A*). Similarly *C. perfringens* sialidase type X was photoaffinity-labeled (*panel B*) and subjected to chemical hydrolysis with CNBr (*panel C*). Proteins were separated on a 10% SDS-polyacrylamide gel (*panels A* and *B*) or a 16.5% Tricine-SDS-polyacrylamide gel (*panel C*). *Panel A*: *lane 1*, *C. perfringens* sialidase type VI (60 μg, 0.1 unit stained with Coomassie Brilliant Blue; *lane 2*, *C. perfringens* sialidase type VI (60 μg, 0.1 unit), photoaffinity-labeled with [^{125}I]IASA-NeuAc2en; *lanes 3–5*, as *lane 2* except that labeling was performed in the presence of different concentrations of NeuAc2en. *Panel B*: *lane 1*, *C. perfringens* sialidase type X (1.3 μg, 0.25 unit, stained with Coomassie Brilliant Blue; *lane 2*, *C. perfringens* sialidase type X (1.3 μg, 0.25 unit), photoaffinity-labeled with [^{125}I]IASA-NeuAc2en; *lane 3*, as *lane 2* except that labeling was performed in the presence of 10 mM NeuAc2en. *Panel C*: *lane 1*, CNBr cleavage products of *C. perfringens* sialidase type X (20 μg) stained with Coomassie Brilliant Blue; *lanes 2* and *3*, as *panel B* except that photoaffinity-labeled proteins were digested with CNBr.

between specific and nonspecific labeling, we have also performed the labeling reaction in the presence of unlabeled NeuAc2en. Only specific labeling is expected to decrease due to competition between NeuAc2en and the photoaffinity probe for the active site. As shown in Fig. 4*A* (*lanes 3–5*), labeling of the 72-kDa protein is strongly reduced with increasing concentrations of NeuAc2en. In contrast nonspecifically labeled proteins, seen on overexposed autoradiograms, are hardly competed out (data not shown). Similar results were obtained when reactions were performed in the presence of 50 mM β-mercaptoethanol, a well known scavenger used to minimize nonspecific labeling by aryl azide photoaffinity probes (data not shown). We conclude that the 72-kDa protein is the *C. perfringens* sialidase, specifically labeled in the active site by [^{125}I]IASA-NeuAc2en.

We have investigated whether ASA-NeuAc2en can be used to identify within the sialidase polypeptide regions that are part of the active site. Photoaffinity-labeled *C. perfringens* sialidase was chemically hydrolyzed with CNBr to generate peptides. To simplify the peptide pattern of the Coomassie-stained enzyme we have used in this experiment *C. perfringens* sialidase type X. This form is purified from the type VI preparation. Fig. 4*B*, *lane 1*, shows the purity of the sialidase. The band migrating just in front of the 72-kDa protein most likely represents a degradation product of the latter since *C. perfringens* sialidase is known to be very susceptible to proteolytic degradation (1). After CNBr treatment of this preparation, peptides with molecular masses of 27.2, 13.3, 11.4, 10.4, and 7.7 kDa were the major cleavage products observed (Fig. 4*C*, *lane 1*). As expected on the basis of the former experiment, photoaffinity labeling of *C. perfringens* sialidase type X results in labeling of the 72-kDa protein (Fig. 4*B*, *lane 2*). Labeling is shown to be specific since it is decreased in the presence of NeuAc2en (Fig. 4*B*, *lane 3*). In comparison with the prior experiment in which type VI sialidase was used, the increase in signal intensity is in agreement with the higher input activity. After CNBr cleavage of the photoaffinity-labeled preparations, only the 27.2- and 13.3-kDa polypeptides contain label (Fig. 4*C*, *lanes 2* and *3*). We have repeated CNBr cleavage on photoaffinity-labeled proteins and peptides excised from the dried exposed gel to exclude that the two labeled peptides are the result of partial hydrolysis. The 72-kDa protein again gave rise to labeled peptides with molecular masses of 27.2 and 13.3 kDa while the separate peptides were not further degraded (data not shown). We conclude that the 27.2- and 13.3-kDa peptides of the 72-kDa sialidase protein contain regions that are involved in formation of the active site.

DISCUSSION

In this study we present the radioiodinatable sialic acid derivative ASA-NeuAc2en as a photoaffinity probe for the active site labeling of sialidases. We have chosen a NeuAc2en derivative since NeuAc2en is the most potent sialidase inhibitor known (16). Introduction of the bulky aryl azide group at C atom 9 of NeuAc2en did not affect the affinity of the compound for *C. perfringens* sialidase. The applicability of ASA-NeuAc2en as a photoaffinity probe is demonstrated by labeling of the *C. perfringens* sialidase. In partially purified as well as in highly purified preparations, a 72-kDa protein was labeled. The extent of labeling was proportional with enzyme activity. Incorporation of the photoaffinity probe occurred specifically at the active site of the sialidase since it is inhibited by addition of the competitive inhibitor NeuAc2en. The observed molecular mass of the photoaffinity-labeled *C. perfringens* sialidase is in good agreement with the 69 kDa reported for the purified enzyme (17). ASA-NeuAc2en is also a powerful probe for the identification of regions within sialidase polypeptides involved in formation of the active site. After CNBr cleavage of photoaffinity-labeled *C. perfringens* sialidase, specifically labeled peptides containing active site sequences could be readily determined. Since the covalently linked probe can even stand the harsh conditions of CNBr treatment, further proteolytic fine mapping should be possible. This demonstrates the potential use of ASA-NeuAc2en in localization of the active site at the amino acid level when sequence data are available.

This is the first time that a photoaffinity probe has been successfully used for labeling of the active site of a sialidase. Recently, other photoreactive analogs of NeuAc2en have been synthesized (18, 19). However, in contrast to the radioiodinatable ASA-NeuAc2en, these compounds cannot easily be obtained in a radiolabeled form.

In principle, structural and functional aspects of other

sialidases in bacteria, viruses, and higher animals can be studied with ASA-NeuAc2en. Initial experiments with mammalian sialidases are hampered by the fact that their specific activity is low in comparison with the bacterial enzyme. Since equivalent amounts of enzyme activity require protein levels that exceed the amount of protein that can be analyzed by gel electrophoresis, photoaffinity labeling should in this case be combined with partial purification of the enzyme.

The relative ease at which ASA-NeuAc2en and its radioiodinated counterpart can be prepared offers possibilities for the synthesis of other ASA-sialic acid derivatives. Substrates, competitive inhibitors, or ligands with a high affinity for any sialic acid binding protein can be coupled to NHS-ASA, providing that a free amino group is present (or can be introduced) at a position not essential for recognition. Recent studies have revealed that ASA-NeuAc2en can be activated to CMP-ASA-NeuAc2en.[3] The use of photoreactive sialic acid derivatives will facilitate research on other proteins involved in sialic acid metabolism like acylneuraminate pyruvate lyases (20), CMP-β-NeuAc hydrolases (21), sialyl transferases (22), and the lysosomal sialic acid transporter (9).

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REFERENCES

1. Corfield, A. P., and Schauer, R. (1982) in *Sialic Acids, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 195–262, Springer-Verlag, New York
2. Reutter, W. Köttgen, E., Bauer, C., and Gerok, W. (1982) in *Sialic Acids, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 263–305, Springer-Verlag, New York
3. O'Brien, J. S. (1982) in *Genetic Errors of Glycoprotein Metabolism* (Durand, P., and O'Brien, J. S., eds) pp. 33–48, Springer-Verlag, New York
4. Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W. S., and Wharton, C. (1981) in *Sialidases and Sialidoses: Perspectives in Inherited Metabolic Diseases* (Tettamanti, G., Durand, P., and Di Donato, S., eds) Vol. 4, pp. 379–395, Ermes, Milan, Italy
5. Conzelmann, E., and Sandhoff, K. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.* **60**, 89–217
6. van der Horst, G. T. J., Galjart, N. J., d'Azzo, A., Galjaard, H., and Verheijen, F. W. (1989) *J. Biol. Chem.* **264**, 1317–1322
7. Shanahan, M. F., Wadzinski, B. E., Lowndes, J. M., and Ruoho, A. E. (1985) *J. Biol. Chem.* **260**, 10897–10900
8. Ji, T. H., and Ji, I. (1982) *Anal. Biochem.* **121**, 286–289
9. Mancini, G. M. S., de Jonge, H. R., Galjaard, H., and Verheijen, F. W. (1989) *J. Biol. Chem.* **264**, 15247–15254
10. Hunter, W. M., and Greenwood, F. C. (1962) *Nature* **194**, 495–496
11. Carrey, E. A. (1989) in *Protein Structure, A Practical Approach* (Creighton, T. E., ed) pp. 117–144, IRL Press Ltd., Oxford
12. Laemmli, U. K. (1970) *Nature* **227**, 680–685
13. Schägger, H., and Von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
14. Warner, T. G., and O'Brien, J. S. (1979) *Biochemistry* **18**, 2783–2787
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
16. Meindl, P., and Tuppy, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1088–1092
17. Nees, S., Veh, R. W., and Schauer, R. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1027–1042
18. Warner, T. G. (1987) *Biochem. Biophys. Res. Commun.* **148**, 1323–1329
19. Warner, T. G., and Lee, L. A. (1988) *Carbohydr. Res.* **176**, 211–218
20. Shukla, K., and Schauer, R. (1986) *Anal. Biochem.* **158**, 158–164
21. Kean, E. L., and Bighouse, K. J. (1974) *J. Biol. Chem.* **249**, 7813–7823
22. Paulson, J. C., Beranek, W. E., and Hill, R. L. (1977) *J. Biol. Chem.* **252**, 2356–2362

[3] P. Mirelis and R. Brossmer, unpublished results.

Publication 3

Photoaffinity labeling of the lysosomal neuraminidase from bovine testis

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Photoaffinity labeling of the lysosomal neuraminidase from bovine testis

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ASA-NeuAc2en, a photoreactive arylazide derivative of sialic acid, is shown to be a powerful competitive inhibitor of lysosomal neuraminidase from bovine testis ($K_i \approx 21$ μM). Photoaffinity labeling and partial purification of preparations containing this lysosomal neuraminidase activity result in specifically and non-specifically labeled polypeptides. Only labeling in a 55 kDa polypeptide is found to be specific, since it could be prevented by the competitive neuraminidase inhibitor NeuAc2en. We conclude that the 55 kDa polypeptide in the bovine testis β-galactosidase/neuraminidase/protective protein complex contains the catalytic site of neuraminidase.

Neuraminidase; Sialidase; Lysosomal; Photoaffinity labeling

1. INTRODUCTION

In the inherited metabolic disorder sialidosis the intralysosomal degradation of sialoglycoconjugates is impaired due to a deficiency of the acid hydrolase neuraminidase (sialidase EC 3.2.1.18) [1-3]. In galactosialidosis a combined deficiency of neuraminidase and β-galactosidase exists [4,5]. The latter disease is caused by a mutation in the 'protective protein', which protects β-galactosidase from intralysosomal degradation and is essential for neuraminidase activity [6]. No information is available about the molecular defect in sialidosis.

Active neuraminidase has been isolated from bovine testis and human placenta as a high molecular mass complex with β-galactosidase and the protective protein [7,8]. Partial disruption of the complex with KSCN results in the dissociation of the neuraminidase polypeptide from the complex. The unassociated neuraminidase polypeptide has no enzymatic activity [9]. Reconstitution experiments have shown that, in vitro, association of the neuraminidase polypeptide with the protective protein generates a labile form of neuraminidase activity. This activity is stabilized by β-galactosidase [10]. Most likely the neuraminidase polypeptide contains the catalytic site.

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Abbreviations: ASA-NeuAc2en, 5-*N*-acetyl-9-(4-azidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid; IASA-NeuAc2en, 5-*N*-acetyl-9-(4-iodoazidosalicoylamido)-2-deoxy-2,3-didehydroneuraminic acid; NeuAc2en, 2-deoxy-2,3-didehydro-5-*N*-acetylneuraminic acid; MU-NeuAc, 2-α-(4-methylumbelliferyl)-5-*N*-acetylneuraminic acid; SDS, sodium dodecyl sulfate

To investigate the catalytic site of neuraminidases, we have recently synthesized the radioiodinatable, photoreactive affinity probe ASA-NeuAc2en. This probe was successfully used to label the active site of a bacterial neuraminidase [11]. In the present study we have used ASA-NeuAc2en to label the neuraminidase polypeptide in the lysosomal β-galactosidase/neuraminidase/protective protein complex from bovine testis.

2. MATERIALS AND METHODS

2.1. *Materials*

ASA-NeuAc2en and [^{125}I]IASA-NeuAc2en were prepared as described previously [11]. The β-galactosidase/neuraminidase/protective protein complex was purified from bovine testis as reported by Verheijen et al. [7].

2.2. *Photoaffinity labeling and further purification of bovine testis neuraminidase*

[^{125}I]IASA-NeuAc2en (150 μl; 25 μM; specific activity 4.7 Ci/mmol) was dried under N_2 in absence or presence of 20 μl 100 mM NeuAc2en. The dried material was dissolved in 200 μl bovine testis complex (200 μg; specific activity 113 mU/mg) in 20 mM sodium acetate, 100 mM NaCl, pH 5.2 (Buffer A), containing 1% Triton X-100 and incubated for 5 min at room temperature (final concentration of [^{125}I]IASA-NeuAc2en 18.75 μM). After cooling on ice for 30 s and addition of β-mercaptoethanol (final concentration 100 mM), samples were exposed to 254 nm UV-light for 1 min at a distance of 1 cm. Unbound label was removed by rapid gel filtration through a Sephadex G-50 medium spin column [12], equilibrated with buffer A containing 1% Triton X-100. Under these conditions, no detectable UV-induced protein-protein crosslinking or ASA-NeuAc2en-specific inactivation of neuraminidase was observed.

After photoaffinity labeling, neuraminidase was further purified by sucrose density gradient centrifugation on a linear 20-40% sucrose gradient in buffer A (15 h; 150 000 × *g*; 4°C). Fractions containing neuraminidase activity were pooled. Protein was precipitated with 10% TCA and analyzed on a 11% SDS-polyacrylamide gel as described by Laemmli [13]. Photoaffinity-labeled proteins were visualized by autoradiography of the dried gel.

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2.3. *Enzymatic assays*

Neuraminidase and β-galactosidase activities were measured with the corresponding 4-methylumbelliferyl substrates as described [14]. Kinetic experiments were performed as previously reported [11] in either neuraminidase assay buffer (125 mM sodium acetate, pH 4.3) or in buffer A. Protein concentrations were determined according to Lowry et al. [15].

3. RESULTS AND DISCUSSION

To generate optimal conditions for successful photoaffinity labeling of a mammalian lysosomal neuraminidase two considerations were made. First, mammalian neuraminidases are known to be very labile [16]. We have performed our experiments with the β-galactosidase/neuraminidase/protective protein complex isolated from bovine testis [7], since among the purified lysosomal neuraminidases it is the most stable form available. Secondly, photoaffinity labeling experiments with complex protein mixtures are often hindered by a large extent of non-specific labeling [17]. To reduce non-specific labeling we have used the scavenger β-mercaptoethanol [17]. To increase the ratio between specifically and non-specifically labeled proteins we have partially purified the neuraminidase after the labeling reaction.

ASA-NeuAc2en is a derivative of NeuAc2en, a potent competitive inhibitor of mammalian neuraminidases [18]. To investigate whether introduction of the photoreactive group in NeuAc2en has reduced its affinity for the bovine testis neuraminidase, K_i values for ASA-NeuAc2en and NeuAc2en were compared in kinetic experiments with the purified complex. Under standard assay conditions the K_m for 4-MU-NeuAc is 115 μM. The K_i values for ASA-NeuAc2en and NeuAc2en are 21 and 16 μM, respectively (Fig. 1A). This demonstrates that despite the presence of the arylazide group ASA-NeuAc2en is efficiently recognized by the bovine testis neuraminidase.

To avoid the risk of protein loss due to buffer changes and pH shifts, we have tested whether the labeling reaction can be performed in the buffer used for the purification of the complex. When kinetic experiments were performed in buffer A (Fig. 1B), the affinity of bovine testis neuraminidase for 4-MU-NeuAc (K_m 104 μM), ASA-NeuAc2en (K_i 25 μM), and NeuAc2en (K_i 16 μM) is not altered. Also, addition of the scavenger β-mercaptoethanol had no effect on the affinity (data not shown). The ability to perform the photoaffinity labeling reaction in buffer A greatly facilitates the further purification of neuraminidase after labeling.

For glycoprotein preparations from human placenta sucrose density gradient centrifugation has been used to

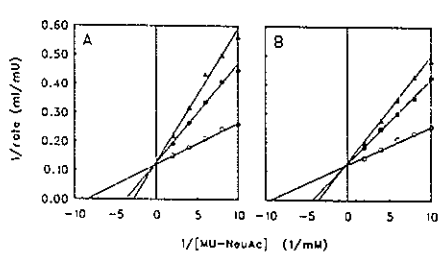

Fig. 1 Inhibition kinetics of lysosomal bovine testis neuraminidase with NeuAc2en and ASA-NeuAc2en. Purified bovine testis complex (8 mU/ml) was incubated with varying concentrations of 4-MU-NeuAc at pH 4.3 (panel A) or 5.2 (panel B) in absence of inhibitor (○) or in presence of 30 μM NeuAc2en (▲) or ASA-NeuAc2en (●). The enzyme activity is plotted as double reciprocal against the substrate concentration.

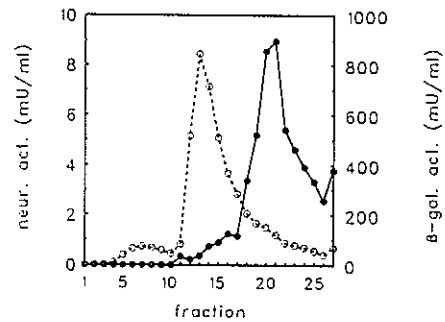

Fig. 2 Sucrose density centrifugation of 200 μg purified bovine testis β-galactosidase/neuraminidase/protective protein complex on a 20–40% linear sucrose gradient. (●——●) Neuraminidase activity, (○---○) β-galactosidase activity.

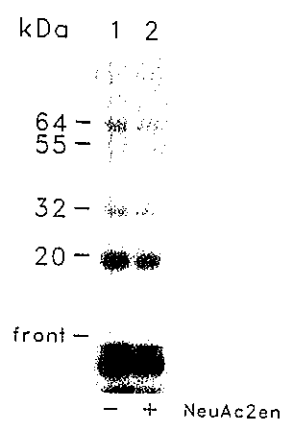

Fig. 3 Photoaffinity labeling of bovine testis neuraminidase. Bovine testis β-galactosidase/neuraminidase/protective protein complex (200 μg) was photoaffinity-labeled with [^{125}I]ASA-NeuAc2en. Neuraminidase was partially purified by sucrose density centrifugation and analyzed on a 11 % SDS-polyacrylamide gel. Lane 1: labeling performed in absence of inhibitor; lane 2: labeling performed in presence of 10 mM NeuAc2en.

separate complexes with neuraminidase and β-galactosidase activity from complexes only containing β-galactosidase activity [8]. Fig. 2 shows the distribution of neuraminidase and β-galactosidase activity after centrifugation of 200 μg bovine testis complex on a 20–40% linear sucrose gradient. With this method the neuraminidase activity can be separated from the majority of β-galactosidase activity.

Purified bovine testis complex was photoaffinity-labeled with [^{125}I]IASA-NeuAc2en and neuraminidase was further purified by sucrose density centrifugation. After SDS-polyacrylamide gel electrophoresis, radiolabeled proteins with molecular masses of 64, 55, 32 and 20 kDa were observed (Fig. 3, lane 1). The pattern corresponds with that of the purified complex after Coomassie staining [7]. The 64 kDa protein is β-galactosidase, whereas the 32 and 20 kDa proteins are the two polypeptides of the heterodimeric protective protein [7,19]. When labeling is performed in the presence of the competitive inhibitor NeuAc2en at a concentration that prevents specific labeling (Fig. 3, lane 2), only labeling of the 55 kDa protein is prevented. Identical results were obtained when total glycoprotein preparations from bovine testis were used, providing that after labeling neuraminidase was further purified by isolation of the complex and sucrose density gradient centrifugation (data not shown). This demonstrates that ASA-NeuAc2en can also be used with less pure neuraminidase preparations. We conclude that the 55 kDa protein is the neuraminidase polypeptide, specifically labeled at the active site. This protein is smaller than the 66 kDa neuraminidase polypeptide observed in human placenta [10]. A comparative biochemical study of both polypeptides is in progress.

After the successful application of ASA-NeuAc2en for the labeling of a bacterial neuraminidase [11], we have now extended its use for the labeling of a mammalian lysosomal neuraminidase. ASA-NeuAc2en enables further localization of catalytic site sequences at the peptide level [11]. This opens new possibilities for obtaining detailed information about the active site of the lysosomal neuraminidase and the molecular defect in sialidosis.

REFERENCES

[1] Cantz, M., Gehler, J. and Spranger, J. (1977) Biochem. Biophys. Res. Commun. 74, 732–738.
[2] Lowden, J.A. and O'Brien, J.S. (1979) Am. J. Hum. Genet. 31, 1–18.
[3] O'Brien, J.S. (1982) in: Genetic Errors of Glycoprotein Metabolism (Durand, P. and OBrien, J.S. eds) pp. 33–48, Springer.
[4] Wenger, D.A., Tarby, T.J. and Wharton, C. (1978) Biochem. Biophys. Res. Commun. 82, 589–595.
[5] Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W.S. and Dodson, W.E. (1981) in: Sialidases and Sialidoses: Perspectives in Inherited Metabolic Diseases (Tettamanti, G., Durand, P. and Di Donato, S. eds) vol. 4, pp. 379–395. Ermes, Milan, Italy.
[6] d'Azzo, A., Hoogeveen, A.T., Reuser, A.J.J., Robinson, D. and Galjaard, H. (1982) Proc. Natl. Acad. Sci. USA 79, 4535–4539.
[7] Verheijen, F.W., Brossmer, R. and Galjaard, H. (1982) Biochem. Biophys. Res. Commun. 108, 868–875.
[8] Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985) Eur. J. Biochem. 149, 315–321.
[9] Verheijen, F.W., Palmeri, S. and Galjaard, H. (1987) Eur. J. Biochem. 162, 63–67.
[10] van der Horst, G.T.J., Galjart, N.J., d'Azzo, A., Galjaard, H. and Verheijen, F.W. (1989) J. Biol. Chem. 264, 1317–1322.
[11] van der Horst, G.T.J., Mancini, G.M.S., Brossmer, R., Rose, U and Verheijen, F.W. (1990) J. Biol. Chem. 265, 10801–10804.
[12] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Galjaard, H. (1980) Genetic Metabolic Diseases: Early Diagnosis and Prenatal Analysis, Elsevier, Amsterdam.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Conzelmann, E. and Sandhoff, K. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 60, 89–217.
[17] Bayley, H. (1983) in: Photogenerated Reagents in Biochemistry and Molecular Biology (Work, T.S. and Burdon, R.H. eds) Lab. Techn. in Biochem. and Mol. Biol., vol. 12, pp. 66–111, Elsevier, Amsterdam.
[18] Corfield, A.P., Michalski, J.C. and Schauer, R. (1981) in: Sialidases and Sialidoses: Perspectives in Inherited Metabolic Diseases (Tettamanti, G., Durand, P. and Di Donato, S. eds) vol. 4, pp. 1–70, Ermes, Milan, Italy.
[19] Galjart, N.J., Gillemans, N., Harris, A., van der Horst, G.T.J., Verheijen, F.W., Galjaard, H. and d'Azzo, A. (1988) Cell 54, 755–764.

Publication 4

Partial purification of the lysosomal neuraminidase polypeptide from bovine testis via a reconstitution assay and determination of active site residues
submitted (1993)

PARTIAL PURIFICATION OF THE LYSOSOMAL NEURAMINIDASE POLYPEPTIDE FROM BOVINE TESTIS VIA A RECONSTITUTION ASSAY AND DETERMINATION OF ACTIVE SITE RESIDUES

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Summary

The bovine testis lysosomal neuraminidase polypeptide was partially purified from the dissociated ß-galactosidase/neuraminidase/protective protein complex by removal of ß-galactosidase and the protective protein. During purification, the neuraminidase polypeptide, which in unassociated state is catalytically inactive, was monitored via a reconstitution assay. Via amino acid sequencing of the remaining proteins in the partially purified preparation, a 55 kDa protein was left as the only candidate neuraminidase polypeptide.

Once dissociated from the complex, the bovine testis neuraminidase polypeptide demonstrates a similar behaviour as the human placental polypeptide. It was able to reassociate with bovine testis as well as with human placental ß-galactosidase and protective protein. The neuraminidase activity generated in the newly formed bovine and bovine/human hybrid complexes could be stabilized by incubation at 37 °C.

Chemical modification of bovine testis lysosomal neuraminidase activity with side chain modifying agents, prevented by addition of competitive inhibitors of neuraminidase, demonstrated that histidine and acidic amino acids are involved in the catalytic mechanism of lysosomal neuraminidase.

Introduction

Lysosomal neuraminidase (sialidase, EC 3.2.1.18) catalyzes the hydrolysis of terminal, α-glycosidically linked sialic acid from a variety of oligosaccharides, glycoproteins and synthetic substrates [1]. With the assistance of saposin B (sulfatide activator protein) the enzyme is also capable of degrading water-insoluble glycolipid substrates [2]. In man a

Abbreviations used: DEP: diethylpyrocarbonate; EDC: 1-ethyl-3-(3dimethylaminopropyl)carbodiimide; NeuAc: 5-N-acetylneuraminic acid; NeuAc2en: 2-deoxy-2,3-didehydro-5-N-acetylneuraminic acid; PAPS-Gal-Seph.: p-aminophenyl-thiogalactoside-CH-Sepharose; SDS: sodium dodecyl sulfate; HP GP^{neur}: human placental glycoproteins, lacking the neuraminidase polypeptide; BT $compl.^{pII}$: sucrose gradient peak II from purified bovine testis complex.

deficiency of lysosomal neuraminidase is associated with two inherited lysosomal storage disorders. In sialidosis only neuraminidase is affected [4,5], whereas in galactosialidosis, clinically resembling sialidosis, a combined deficiency of lysosomal neuraminidase and ß-galactosidase exists [6-8]. Galactosialidosis is caused by a deficient "protective protein" which, in addition to its cathepsin A-like carboxypeptidase activity, is required for neuraminidase activity and prevents rapid intralysosomal degradation of ß-galactosidase [9-14]. In contrast to galactosialidosis, the molecular defect in sialidosis has not been elucidated yet.

The membrane bound character, low abundance and low stability of lysosomal neuraminidase largely obstructed the purification of the enzyme. Lysosomal neuraminidase from bovine testis and human placenta has been shown to copurify with lysosomal ß-galactosidase and its protective protein as a high molecular mass complex [15,16]. Characterization of the human placental complex, typically requiring concentration dependent association of the separate components during purification, revealed that neuraminidase activity originates from subunit association of a lysosomal neuraminidase polypeptide with the protective protein. ß-Galactosidase is required for stability of the neuraminidase activity [16-18]. The human placental neuraminidase polypeptide has been immunologically identified as a 66 kDa protein [18], whereas the bovine testis protein was recognized as a 55 kDa protein by photoaffinity labeling with a specially designed photoreactive sialic acid analogue [19,20]. Only cDNA's encoding ß-galactosidase and the protective protein have been cloned thusfar, which largely extended knowledge about the structure and function of the latter two proteins [14,21-25].

The present study describes the partial purification of the lysosomal neuraminidase polypeptide after dissociation of the bovine testis ß-galactosidase/neuraminidase/protective protein complex. As dissociation of the complex results in the loss of neuraminidase activity, a reconstitution assay was developed to detect the catalytically inactive protein. In addition, amino acids involved in the catalytic mechanism of lysosomal neuraminidase are determined via chemical modification of the protein, which will facilitate the identification of active site residues.

Materials and methods

Purification procedures

Procedures for the isolation of bovine testis glycoproteins by Concanavalin A-Sepharose chromatography (Sigma) and subsequent purification of the ß-galactosidase/ neuraminidase/protective protein complex by chromatography on a PAPS-Gal-Seph. column have been described before [15]. Preparations of the purified complex, dissolved in 20 mM

Na-acetate, 100 mM NaCl (pH 5.2), contained approximately 90-120 mU/mg neuraminidase and 7000-9000 mU/mg ß-galactosidase at a protein concentration of 1 mg/ml.

In initial experiments, the effect of increased pH on neuraminidase activity was investigated by 10-fold dilution of bovine testis glycoproteins in 20 mM Na-phosphate, 100 mM NaCl (pH 8.0) or 20 mM sodium acetate, 100 mM NaCl (pH 5.2), followed by incubation at 37 °C for various times. Standard conditions for the dissociation of the purified bovine testis complex included addition of an equal volume of 200 mM sodium phosphate, 100 mM NaCl (pH 8.0), incubation at 37 °C for 15 min and adjustment of the pH to 5.2 with 500 mM sodium acetate. Under these conditions more than 95 % of neuraminidase activity is lost, whereas ß-galactosidase activity is hardly affected.

The dissociated protein fraction was separated from ß-galactosidase and the majority of protective protein by ß-galactosidase specific affinity chromatography. Purified complex (5 ml), dissociated as described above, was diluted in 20 mM sodium acetate, 100 mM NaCl (pH 5.2) to a final volume of 50 ml and recirculated twice on a 6 ml PAPS-Gal-Seph. column, equilibrated with the same buffer. The column was washed with 10 volumes of 20 mM sodium acetate, 100 mM NaCl (pH 5.2). The unretained fraction was concentrated to 5 ml on an Amicon Ultrafiltration cell equipped with a PM 10 filter and dialyzed against 20 mM sodium acetate, 100 mM NaCl (pH 5.2). The column was washed with 10 volumes of 20 mM sodium acetate, 100 mM NaCl (pH 5.2) and 20 mM sodium acetate, 1 M NaCl (pH 5.2). The retained fraction, recovered by elution of the column with 10 volumes 20 mM sodium acetate, 0.5 M NaCl, 100 mM γ-galactonolactone, was concentrated to 5 ml and dialyzed as described above.

A human placental glycoprotein preparation devoid of the lysosomal neuraminidase polypeptide (HP GP^{-neur}) was obtained by immunoprecipitation of the protein from an unconcentrated glycoprotein preparation, containing the components of the complex in a non-associated state [18].

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed on 20-40 % (w/v) linear sucrose gradients (5 ml) in 20 mM sodium acetate, 100 mM NaCl (pH 5.2) as described by Verheijen *et al.* [16]. After centrifugation, 250 μl fractions were collected and assayed for neuraminidase and/or ß-galactosidase activity. Pooled sucrose gradient fractions designated for reconstitution assays were first dialyzed for 16 h against 1000 volumes 20 mM sodium acetate, 100 mM NaCl (pH 5.2).

Reconstitution of neuraminidase activity

Column fractions were mixed with human placental glycoproteins lacking the

neuraminidase polypeptide (HP GP^{neur}, 5 mg protein/ml) or with sucrose gradient purified peak II from bovine testis complex (BT $compl.^{pII}$, 0.1 mg protein/ml) in a final volume of 1.5-2 ml 20 mM sodium acetate, 100 mM NaCl (pH 5.2). Amounts are indicated in the text. After subsequent concentration to 50 μl on Centricon 10 microconcentrators according to the specifications of the manufacturer (Amicon), neuraminidase activity is measured before (activation) and after incubation of the preparation at 37 °C for 90 min (stabilization). Similarly, individual components were treated as described to determine background neuraminidase activity in non-reconstituted samples.

Polyacrylamide gel electrophoresis and protein sequence analysis

SDS-polyacrylamide gel electrophoresis under reducing conditions was performed on 10 % gradient gels according to Laemmli [26]. For NH_2-terminal sequence analysis of partially purified lysosomal neuraminidase polypeptide preparations, proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted to Problott (Applied Biosystems) membranes [27]. Excised filter pieces were used for automated Edman degradation on an Applied Biosystems 473A Protein Sequencer.

Amino acid side chain modification

Bovine testis glycoproteins (320 μg) were incubated with various concentrations DEP (Sigma, 10 x stock in 100 % ethanol) at 20 °C in absence or presence of NeuAc or NeuAc2en (final volume 100 μl in 20 mM sodium acetate, 100 mM NaCl, pH 5.2). Similarly, bovine testis glycoproteins (160 μg) were incubated with various concentrations EDAC (Sigma, 10 x stock in H_20) at 20 °C in presence or absence of NeuAc or NeuAc2en (final volume 100 μl in 20 mM sodium phosphate, 100 mM NaCl, pH 5.2). After 30 min, modifying agents were removed by rapid gel filtration through a 1 ml Sephadex G-50 medium spin column [28] equilibrated with 20 mM sodium acetate, 100 mM NaCl (pH 5.2) and enzymatic activities were measured. DEP modification of histidine residues was reversed by incubation of 80 μl modified glycoproteins with 20 μl 1.5 M hydroxylamine (in 20 mM sodium phosphate pH 7.0) at 20 °C. After 30 min glycoproteins were recovered by rapid gel filtration as described above.

Assays

Neuraminidase and ß-galactosidase activities were measured with the corresponding 4-methylumbelliferyl substrates (Sigma) as described [29]. Activities are expressed in milliunits and one unit is defined as the amount of enzyme releasing 1 μmole of 4-methyl-umbelliferone/min at 37 °C. Protein concentrations were determined according to Lowry et al. [30].

Results

Partial purification of the bovine testis lysosomal neuraminidase polypeptide

Mammalian lysosomal neuraminidase activity can be purified as a high molecular mass complex with ß-galactosidase and the protective protein and results from association of the protective protein with a lysosomal neuraminidase polypeptide. Further purification of the lysosomal neuraminidase polypeptide from the purified ß-galactosidase/neuraminidase/ protective protein complex therefore requires dissociation of the complex. As the unassociated neuraminidase polypeptide is catalytically inactive, a reconstitution assay must be developed to monitor the protein during further purification.

Treatment of bovine testis glycoproteins with 1.5 M KSCN at 0 °C, an approach known to inactivate the human placental neuraminidase by dissociation of the complex [17], did not result in dissociation of the bovine testis complex (data not shown). However, neuraminidase activity was rapidly lost upon incubation at pH 8.0 and 37 °C (fig. 1). Sucrose density gradient centrifugation experiments were performed to analyze the effect of incubation at increased pH on the composition of the complex. The purified complex consists of the ß-galactosidase/protective protein multimer (peak II) with small amounts of the ß-galactosidase/neuraminidase/protective protein multimer (peak III) and unassociated ß-galactosidase (peak I) (fig. 2a). After incubation at pH 8, resulting in the complete loss of neuraminidase activity, ß-galactosidase is exclusively recovered at peak I (fig. 2b), demonstrating that the complex is completely dissociated.

On basis of our knowledge on the placental neuraminidase, reconstitution of neuraminidase activity is expected to occur when fractions containing the catalytically inactive neuraminidase polypeptide are concentrated in the presence of a source containing ß-galactosidase and protective protein, but lacking the neuraminidase polypeptide. Previously,

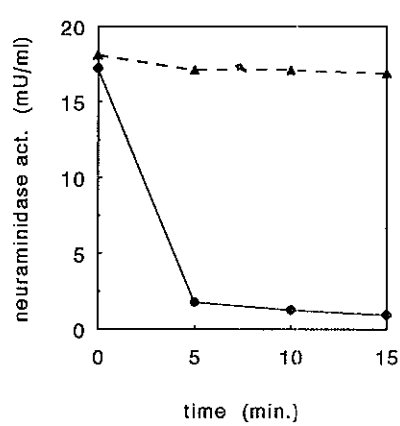

Fig. 1. Inactivation of bovine testis lysosomal neuraminidase at increased pH
Bovine testis glycoproteins, containing 190 mU/ml neuraminidase, were 10-fold diluted in buffers containing 20 mM sodium phosphate, 100 mM NaCl (pH 8.0; ●) or 20 mM sodium acetate, 100 mM NaCl (pH 5.2; ▲) and incubated at 37 °C for various times.

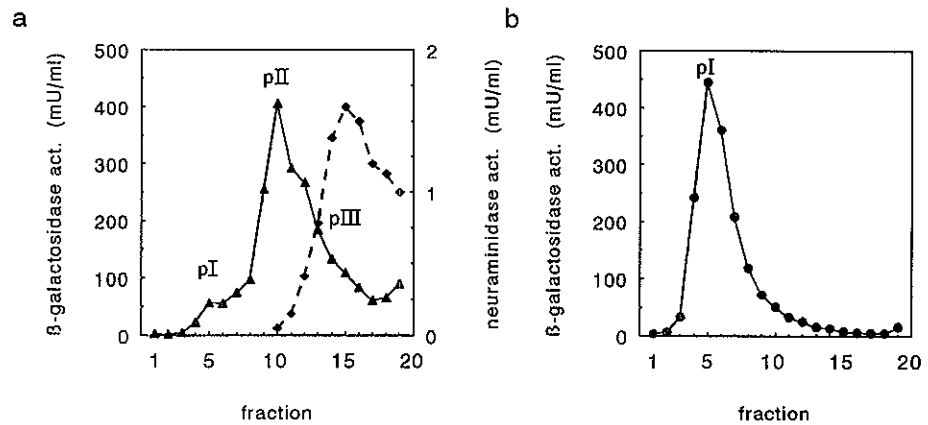

Fig. 2. Dissociation of the complex at increased pH
Distribution of ß-galactosidase (▲, ●) and neuraminidase (◆) activity after sucrose density centrifugation of 60 µl untreated purified bovine testis complex (a) or 60 µl purified bovine testis complex, dissociated under standard conditions (b).

we have described the preparation of a human placental glycoprotein preparation devoid of the neuraminidase polypeptide. The applicability of such a preparation for detection of the bovine testis neuraminidase polypeptide relies on the formation of hybrid complexes. The reconstitution assay was used to monitor the inactive bovine testis neuraminidase polypeptide during removal of ß-galactosidase by PAPS-Gal-Seph. chromatography of the dissociated complex. Approximately 15 % (390 µg) of the recovered protein and less than 0.1 % of the original ß-galactosidase activity was present in the unretained fraction. By employing the reconstitution assay, a considerable amount of neuraminidase was detected in the unretained fraction. The individual components of the reconstitution system were insufficient to generate neuraminidase activity (table 1a). Incubation of this reconstituted neuraminidase activity at 37 °C for 90 min resulted in a 3-fold increase in neuraminidase activity, which after sucrose

Table 1. Reconstitution of neuraminidase activity
Purified bovine testis complex was dissociated under standard conditions and subjected to PAPS-Gal-Seph. chromatography.
a) Reconstitution of the unretained and retained fraction (150 µl) with human placental glycoproteins lacking the neuraminidase polypeptide (1 ml); b) Reconstitution of the unretained fraction (125 µl) with sucrose gradient purified peak II from bovine testis purified complex (400 µl).

	sample	neur. act. (mU/ml) activated	stabilized
a	unretained	0.23	0.32
	retained	<0.01	<0.01
	HP GP^{-neur}	<0.01	0.07
	unretained + HP GP^{-neur}	11.81	34.79
	retained + HP GP^{-neur}	0.98	3.41
b	unretained	0.78	0.89
	BT compl.	0.98	0.74
	unretained + BT compl.^{pII}	9.34	21.38

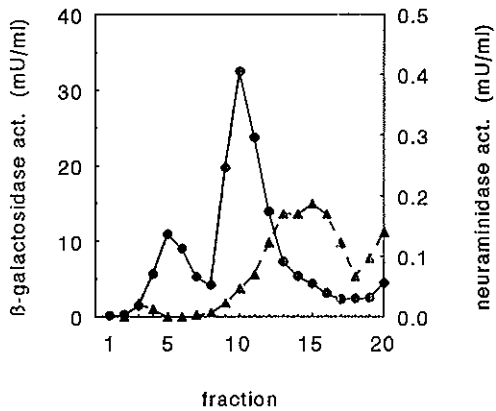

Fig. 3. Sucrose density centrifugation of the bovine/human hybrid complex
Distribution of the ß-galactosidase (●) and neuraminidase (▲) activity after sucrose density centrifugation of 30 μl reconstituted bovine/human hybrid ß-galactosidase/neuraminidase/protective protein complex.

density centrifugation was shown to be associated with the high density multimeric form of ß-galactosidase (fig. 3). This demonstrates that the reconstitution assay can be used to monitor the inactive bovine testis neuraminidase polypeptide and that the bovine testis neuraminidase in the bovine/human hybrid complex behaves identical to its human placental counterpart with respect to generation and stabilization of neuraminidase activity. Moreover, the dissociated bovine testis neuraminidase polypeptide can be partially purified by PAPS-Gal-Seph. chromatography. An estimation of the relative specific activities reveals a 58-fold enrichment of the bovine testis neuraminidase polypeptide in the unretained over the retained fraction.

Isolation of the neuraminidase depleted human placental glycoprotein preparation is laborious and yields relative small quantities, whereas large amounts are required for further chromatographic purification steps. Table 1b shows that peak II from purified bovine testis complex (pooled fractions 8-11, fig. 2a) can also be used for reconstitution assays. Since these sucrose density centrifugation fractions are easily prepared, they form an excellent substitute for the human placental neuraminidase polypeptide deficient glycoprotein preparation in large scale purification experiments

The protein pattern of the partially purified bovine testis neuraminidase polypeptide preparation is shown in figure 4a. In addition to the 64, 32 and 20 kDa proteins, representing ß-galactosidase and the polypeptides of the protective protein, three other proteins with molecular masses around 75, 55 and 42 kDa are observed. Despite the ease at which the inactive neuraminidase polypeptide could be detected with our reconstitution assay, additional chromatographic procedures like gelpermeation, ionexchange, hydrophobic interaction, or hydroxylapatite chromatography SDS-polyacrylamide gel electrophoresis did not reveal a significant further separation of these proteins (data not shown). We have determined the

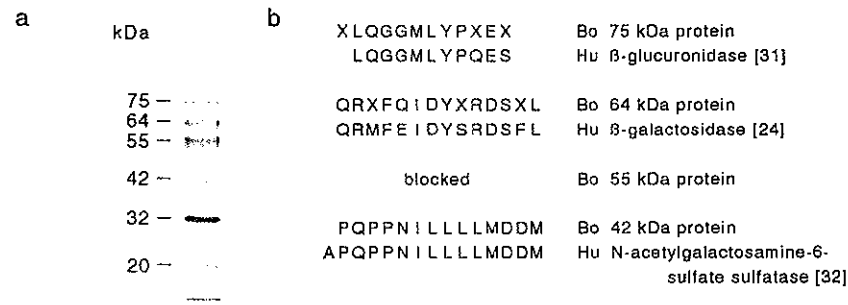

Fig. 4. Protein composition and NH_2-terminal amino acid sequencing of the partially purified bovine testis lysosomal neuraminidase polypeptide preparation
The lysosomal neuraminidase polypeptide was partially purified from the dissociated bovine testis ß-galactosidase/neuraminidase/protective protein complex by PAPS-Gal-Seph. chromatography. Panel a: SDS-polyacrylamide gel electrophoresis of the partially purified preparation on a 10 % gel. Proteins were stained with Coomassie Brilliant Blue. Panel b: NH_2-terminal sequence of proteins in the partially purified preparation and homology to known proteins.

NH_2-terminal amino acid sequence of the remaining proteins in the neuraminidase polypeptide enriched preparation. As shown in figure 4b, all proteins could be identified as known lysosomal proteins except for the 55 kDa protein which appeared to be blocked.

Identification of active site residues

To delineate the type of amino acids involved in the catalytic mechanism of the lysosomal neuraminidase, we have performed chemical modification experiments. In this type of experiments, substrate-protectable inactivation of an enzyme by amino acid side-chain specific reagents indicates that this particular residue is located in or near the active site. As the lysosomal neuraminidase is only active at sufficiently low pH, our choice of modifying agents was limited to DEP, specific for histidines and EDAC, specific for carboxylic amino acids. Incubation of bovine testis lysosomal neuraminidase with DEP resulted in a concentration dependent inactivation of neuraminidase activity (fig. 5a). DEP also reacts to some extend with cysteine, lysine and tyrosine residues, but modification of these amino acids is not reversed by hydroxylamine treatment. After complete inactivation of neuraminidase with DEP, 25 % of the initial neuraminidase activity could be restored by treatment with 300 mM hydroxylamine, indicating that histidine residues had been modified. The lack of complete restoration of activity may be explained by dissociation of the complex at pH 7. DEP treatment of lysosomal neuraminidase in the presence of the competitive inhibitors NeuAc (K_i=6.3 mM) and NeuAc2en (K_i=13 μM) prevented the modification of histidine residues in a concentration and affinity dependent way (fig. 5b). Similarly,

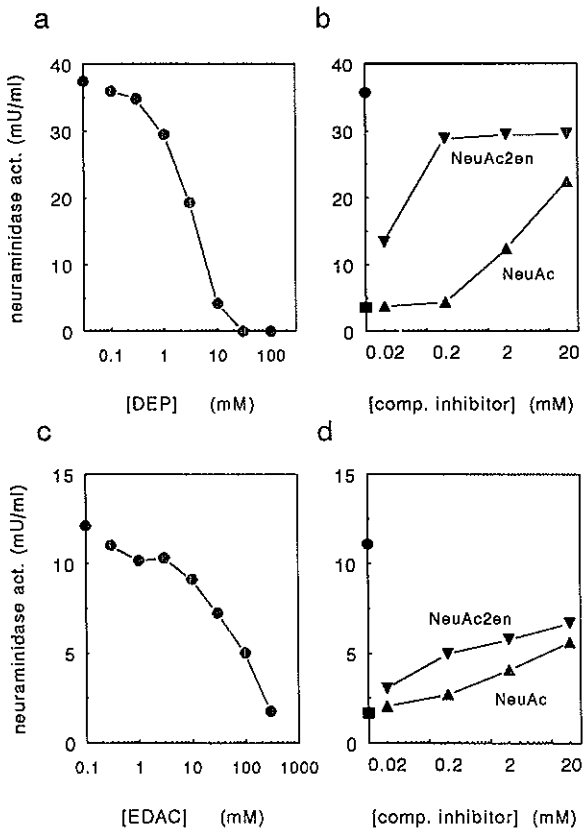

Fig. 5. Chemical modification of lysosomal neuraminidase from bovine testis
Bovine testis glycoprotein preparations containing active lysosomal neuraminidase were treated with amino acid modifying agents as described in the "Materials and methods" section. Shown are the residual neuraminidase activities (●) after incubation with various concentrations DEP (panel a) or EDAC (panel c) and the protection of neuraminidase activity against inactivation with 10 mM DEP (panel b) or 300 mM EDAC (panel d) by various concentrations NeuAc (▲) or NeuAc2en (▼). Unmodified neuraminidase activities (●) and neuraminidase activities, modified in absence of protecting agents (■) are indicated on the y-ordinate.

NeuAc/NeuAc2en protectable modification of carboxylic amino acid residues with EDAC resulted in the loss of neuraminidase activity (fig. 5c and d). From these data we conclude that histidine and aspartic/glutamic acid residues are involved in the catalytic mechanism of lysosomal neuraminidase.

Discussion

In the present study we have partially purified the lysosomal neuraminidase polypeptide from bovine testis via dissociation of the purified ß-galactosidase/neuraminidase/ protective protein complex at increased pH and removal of ß-galactosidase and most of the protective protein by ß-galactosidase specific affinity chromatography. The inactive neuraminidase polypeptide was successfully monitored via a reconstitution assay. In addition to small amounts of ß-galactosidase and the protective protein, the partially purified lysosomal neuraminidase protein, was shown to contain proteins with molecular masses around 75, 55 and 42 kDa. These proteins could not be further separateded by conventional chromatographic procedures. NH_2-terminal amino acid sequencing has identified the 75 kDa protein as ß-glucuronidase and the 42 kDa protein as N-acetylgalactosamine-6-sulfate sulfatase. Since the corresponding enzymatic activities were not shown to copurify with the complex, we consider these proteins as contaminants. The 55 kDa protein appears to be blocked. The molecular mass of this protein matches that of the bovine testis neuraminidase polypeptide, previously identified by photoaffinity labeling [20], and preliminary sequencing results obtained with tryptic peptides of the 55 kDa protein revealed no homology to known proteins. Therefore, we conclude that the latter protein is the bovine testis lysosomal neuraminidase polypeptide. Detailed sequence analysis of the 55 kDa protein, currently underway, will form the basis for the cloning of the mammalian neuraminidase polypeptide.

Our reconstitution experiments also reveal that the dissociated 55 kDa bovine testis neuraminidase polypeptide parallels the behaviour of the human placental 66 kDa neuraminidase polypeptide with respect to its activation and stabilization properties. The formation of hybrid complexes demonstrates that the bovine polypeptide is able to substitute for its human placental counterpart. We have also shown that the bovine testis neuraminidase polypeptide associates with free human placental ß-galactosidase and protective protein as well as with preexisting bovine testicular ß-galactosidase/protective protein multimers. Previous studies have shown that ß-galactosidase in human placenta is able to associate with preexisting neuraminidase polypeptide/protective protein complexes [18]. Taken together, these data indicate that the generation of active stabile neuraminidase does not require a vast order of association events. Besides, association of the neuraminidase polypeptide to ß-galactosidase linked protective protein confirms the presence of separate binding sites on the protective protein for the two hydrolases, as previously suggested [18].

To get insight in the catalytic mechanism of the lysosomal neuraminidase, we have performed chemical modification experiments. NeuAc and NeuAc2en protectable inactivation of the enzyme with DEP and EDAC demonstrated that histidine and carboxylic amino acid residues are involved in neuraminidase activity. In vitro mutagenesis experiments with

influenza virus neuraminidase, performed on basis of the existing knowledge about conserved amino acid residues and the three dimensional structure of the active neuraminidase molecule, have resulted in the proposal of a catalytic mechanism for the viral enzyme [33]. A histidine residue (His274) is proposed to transfer its proton to a nearby glutamic acid (Glu276) residue which in turn uses this proton to open the glycosidic bond between the sialic acid residue and the adjacent sugar. Our chemical modifiction results suggest that lysosomal neuraminidase may very well act via a similar reaction mechanism. In addition, these findings may facilitate the mutational analysis of the active site of lysosomal neuraminidase and the identification of amino acid substitutions that affect the kinetic parameters of the enzyme in sialidosis patients.

References

1 Corfield, A.P., Michalski, J.C. and Schauer, R. (1981) in Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 3-70, Edi. Ermes, Milan

2 Fingerhut, R., van der Horst, G.T.J., Verheijen, F.W. and Conzelmann, E. (1992) Eur. J. Biochem. **208**, 623-629

3 Cantz, M., Gehler, J. and Spranger, J. (1977) Biochem. Biophys. Res. Commun. **74**, 732-738

4 Beaudet, A.L. and Thomas, G.H. (1989) in The metabolic basis of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. eds.), pp. 1603-1621, McGraw-Hill, New York

5 Wenger, D.A., Tarby, T.J. and Wharton, C. (1978) Biochem. Biophys. Res. Commun. **82**, 589-595

6 Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W.S. and Dodson, W.E. (1981) in Sialidases and sialidoses (Tettamanti, G. Durand, P. and Di Donato, S. eds.), Perspectives in inherited metabolic diseases, Vol.4 pp. 379-395, Ermes, Milan

7 O'Brien, J.S. (1989) in The metabolic basis of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. eds.), pp. 1797-1806, McGraw-Hill, New York

8 d'Azzo, A., Hoogeveen, A.T., Reuser, A.J., Robinson, D. and Galjaard, H. (1982) Proc. Natl. Acad. Sci. USA **79**, 4535-4539

9 Hoogeveen, A., d'Azzo, A., Brossmer, R. and Galjaard, H. (1981) Biochem. Biophys. Res. Commun. **103**, 292-300

10 Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1983) J. Biol. Chem. **258**, 12143-12146

11 Kase, R., Itoh, K., Takiyama, N., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) Biochem. Biophys. Res. Commun. **172**, 1175-1179

12 Tranchemontagne, J., Michaud, L. and Potier, M. (1990) Biochem. Biophys. Res. Commun. **168**, 22-29

13 Hoogeveen, A.T., Verheijen, F.W., d'Azzo, A. and Galjaard, H. (1980) Nature **285**, 500-502

14 Galjart, N.J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E.J. and d'Azzo, A. (1991) J. Biol. Chem. **266**, 14754-14762

15 Verheijen, F.W., Brossmer, R. and Galjaard, H. (1982) Biochem. Biophys. Res. Commun. **108**, 868-875

16 Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985) Eur. J. Biochem. **149**, 315-322

17 Verheijen, F.W., Palmeri, S. and Galjaard, H. (1987) Eur. J. Biochem. **162**, 63-67
18 van der Horst, G.T.J., Galjart, N.J., d'Azzo, A., Galjaard, H. and Verheijen, F.W. (1989) J. Biol. Chem. **264**, 1317-1322
19 van der Horst, G.T.J., Mancini, G.M.S., Brossmer, R., Rose, U. and Verheijen, F.W. (1990) J. Biol. Chem. **265**, 10801-10894
20 van der Horst, G.T.J., Rose, U., Brossmer, R. and Verheijen, F.W. (1990) FEBS Lett. **277**, 42-44
21 Galjart, N.J., Gillemans, N., Harris, A., van der Horst, G.T.J., Verheijen, F.W., Galjaard, H. and d'Azzo, A. (1988) Cell **54**, 755-764
22 Oshima, A., Tsuji, A., Nagao, Y., Sakuraba, H. and Suzuki, Y. (1988) Biochem. Biophys. Res. Commun. **157**, 238-244
23 Morreau, H., Galjart, N.J., Gillemans, N., Willemsen, R., van der Horst, G.T.J. and d'Azzo, A. (1989) J. Biol. Chem. **264**, 20655-20663
24 Morreau, H., Galjart, N.J., Willemsen, R., Gillemans, N., Zhou, X.Y. and d'Azzo, A. (1992) J. Biol. Chem. **267**, 17949-17956
25 Itoh, K., Takiyama, N., Kondoh, K., Sano, A., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) J. Biol. Chem. **268**, 1180-1186
26 Laemmli, U.K. (1970) Nature **227**, 680-685
27 Aebersold, R.H., Teplow, D.B., Hood, L.E. and Kent, S.B.H. (1986) J. Biol. Chem. **261**, 4229-4238
28 Penefsky, H.S. (1977) J. Biol. Chem. **252**, 2891-2899
29 Galjaard, H. (1980) in Genetic metabolic diseases: Early diagnosis and prenatal analysis. Elsevier/North-Holland, Amsterdam
30 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275
31 Oshima, A., Kyle, J.W., Miller, R.D., Hoffmann, J.W., Powell, P.P., Grubb, J.H., Sly, W.S., Tropak, M., Guise, K.S. and Gravell, R.A. (1987) Proc. Natl. Acad. Sci. USA **84**, 685-689
32 Tomatsu, S. *et al.* (1991) Biochem. Biophys. Res. Commun. **181**, 677-683
33 Lentz, M.R., Webster, R.G. and Air, G.M. (1987) Biochemistry **26**, 5351-5358

Publication 5

Degradation of gangliosides by the lysosomal sialidase requires an activator protein
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Degradation of gangliosides by the lysosomal sialidase requires an activator protein

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Lysosomal sialidase, which was formerly believed to degrade only water-soluble substrates but not glycolipids, cleaves ganglioside substrates II^{3}Neu*N*Ac-LacCer, IV^{3}Neu*N*Ac, II^{3}Neu*N*Ac-GgOse$_4$Cer, IV^3 Neu*N*Ac, II^3(Neu*N*Ac)$_2$-GgOse$_4$Cer when these are dispersed either with an appropriate detergent (taurodeoxycholate) or with the sulfatide activator protein, a physiologic lipid solubilizer required for the lysosomal hydrolysis of other glycolipids by water-soluble hydrolases. In the presence of the activator protein, time and protein dependence were linear within wide limits, while the detergent rapidly inactivated the enzyme.

The disialo group of the b-series gangliosides was only poorly attacked by the enzyme when the lipids were dispersed with the activator protein, whereas in the presence of the detergent, they were hydrolyzed as fast as terminal sialic acid residues.

With the appropriate assay method, significant ganglioside sialidase activity could be demonstrated in the secondary lysosome fraction of normal skin fibroblasts but not of sialidosis fibroblasts.

Our results support the notion that there is only one lysosomal sialidase, which degrades both the water-soluble and the membrane-bound sialyl glycoconjugates.

The individual steps in the lysosomal catabolism of glycolipids are generally well established and the enzymes involved have been identified and characterized. One of the few exceptions to this rule is the removal of *N*-acylneuraminic acid (sialic acid) residues from gangliosides. The only sialidase found in lysosomes was so far believed to act only on water-soluble substrates ('oligosaccharide sialidase') but not on glycolipids [1–3]. The lack of ganglioside accumulation in the brains of patients with a deficiency of lysosomal sialidase ('sialidosis') [4, 5] seemed to corroborate the notion that this enzyme does not play any significant role in ganglioside catabolism. A specific ganglioside-degrading sialidase had been demonstrated in many tissues but this activity was consistently found to be localized in the plasma membrane [2, 3] (for reviews on sialidases, see [6, 7]). Earlier reports on the deficiency of a specific ganglioside sialidase in patients with mucolipidosis type IV [8–10] were disproved later [11–13].

Significant ganglioside storage was meanwhile found in non-neural tissues of sialidosis patients [5] and accumulation of ganglioside II^{3}Neu*N*Ac-LacCer (G_{M3}) was observed in cultured sialidosis fibroblasts after feeding radiolabeled ganglioside II^{3}Neu*N*Ac-GgOse$_4$Cer (G_{M1}) [14], indicative of a broader role of lysosomal sialidase. It was also shown that, in the presence of appropriate detergents, this enzyme acts on ganglioside substrates, too [12, 15]. Such detergents are, however, not present in lysosomes. Instead, the interaction between water-soluble enzymes and their membrane-bound glycolipid substrates is frequently mediated by more or less specific lipid-solubilizing proteins, so-called 'activator proteins' (for reviews, see [16–18]). Two proteins of this type have been well characterized. One is highly specific for the hydrolysis of ganglioside II^{3}Neu*N*Ac-GgOse$_3$Cer (G_{M2}) and closely related glycolipids by β-hexosaminidase A [19]. The other has a much broader substrate and enzyme specificity. Originally, it was isolated as a cofactor for the degradation of sulfatide by arylsulfatase A [20], and was therefore named 'sulfatide activator'. *In vitro*, it also accelerates the hydrolysis of ganglioside G_{M1} by β-galactosidase and of globotriaosylceramide by α-galactosidase A [21, 22]. Its physiological role, other than in sulfatide catabolism, is not yet clear. The inherited deficiency of this protein leads to a variant of metachromatic leukodystrophy [23].

Preliminary feeding studies with activator-protein-deficient fibroblasts [24] had indicated that the action of lysosomal sialidase on ganglioside substrates also depends on the presence of the sulfatide activator. We have now studied the interaction of lysosomal sialidase with various ganglioside substrates in the presence of this activator protein. The results clearly show that this enzyme does indeed degrade gangliosides and that the sulfatide activator is required to mediate the interaction with the substrate.

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Abbreviations. Cer, ceramide or *N*-acylsphingosine; 4-MeUmb, 4-methylumbelliferone; Con A, concanavalin A; ganglioside G_{M3}, II^{3}Neu*N*Ac-LacCer; ganglioside G_{D3}, II^3(Neu*N*Ac)$_2$-LacCer; ganglioside G_{M2}, II^{3}Neu*N*Ac-GgOse$_3$Cer; ganglioside G_{D2}, II^3(Neu*N*Ac)$_2$-GgOse$_3$Cer; ganglioside G_{M1}, II^{3}Neu*N*Ac-GgOse$_4$Cer; ganglioside G_{D1a}, IV^{3}Neu*N*Ac, II^{3}Neu*N*Ac-GgOse$_4$Cer; ganglioside G_{D1b}, II^3(Neu*N*Ac)$_2$-GgOse$_4$Cer; ganglioside G_{T1b}, IV^{3}Neu*N*Ac, II^3(Neu*N*Ac)$_2$-GgOse$_4$Cer.

Enzymes. Lysosomal sialidase, *N*-acylneuraminyl hydrolase (EC 3.2.1.18).

MATERIALS AND METHODS

Reagents were purchased from the following companies: Dulbecco's minimum essential medium from Flow; fetal calf serum and trypsin from Boehringer; DEAE-Sepharose CL-6B and Percoll from Pharmacia; *Clostridium perfringens* sialidase, sodium taurodeoxycholate, sodium cholate, AMP, Triton X-100, 4-methylumbelliferone (4-MeUmb) and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (4-MeUmb-Glc*N*Ac) from Sigma Chemie; 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (4-MeUmbNeuNAc) from Melford Laboratories; diagnostic X-ray film X-OMAT™ AR from Kodak; phosphate determination kit from Serva; precoated thin-layer plates (Kieselgel 60) from Merck; radiochemicals from Amersham Buchler. All other reagents were analytical grade or of the highest purity available.

Gangliosides G_{M3}, IV^{3}Neu*N*Ac, II^{3}Neu*N*Ac-GgOse$_4$Cer (G_{D1a}), II^3(Neu*N*Ac)$_2$-GgOse$_4$Cer (G_{D1b}) and IV^{3}Neu*N*Ac, II^3(Neu*N*Ac)$_2$-GgOse$_4$Cer (G_{T1b}), isolated from calf brain [25], ^{3}H-labelled by catalytic reduction of the sphingosine double bond with [^{3}H]$NaBH_4$ [26] and separated by a combination of ion-exchange chromatography and chromatography on Iatrobeads 6RS-8060 [27, 28], were kindly supplied by G. Scheel. They were repurified by preparative thin-layer chromatography, if necessary. Specific radioactivities were as follows: G_{M3}, 3800; G_{D1a}, 630; G_{D1b}, 300; G_{T1b}, 280 GBq/mol.

Lysosomal sialidase was purified from human placenta by chromatography on Concanavalin-A – Sepharose ('Con-A fraction') and affinity chromatography on *p*-aminophenyl-β-D-thiogalactoside – Sepharose as described [29]. The partially purified Con-A fraction was used for most experiments. Sulfatide activator protein and G_{M2}-activator protein were purified from human liver and kidney, respectively, as described previously [16, 17].

Human skin fibroblasts from the various patients and from normal controls were grown in monolayers to confluency in 175-cm^2 flasks as previously described [30]. For subcellular fractionation of fibroblasts, the cells from two flasks were harvested by scraping in 10 ml 150 mM NaCl and 10 mM sodium phosphate, pH 7.4. Homogenization of the cells, fractionation in a self-forming Percoll gradient and assay of the marker enzymes β-hexosaminidase (lysosomes) and 5'-nucleotidase (plasma membrane) were performed as previously described [31].

Assay of sialidase activity with the synthetic 4-MeUmbNeu*N*Ac as substrate [28]

Appropriately diluted enzyme samples were incubated with 0.2 mM 4-MeUmbNeu*N*Ac in a total volume of 100 µl 35 mM sodium acetate, pH 4.5, for 60 min. The reaction was terminated by the addition of 0.5 ml 0.2 M Na_2CO_3 and 0.2 M glycine and liberated 4-MeUmb was quantified fluorimetrically (excitation at 366 nm, emission at 440 nm).

Enzymatic degradation of ganglioside substrates

A solution of the respective ganglioside (1 nmol) in methanol was added to the incubation vial and the solvent evaporated under a stream of N_2. Buffer, enzyme and other additions were to a final volume of 50 µl 50 mM acetate, pH 4.5. After incubation at 37 °C for the times indicated, the assay mixtures were put on ice and immediately loaded onto 0.5-ml columns of DEAE – Sepharose equilibrated with methanol. Products were separated from unreacted substrates by an adaptation of a previously published method [27, 28]. When G_{M3} was the substrate, products were eluted with 4 ml methanol, then the remaining G_{M3} with 4 ml 15 mM ammonium acetate in methanol, and their respective radioactivities were quantified by liquid scintillation counting. For the disialogangliosides as substrates, ammonium acetate concentrations in the respective eluents were 15 mM for products and 40 mM for substrates; for G_{T1b} they were 40 mM for products and 100 mM for the substrate. All assays were performed at least in duplicate.

Identification of the degradation products of ganglioside G_{D1b}

Assays containing 12 nmol [^{3}H]ganglioside G_{D1b}, 86 µg partially purified sialidase (Con-A fraction) and other additions as given in the legend of Fig. 5, were incubated in a total volume of 200 µl 50 mM acetate, pH 4.5, for 20 h. The mixtures were then lyophilized, resuspended in methanol and applied to a thin-layer plate. The plates were developed in chloroform/methanol/15 mM aqueous $CaCl_2$ (60:35:8, by vol.) and the radioactive spots were visualized by fluorography [32].

Protein determination

Protein was measured with the dye-binding method of Bradford [33].

RESULTS

The degradation of several gangliosides (G_{M3}, G_{D1a}, G_{D1b} and G_{T1b}) by a partially purified preparation of lysosomal sialidase (Con-A fraction) was measured in the presence of either sodium taurodeoxycholate (Fig. 1a) or purified sulfatide activator protein (Fig. 1b). With the detergent as solubilizer, stimulation of the reaction was only observed at concentrations above the critical micellar concentration of taurodeoxycholate (0.8 mM at pH 4.5 [34]), with optimal effect at 2 – 2.5 mM. At even higher detergent concentrations, reaction rates decreased again, presumably due to a more rapid inactivation of the enzyme, as indicated by the time course at different detergent concentrations (Fig. 2a). Ganglioside G_{M3} was hydrolyzed quite rapidly, while the degradation of higher gangliosides was generally much slower.

In the presence of the activator protein, reaction rates increased almost linearly with the amount of activator added (Fig. 1b). Within the activator concentration range tested, hydrolysis of G_{M3} was still slower (approximately 20%) than at the optimal concentration of sodium taurodeoxycholate, but since it was still in the linear range, much higher reaction rates would probably be attainable with higher activator concentrations, whereas with the detergent, the maximum had been reached. The turnover of G_{D1a} and G_{T1b} was already in the same range as that obtained with the detergent. Surprisingly, G_{D1b} was not degraded by lysosomal sialidase under these conditions. The experiment with G_{M3} and sulfatide activator was repeated with an affinity-purified sialidase preparation (Fig. 1c), to show that indeed lysosomal sialidase and not some contaminating non-lysosomal enzyme, was responsible for the reaction.

At the optimal detergent concentration, the reaction initially proceeded quite fast, but slowed down very soon and ceased completely after 2 – 3 h (Fig. 2a). Addition of more enzyme after 6 h resulted in additional ganglioside hydrolysis.

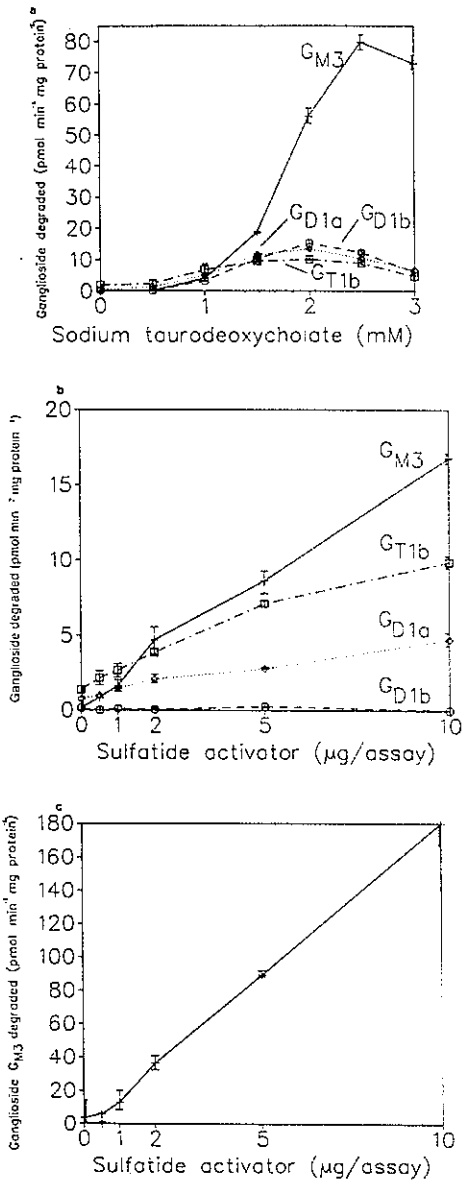

Fig. 1. Degradation of ganglioside substrates by lysosomal sialidase. The [^{3}H]ganglioside substrates (1 nmol/assay) were incubated with partially purified sialidase (Con-A fraction, 86 μg protein) (a, b) or affinity purified enzyme (1.4 μg protein) (c) with the indicated concentrations of sodium taurodeoxycholate (a) or sulfatide activator protein (b, c), in a total volume of 50 μl 50 mM sodium acetate, pH 4.5, for 1 h (a, b) or 5 h (c) at 37°C. Products were separated from unreacted substrate by ion-exchange chromatography as described in the Materials and Methods section.

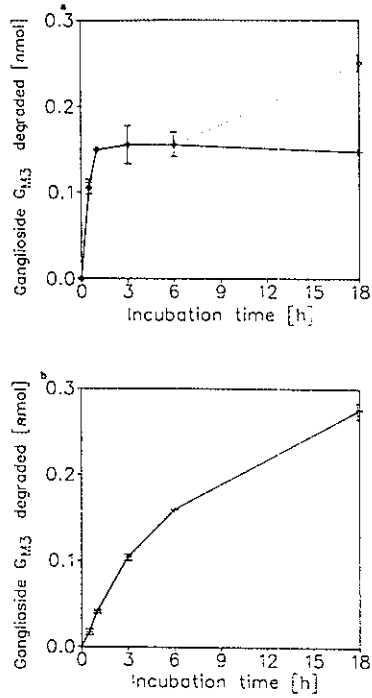

Fig. 2. Degradation of ganglioside G_{M3} by lysosomal sialidase as a function of time. (a) [^{3}H]Ganglioside G_{M3} (1 nmol/assay) was incubated with partially purified sialidase (Con-A fraction, 21.5 μg protein) with 2 mM sodium taurodeoxycholate in a total volume of 50 μl 50 mM sodium acetate, pH 4.5, at 37°C for the times indicated. In one series of assays, an additional 21.5 μg enzyme in 5 μl of the same buffer was added after 6 h (dotted line). Products were separated from unreacted substrate by ion-exchange chromatography as described in the Materials and Methods section. (b) Assays were as in (a) but with 86 μg enzyme preparation and with 5 μg sulfatide activator protein instead of detergent.

indicating that denaturation of the enzyme and not substrate depletion (or product inhibition) was responsible for the observed time course. In the presence of the activator protein, the reaction was linear with time for some 3 h and an appreciable activity was still observed even after 18 h (Fig. 2b).

The activator-protein-stimulated reaction had a pH optimum around pH 4.3 (Fig. 3a) and was inhibited by higher ionic strength (Fig. 3b), similar to other reactions of this type [35, 36].

In subcellular fractions of human skin fibroblasts, the sialidase activity with the synthetic 4-MeUmbNeuAc as substrate co-distributed exactly with the marker enzyme β-hexosaminidase (Fig. 4a). When degradation of ganglioside G_{M3} was measured in the presence of taurodeoxycholate as detergent, the activity was found to be distributed almost evenly between lysosomal and light-membrane (plasma membrane) fractions, whereas, with the neutral detergent Triton X-100, activity was only found in the plasma-membrane fractions (Fig. 4b), confirming the findings of Lieser et al. [12].

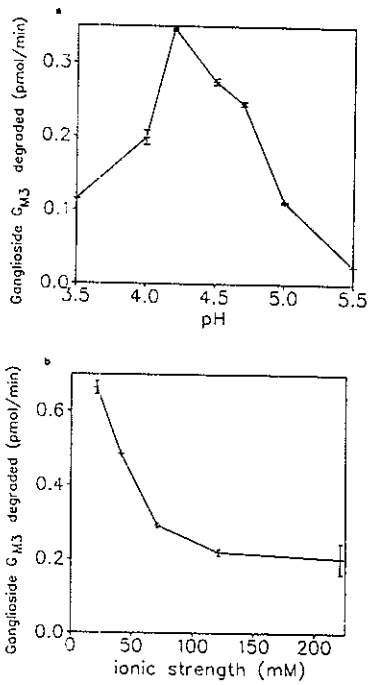

Fig. 3. Degradation of ganglioside G_{M3} by lysosomal sialidase as a function of pH (a) and ionic strength (b). (a) [^{3}H]Ganglioside G_{M3} (1 nmol/assay) was incubated with partially purified sialidase (Con-A fraction, 43 μg protein) and 5 μg sulfatide activator protein in a total volume of 50 μl 50 mM sodium formate (pH 3.5 and 4.0) or sodium acetate (other pH values) at the indicated pH and 37°C for 3.5 h. Appropriate concentrations of NaCl were included to adjust the ionic strength to 50 mM in all assays. (b) Assay mixtures with [^{3}H]ganglioside G_{M3} (1 nmol), partially purified sialidase (Con-A fraction, 43 μg protein), 5 μg sulfatide activator protein and 0–200 mM NaCl in a total volume of 50 μl 50 mM sodium acetate, pH 4.5, were incubated at 37°C for 2 h. In both experiments, products were separated from unreacted substrate by ion-exchange chromatography as described in the Materials and Methods section.

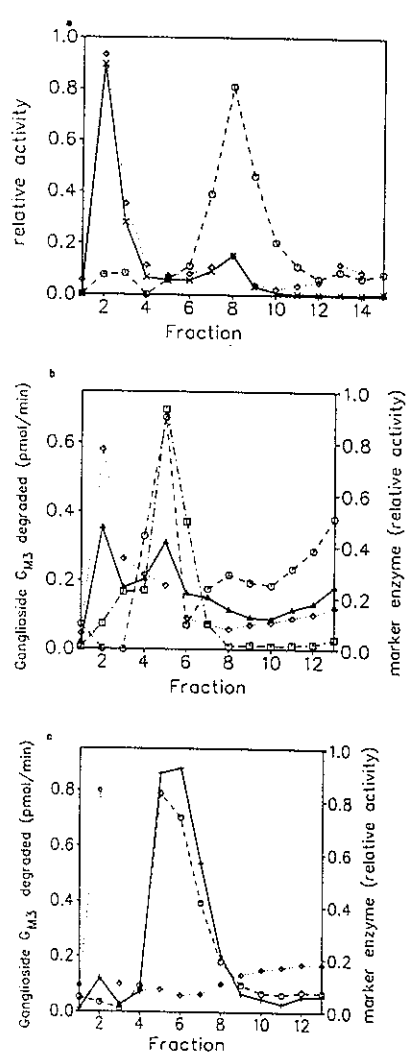

Fig. 4. Subcellular distribution of sialidase activity. Cultured skin fibroblasts were homogenized and fractionated on a self-forming Percoll density gradient as described in the Materials and Methods. Fractions were assayed for marker enzymes β-hexosaminidase (◇····◇, lysosomes) and 5′-nucleotidase (⊖——⊖, plasma membranes) and for sialidase activity with (a) synthetic 4-MeUmbNeu*N*Ac (×——×), (b) ganglioside G_{M3} in the presence of either 2 mM sodium taurodeoxycholate (△——△) or 0.1% Triton X-100 (□——□) and (c) ganglioside G_{M3} in the presence of 5 μg sulfatide activator protein/assay (+——+).

When the reaction was stimulated with the sulfatide activator instead of detergents, the bulk of activity was again found in the light-membrane fractions, with only a small but significant peak of lysosomal activity (Fig. 4c). Disruption of the membranes by sonication did not increase the reaction rate (not shown), essentially ruling out structural latency of the lysosomal enzyme as an explanation for this rather surprising result. In sialidosis fibroblasts, only the activity in the light-membrane fraction was seen; no activity could be detected in the lysosomes (not shown).

The observation that G_{D1b} is an extremely poor substrate for lysosomal sialidase prompted further studies on the degradation of b-series gangliosides by lysosomal enzymes. Radiolabelled ganglioside G_{T1b} (10 nmol) was incubated for 29 h with 14 μg affinity-purified sialidase preparation and 20 μg activator protein under standard conditions. The products were then separated into monosialogangliosides, disialogangliosides and trisialogangliosides by ion-exchange chromatography. Unreacted trisialoganglioside comprised 88.6% of the total radioactivity, 10.1% was in the disialo fraction

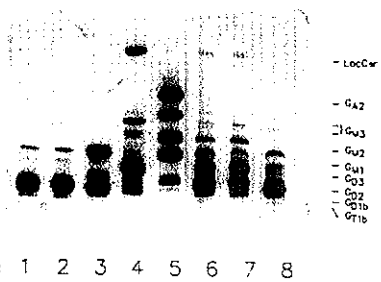

Fig. 5. Degradation of ganglioside G_{D1b} by lysosomal enzymes. [^{3}H]Ganglioside G_{D1b} (12 nmol) was incubated with partially purified lysosomal enzyme preparation (Con-A fraction) and activator proteins as indicated below, in 200 µl 50 mM sodium acetate, pH 4.5, at 37°C for 20 h. After lyophilization, assays were resuspended in methanol and the products were separated by thin-layer chromatography in chloroform/methanol/15 mM aqueous $CaCl_2$ (60:35:8, by vol.). Radioactive spots were visualized by fluorography. Lane 1; control, 86 µg bovine serum albumin; lane 2, control, 86 µg bovine serum albumin, 20 µg sulfatide activator, 6.5 µg G_{M2} activator; lane 3, ganglioside standards; lane 4, 86 µg enzyme protein, 20 µg sulfatide activator, 6.5 µg G_{M2} activator (complete assay); lane 5, ganglioside standards; lane 6, 86 µg enzyme protein, 6.5 µg G_{M2} activator; lane 7, 86 µg enzyme protein, 20 µg sulfatide activator; lane 8, 86 µg enzyme protein. LacCer, lactosylceramide.

and 1.3% in the monosialo fraction. As expected, the latter was mainly ganglioside G_{M2}. (Note that the affinity ligand used for enzyme purification is specific for β-galactosidase and that the sialidase just co-purifies as a complex with β-galactosidase.) When an aliquot of the isolated disialoganglioside fraction was incubated with sialidase from *C. perfringens*, it was converted almost exclusively to ganglioside G_{M2}, indicating that it had mainly consisted of ganglioside G_{D2} (which had been formed from G_{D1b} by the β-galactosidase). Thus the $\alpha2\rightarrow3$ disialo linkage seems to be much more resistant to the action of lysosomal sialidase than the terminal sialic acid moiety of ganglioside G_{T1b}.

When ganglioside G_{D1b} was incubated with a Con-A-purified enzyme preparation, which contains essentially all soluble lysosomal enzymes, only very little turnover was observed without addition of the activator proteins (Fig. 5, lane 8). Any one of the two activators, sulfatide activator (Fig. 5, lane 7) or G_{M2} activator (lane 6), stimulated product formation to some extent, the intermediates mainly accumulating being gangliosides II^3(Neu*N*Ac)$_2$-GgOse$_3$Cer (G_{D2}) and II^3(Neu*N*Ac)$_2$-LacCer (G_{D3}). When both activator proteins were added simultaneously (Fig. 5, lane 4), the substrate, ganglioside G_{D1b}, was almost completely hydrolyzed, with mainly ganglioside G_{D3} accumulating as intermediate. Significant amounts of G_{M3} and lactosylceramide were also formed, but the $\alpha2\rightarrow8$ disialo linkage seemed to be the bottle neck of the degradation of b-series gangliosides.

DISCUSSION

For the lysosomal degradation of glycoconjugates, there is, with only few exceptions, only one glycosidase for each anomer of each sugar residue. The same enzyme is usually responsible for the hydrolysis of glycosaminoglycans, oligosaccharides, glycopeptides and glycolipids, depending on the occurrence of the respective sugar residue. Most of these enzymes are water-soluble and, as outlined above, cannot directly attack their lipid substrates, which are membrane bound, but require the assistance of so-called activator proteins, which solubilize the lipid. The physiological significance of these activator proteins is demonstrated by the fact that deficiency of such a protein, e.g. in the activator deficiency variant of G_{M2} gangliosidosis ('variant AB') [37] or in a variant form of metachromatic leukodystrophy (sulfatide activator deficiency) [23] leads to the same fatal accumulation of glycolipids as a deficiency of the degrading enzyme in the other variants of these diseases. Patients with sulfatide activator deficiency excrete, in their urinary sediment, not only high amounts of sulfatide but also other glycolipids such as globotriaosylceramide ('Fabry glycolipid') [21], which is a substrate of α-galactosidase, indicating a broader physiologic role of the 'sulfatide activator'. The observation that cultured fibroblasts of these patients accumulate significant amounts of ganglioside G_{M3} when fed with radiolabeled higher gangliosides [24] suggested the possibility that this protein might also be required (or at least very helpful) for the action of lysosomal sialidase on gangliosides. The conflicting results reported on the ability of lysosomal sialidase to degrade gangliosides may then be ascribed to the non-physiological assay conditions used.

The experiments presented here show that lysosomal sialidase ('oligosaccharide sialidase') does degrade ganglioside substrates in the presence of the sulfatide activator. The enzyme's substrate specificity under these conditions is remarkable in two respects. First, the $\alpha2\rightarrow8$ disialo linkage of the b-series gangliosides, which is chemically rather unstable, is quite resistant against enzymatic cleavage in the presence of the activator. This cannot be due to a general inability of the enzyme to attack this kind of linkage, since in the presence of taurodeoxycholate, G_{D1b} is hydrolyzed at similar rates to G_{D1a} and G_{T1b} (Fig. 1a). Given the wide range of glycolipids bound and solubilized by the sulfatide activator [22, 38–40], it also seems unlikely that the activator should not bind this substrate (particularly since it does bind G_{T1b}). It rather appears that the activator protein serves not merely as a solubilizer, but interacts with the enzyme in a specific way, which determines the enzyme's substrate specificity.

Secondly, in the presence of the activator protein, the higher gangliosides G_{D1a} and G_{T1b} are degraded more slowly than G_{M3}, although they are more polar and, probably owing to their better solubility, are attacked to a significant extent in the absence of any activating agent (Fig. 1b). This finding is reminiscent of the case of ganglioside G_{D1a}-Gal*N*Ac, which has the same branched terminal structure as ganglioside G_{M2}, Gal*N*Ac($\beta1\rightarrow4$)[Neu*N*Ac($\alpha2\rightarrow3$)]Gal, but is more polar and is hydrolyzed by hexosaminidase A at a significant rate in the absence of any activating factor. The reaction is not accelerated by the G_{M2} activator (which is nonetheless able to bind and solubilize the lipid) [41]. This observation was readily explained by the highly specific interaction between the G_{M2} activator and hexosaminidase A [19, 42].

When the subcellular distribution of ganglioside sialidase activity is measured with detergent-based assays, the results depend strongly on the type of detergent used. With Triton X-100, the activity is found exclusively on the plasma membrane whereas, with bile salts, a clear peak of lysosomal activity can be distinguished (Fig. 4b; [12]). This difference can be explained by the different physicochemical behaviour of the two enzymes. The plasma membrane sialidase behaves like an integral membrane protein and interacts with its ganglioside

substrates via lateral diffusion within the membrane (or mixed micelles, in the presence of excess detergents) [28]. Its action is stimulated by any agent which increases membrane fluidity and/or accelerates insertion of exogenously added lipids into the membrane. If the detergent concentration exceeds the amount required for maximal fluidization of the membrane, reaction rates decrease again because the volume of the hydrophobic phase, to which both enzyme and substrate are confined, is increased and hence the substrate is diluted [28]. A characteristic of this phenomenon is that the optimal detergent concentration depends almost linearly on the amount of protein (i.e. membrane) [12].

Lysosomal sialidase, in contrast, although being membrane associated to some extent, behaves like a water-soluble enzyme and requires solubilization of its lipid substrates in such a way that the sialic acid residues are exposed. Bile salts fulfil this condition, since they form small mixed micelles [43] from which the oligosaccharide chains of glycolipids protrude into the aqueous phase. At the same time, these detergents denature the enzyme, the rate of inactivation being roughly proportional to the detergent concentration. The observed 'optimal concentration' results from the superposition of these two effects and depends on a number of variables such as pH, ionic strength and incubation time. The Triton type detergents cannot stimulate such a reaction, probably because their polar poly(oxyethylene) chains are long enough to cover the oligosaccharide moieties completely, thus shielding the glycolipid against attack from the water phase.

In the presence of the sulfatide activator, the activity of ganglioside sialidase measured in the lysosomes was much lower than in the plasma membrane fraction. These data can, however, not be taken at face value but are probably strongly distorted by the different physicochemical behaviour of the two enzymes, as mentioned above. The plasma-membrane enzyme is an integral membrane protein and meets its lipid substrates via lateral diffusion within the same membrane [28]. The activator protein promotes insertion of the (micellar) glycolipid into the membrane [44] and thus accelerates the overall reaction considerably. The same phenomenon, insertion of the lipid into a membrane, has the opposite effect in the case of the lysosomal enzyme; extraction of the lipid from a phospholipid bilayer, to solubilize it as activator-lipid complex, is energetically much less favourable than from a micelle [44]. As a consequence, in the presence of membranes, the concentration of the activator-lipid complex (i.e. the actual substrate concentration) is quite small and the enzymatic reaction is slow. Strong inhibition by small amounts of phospholipids or cell membranes is a characteristic finding when the hydrolysis of a glycolipid by a water-soluble glycosidase, in the presence of an activator protein, is studied *in vitro* [36, 38, 44, 45]. (*In vivo*, the activators are concentrated in the lysosome and thus attain concentrations high enough to compensate for the unfavourable equilibrium and to ensure a rapid turnover of the lipid.)

From the information now available, the following pattern emerges for the overall catabolism of gangliosides. Some sialic acid residues may be removed from higher gangliosides by specific enzymes located on the plasma membrane [46, 47]. This does, however, not proceed beyond the stage of G_{M1}, leaving all following steps, including G_{M3}, to lysosomal degradation. Also, intact gangliosides may be ingested by the lysosome before having been attacked by other sialidases, as evidenced by their lysosomal accumulation in several mucopolysaccharidoses [48] and in sialidosis [5]. In any case, lysosomes must possess ganglioside sialidase activity.

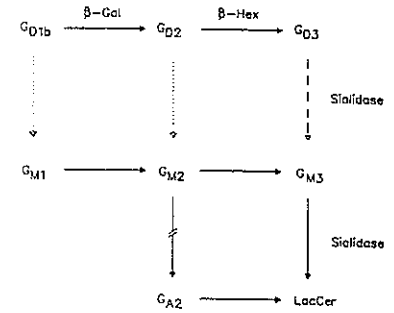

Fig. 6. Proposed pathway of lysosomal ganglioside degradation. LacCer, lactosylceramide; G_{A2}, Gal*N*Ac(β1→4)Gal(β1→4)Glc(β1-1)Cer.

The complete deficiency of lysosomal sialidase activity against both soluble and lipid substrates in sialidosis [11, 12] argues strongly for the existence of only one lysosomal sialidase, which would act on all kinds of glycoconjugate substrates, including glycolipids. Our results show that this enzyme can indeed degrade ganglioside substrates under the conditions that prevail in the lysosome (presence of activator proteins, absence of detergents) so that there is no need to postulate the existence of another lysosomal sialidase specific for gangliosides. The general pathway of lysosomal ganglioside degradation seems to be as outlined in Fig. 6; the b-series gangliosides are mainly converted to G_{D3}, which is then hydrolyzed to G_{M3}. Probably, G_{D3} is not much better as substrate for lysosomal sialidase than G_{D2} or G_{D1b} (unfortunately, we did not have the substrates to verify this) but, being in a critical bottle-neck position, attains a sufficiently high concentration to ensure normal degradation. Plasma membrane sialidase may have a role in this process in nervous tissue.

An obvious paradox that remains to be resolved is that patients with a complete deficiency of lysosomal sialidase (sialidosis) do not show any significant ganglioside accumulation in brain, which has the highest ganglioside concentration and turnover of all organs [49]. The existence of a second lysosomal sialidase, which is specific for gangliosides and is expressed only in brain, cannot be ruled out altogether, but seems very unlikely since all other lysosomal enzymes are ubiquitous and, although there may be some quantitative variation between different cell types, organ-specific expression has not yet been found. A role for the plasma-membrane enzyme in this process is much more likely. Gangliosides, like almost all membrane components, reach the digestive compartment by intracellular membrane flow [50]. The plasma-membrane sialidase, which resides in the same membrane, is quite likely to be endocytosed and delivered to the lysosome along with the gangliosides (and the rest of the membrane). This enzyme is optimally active at an acidic pH of 4–4.5 [51, 52] and may hydrolyze a substantial amount of ganglioside substrates before being degraded itself by proteases. In most cases, a small residual activity of a lysosomal enzyme, usually less than 5% of the normal amounts, is sufficient to cope with normal substrate turnover [53, 54]. In contrast, plasma-membrane sialidase is most highly concentrated in nerve-cell membranes [6, 55] and thus even a small fraction of the enzyme being active in the lysosome may be sufficient to retard or even prevent accumulation of

gangliosides in the brain. Since plasma-membrane sialidase is not entirely specific for gangliosides, but also attacks water-soluble substrates [56–58], this hypothesis would also explain why, in sialidosis patients, the accumulation of water-soluble bound sialic acid is much lower in brain than in other tissues [5].

We are greatly indebted to Dr. G. Scheel for supplying us with tritiated ganglioside substrates, and to R. Hurwitz and Dr. F. Sarmientos for their gifts of purified activator proteins. The skillful assistance of Claudia Mies and Gabriele Weiss in the cell-culture work is also gratefully acknowledged. Our sincere thanks are due to Prof. K. Sandhoff for his continuous support and for helpful discussions.

REFERENCES

1. Sander, M., Veh, R. & Schauer, R. (1979) in *Glycoconjugates 1977* (Gregory, J. D. & Jeanloz, R. W., eds) pp. 927–931, Academic Press, New York.
2. Zeigler, M. & Bach, G. (1981) *Biochem. J. 198*, 505–508.
3. Van Dessel, G., De Wolf, M., Lagrou, A., Hilderson, H. & Dierick, W. (1984) *J. Biochem. (Tokyo) 96*, 937–947.
4. Bérard, M., Toga, M., Bernard, R., Dubois, D., Mariani, R. & Hassoun, J. (1968) *Pathol. Eur. 3*, 172–183.
5. Ulrich-Bott, B., Klem, B., Kaiser, R., Spranger, J. & Cantz, M. (1987) *Enzyme 38*, 262–266.
6. Veh, R. W. & Sander, M. (1981) In *Perspectives in inherited metabolic diseases* (Tettamanti, G., Durand P. & DiDonato, S., eds) vol. 4, pp. 71–109, Edizione Ermes, Milano.
7. Conzelmann, E. & Sandhoff, K. (1987) *Adv. Enzymol. 60*, 89–216.
8. Bach, G., Zeigler, M., Schaap, T. & Kohn, G. (1979) *Biochem. Biophys. Res. Commun. 90*, 1341–1347.
9. Caimi, L., Tettamanti, G., Berra, B., Omodeo Sale, F., Borrone, C., Gatti, R., Durand, P. & Martin, J. J. (1982) *J. Inherited Metab. Dis. 5*, 218–224.
10. Ben-Yoseph, Y., Momoi, T., Hahn, L. C. & Nadler, H. L. (1982) *Clin. Genet. 21*, 374–381.
11. Zeigler, M., Sury, V. & Bach, G. (1989) *Eur. J. Biochem. 198*, 505–508.
12. Lieser, M., Harms, E., Kern, H., Bach, G. & Cantz, M. (1989) *Biochem. J. 260*, 69–74.
13. Schneider-Jakob, H. R. & Cantz, M. (1991) *Biol. Chem. Hoppe-Seyler 372*, 443–450.
14. Mancini, G. M. S., Hoogeveen, A. T., Galjaard, H., Månsson, J. E. & Svennerholm, L. (1986) *Hum. Genet. 73*, 35–38.
15. Hiraiwa, M., Uda, Y., Nishizawa, M., & Miyatake, T. (1987) *J. Biochem. 101*, 1273–1279.
16. Conzelmann, E. & Sandhoff, K. (1987) in *Methods of biochemical analysis* (Glick, D., ed.) vol. 32, pp. 1–23, Wiley, New York.
17. Conzelmann, E. & Sandhoff, K. (1987) *Methods Enzymol. 138*, 792–815.
18. Conzelmann, E. (1989) In *Molecular basis of membrane-associated diseases* (Azzi, A., Drahota, Z. & Papa, S., eds) pp. 379–394, Springer Verlag, Berlin.
19. Conzelmann, E. & Sandhoff, K. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1837–1849.
20. Fischer, G. & Jatzkewitz, H. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 605–613.
21. Li, S.-C., Kihara, H., Serizawa, S., Li, Y.-T., Fluharty, A. L., Mayes, J. S. & Shapiro, L. J. (1985) *J. Biol. Chem. 260*, 1867–1871.
22. Vogel, A., Fürst, W., Abo-Hashish, M. A., Lee-Vaupel, M., Conzelmann, E. & Sandhoff, K. (1987) *Arch. Biochem. Biophys. 259*, 627–638.
23. Stevens, R. L., Fluharty, A. L., Kihara, H., Kaback, M. M., Shapiro, L. J., Marsh, B., Sandhoff, K. & Fischer, G. (1981) *Am. J. Hum. Genet. 33*, 900–906.
24. Conzelmann, E., Lee-Vaupel, M. & Sandhoff, K. (1988) in *Lipid storage disorders: biological and medical aspects* (Salvayre, R., Douste-Blazy, L. & Gatt, S., eds) *NATO ASI Series A*, vol. 150, pp. 323–332, Plenum, New York.
25. Svennerholm, L. (1972) *Methods Carbohydr. Chem. 6*, 464–474.
26. Schwarzmann, G. (1978) *Biochim. Biophys. Acta 529*, 106–114.
27. Momoi, T., Ando, S. & Nagai, Y. (1976) *Biochim. Biophys. Acta 441*, 488–497.
28. Scheel, G., Acevedo, E., Conzelmann, E., Nehrkorn, H. & Sandhoff, K. (1982) *Eur. J. Biochem. 127*, 245–253.
29. Verheijen, F., Palmeri, S. & Galjaard, H. (1987) *Eur. J. Biochem. 162*, 63–67.
30. Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U. & Sandhoff, K. (1985) *Eur. J. Biochem. 149*, 247–255.
31. Banerjee, A., Burg, J., Conzelmann, E., Carroll, M. & Sandhoff, K. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 347–356.
32. Randerath, K. (1970) *Anal. Biochem. 34*, 188–205.
33. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
34. Conzelmann, E. (1979) Ph. D. Thesis, Ludwig-Maximilians-Universität, München.
35. Fischer, G. & Jatzkewitz, H. (1978) In *Enzymes of lipid metabolism* (Gatt, S., Freysz, L. & Mandel, P., eds), pp. 573–582, Plenum, New York.
36. Conzelmann, E., Kytzia, H.-J., Navon, R. & Sandhoff, K. (1983) *Am. J. Hum. Genet. 35*, 900–913.
37. Conzelmann, E. & Sandhoff, K. (1978) *Proc. Natl Acad. Sci. USA 75*, 3979–3983.
38. Fischer, G. & Jatzkewitz, H. (1978) *Biochim. Biophys. Acta 528*, 69–76.
39. Wenger, D. A. & Inui, K. (1984) In *Molecular basis of lysosomal storage disorders* (Brady, R. O. & Barranger, J. A., eds) pp. 61–78, Academic Press, New York.
40. Mitsuyama, T., Gasa, S., Nojima, T., Taniguchi, T. & Makita, A. (1985) *J. Biochem. 98*, 605–613.
41. Meier, E. M., Schwarzmann, G., Fürst, W. & Sandhoff, K. (1991) *J. Biol. Chem. 266*, 1879–1887.
42. Kytzia, H.-J. & Sandhoff, K. (1985) *J. Biol. Chem. 260*, 7568–7572.
43. Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
44. Conzelmann, E., Burg, J., Stephan, G. & Sandhoff, K. (1982) *Eur. J. Biochem. 123*, 455–464.
45. Lee-Vaupel, M. & Conzelmann, E. (1987) *Clin. Chim. Acta 168*, 55–68.
46. Sandhoff, K., Schwarzmann, G., Sarmientos, F. & Conzelmann, E. (1987) in *Gangliosides and modulation of neuronal function*, *NATO ASI Series*, vol. H7 (Rahmann, H., ed.) pp. 231–250, Springer Verlag, Berlin.
47. Riboni, L., Prinetti, A., Bassi, R. & Tettamanti, G. (1991) *FEBS Lett. 287*, 42–46.
48. Constantopoulos, G. & Dekaban, A. S. (1978) *J. Neurochem. 30*, 965–970.
49. Svennerholm, L. (1980) in *Structure and function of gangliosides* (Svennerholm, L., Mandel, P., Dreyfus, H. & Urban, P.-F., eds) pp. 533–544, Plenum Press, New York.
50. Schwarzmann, G., Sonderfeld, S., Conzelmann, E., Marsh, D. & Sandhoff, K. (1984) in *Cellular and pathological aspects of glycoconjugate metabolism* (Dreyfus, H., Massarelli, R., Freysz, L. & Rebel, G., eds) *INSERM*, vol. 126, pp. 195–210.
51. Leibovitz, Z. & Gatt, S. (1968) *Biochim. Biophys. Acta 152*, 136–142.
52. Öhman, R., Rosenberg, A. & Svennerholm, L. (1970) *Biochemistry 9*, 3774–3782.
53. Conzelmann, E. & Sandhoff, K. (1983/84) *Dev. Neurosci. 6*, 58–71.
54. Leinekugel, P., Michel, S., Conzelmann, E. & Sandhoff, K. (1992) *Hum. Genet. 88*, 513–523.
55. Carubelli, R., Trucco, R. E. & Caputto, R. (1962) *Biochim. Biophys. Acta 60*, 196–200.
56. Tettamanti, G. & Zambotti, V. (1968) *Enzymologia 35*, 61–74.
57. Sandhoff, K. & Pallmann, B. (1978) *Proc. Natl Acad. Sci. USA 75*, 122–126.
58. Michalski, J.-C., Corfield, A. P. & Schauer, R. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1097–1102.

Summary

Sialic acids, a group of derivatives of the acid amino sugar neuraminic acid, mainly exist in α-glycosidic linkage with other sugars as part of oligosaccharides, glycoproteins and glycolipids and as such are involved in many biological processes. In the catabolic pathway of sialic acid metabolism, the enzyme responsible for the hydrolysis of these α-glycosidic bonds is neuraminidase (sialidase). Neuraminidases have been described in a variety of microorganisms and viruses and are commonly occurring in vertebrates (chapter 1).

According to their subcellular localization, three different mammalian neuraminidases have been distinguished: a membrane bound lysosomal neuraminidase, a membrane bound plasma membrane neuraminidase and a soluble cytosolic neuraminidase. Whereas the lysosomal neuraminidase is indisputably involved in the lysosomal degradation of sialooligosaccharides and sialoglycoconjugates, the exact function of the other two enzymes is not known. In contrast to bacterial and viral neuraminidases, knowledge about the mammalian enzymes is limited. Due to their lability, low abundancy and/or membrane bound character, mammalian neuraminidases have hardly been purified till homogeneity and as a result the corresponding genes have not been cloned yet (chapter 2).

From a medical point of view, the lysosomal neuraminidase is interesting because its deficiency is associated with the disease. The lysosome represents the primary site for intracellular digestion of macromolecules into their original building blocks, which after transport to the cytosol are reutilized or further degraded. For this purpose the lysosome is equipped with a large battery of substrate specific hydrolases, activator proteins and transport proteins. A deficiency of one of these lysosomal proteins results in the storage of undegradable substrates or untransportable reaction products. Massive intracellular accumulation and urinary excretion of sialooligosaccharides as a result of a defective lysosomal neuraminidase is observed in two lysosomal storage disorders. In sialidosis, only lysosomal neuraminidase activity is deficient but the exact molecular nature of the defect has not yet been demonstrated. In galactosialidosis, clinically resembling sialidosis, a combined deficiency of lysosomal neuraminidase and ß-galactosidase exists as a result of a deficient protective protein. The latter protein is a cathepsin A-like carboxypeptidase that, independent of its protease activity, protects ß-galactosidase from enhanced intralysosomal degradation by multimerization with the latter into a high molecular mass complex and acts as a subunit for neuraminidase by association with an otherwise catalytically inactive neuraminidase polypeptide. In fact, a soluble form of lysosomal neuraminidase has been shown to occur in a high molecular mass complex with ß-galactosidase and its protective protein and as such could be purified at high specific activity via a ß-galactosidase specific affinity column (chapter 2 and 4).

Since at least 23 different sialic acids exist in nature and sialic acids are found in different types of compounds (i.e. oligosaccharides, glycoproteins, glycolipids), neuraminidases are confronted with a wide variety of potential substrates. The substrate specificity of viral and bacterial neuraminidases is well documented and with the cloning of their respective genes, the exploration of the structure of the active site has started. In case of influenza A virus neuraminidase even a model for the reaction mechanism has been proposed (chapter 3). For mammalian neuraminidases however, progress has been much slower.

The experimental work, discussed in chapter 4 of this thesis, is focussed on the identification of the mammalian lysosomal neuraminidase protein and the involvement of other proteins in neuraminidase activity, stability and substrate specificity.

The lysosomal neuraminidase polypeptide in purified mammalian ß-galactosidase/ neuraminidase/protective protein complexes was identified via two approaches. **Publication 1** describes the application of a neuraminidase specific antibody for the immunological identification of the human placental neuraminidase polypeptide. Isolation of the human placental neuraminidase typically requires the generation and stabilization of enzymatic activity by concentration of the glycoprotein fraction and incubation at 37 °C respectively. Immunoprecipitation of a 66 kDa protein from the glycoprotein preparation prevented the generation of neuraminidase activity, suggesting that this is the neuraminidase polypeptide that upon association with the protective protein is responsible for catalytic activity. In a second approach, reported in **publication 2**, we have developed a neuraminidase specific affinity probe, not available thusfar. Introduction of a radioiodinatable photoreactive group in the potent competitive neuraminidase inhibitor NeuAc2en rendered a photoaffinity probe for molecular recognition of neuraminidases on the basis of their catalytic activity. The applicability of this probe for the specific labeling of neuraminidases was demonstrated by the successful labeling of the *Clostridium perfringens* neuraminidase. Moreover, CNBr cleavage of the labeled protein demonstrated that our probe may also be used for identification of active site containing regions within the protein. In **publication 3**, the photoaffinity probe was used to label the bovine testis ß-galactosidase/neuraminidase/ protective protein complex. A 55 kDa protein was identified as the bovine testis neuraminidase polypeptide.

The involvement of the protective protein and ß-galactosidase in neuraminidase activity was investigated in **publications 1** and **4**. Via reconstitution assays with human placental glycoprotein preparations, lacking the neuraminidase polypeptide or ß-galactosidase, or containing partially reduced protective protein, all three components of the complex were shown to be required for neuraminidase activity. Although reversible generation of labile

neuraminidase activity only depends on association of the neuraminidase polypeptide with the protective protein, stabilization of this activity by incubation at 37 °C requires the presence of ß-galactosidase. The absence of neuraminidase activity in galactosialidosis fibroblasts and decreased stability of neuraminidase activity in ß-galactosidase deficient G_{M1}-gangliosidosis fibroblasts point to a similar situation *in vivo*. In addition, our experiments suggest that the protective protein contains different binding domains for ß-galactosidase and the neuraminidase polypeptide. In contrast to human placenta, bovine testis homogenates contain a stable ß-galactosidase/neuraminidase/protective protein complex with catalytically active neuraminidase. After dissociation at increased pH, resulting in the loss of neuraminidase activity, the components of the bovine testis complex demonstrated a similar behaviour with respect to generation and stabilization of neuraminidase activity as the corresponding human placental proteins. Moreover, the bovine testis neuraminidase polypeptide is able to substitute its human placental counterpart in a hybrid complex.

Publication 4 also describes the further purification of the bovine testis neuraminidase polypeptide after dissociation of the purified complex, and the determination of amino acid residues involved in the catalytic mechanism of the enzyme. In line with the photoaffinity labeling experiments, amino acid sequencing of the remaining proteins in the purified preparation revealed a thusfar unknown amino acid sequence for the 55 kDa protein. Chemical modification experiments with the bovine testis lysosomal neuraminidase demonstrated that, analogous to influenza virus neuraminidase, histidine and acidic amino acids are involved in the catalytic mechanism of lysosomal neuraminidase.

The substrate specificity of human placental lysosomal neuraminidase towards gangliosides is described in **publication 5**. In the presence of suitable detergents lysosomal neuraminidase has been shown to degrade glycolipid substrates. We demonstrate that in the absence of detergents, thus under conditions mimicking the *in vivo* situation, lysosomal neuraminidase requires the activator protein *sap*-B for the hydrolysis of gangliosides. In contrast to gangliosides G_{M3}, G_{T1b} and G_{D1a}, ganglioside G_{D1b} is hardly cleaved, indicating that the α(2-8) disialo linkage forms the bottleneck in the degradation of the b-series gangliosides.

On the basis of the work presented in this thesis, it is expected that in the near future the cDNA and gene encoding the lysosomal neuraminidase polypeptide will be cloned, which in turn will enable the detailed analysis of the structure and function of this protein

Samenvatting

Siaalzuur, verzamelnaam voor een groep derivaten van het zure amino suiker neuraminezuur, komt voornamelijk voor in α-glycosidisch gebonden vorm als onderdeel van oligosacchariden, glycoproteïnen en glycolipiden en is betrokken bij een groot aantal biologische processen. In de katabole reactieprocessen van het siaalzuur metabolisme wordt de hydrolyse van deze α-glycosidische bindingen verzorgd door het enzym neuraminidase (sialidase). Neuraminidases zijn beschreven in een groot aantal microorganismen en virussen en komen algemeen voor in vertebraten (hoofdstuk 1).

Op grond van hun verdeling in de cel worden in zoogdieren drie verschillende neuraminidases onderscheiden: een membraan gebonden lysosomaal neuraminidase, een membraan gebonden plasma membraan neuraminidase en een oplosbaar cytosolisch neuraminidase. Terwijl het lysosomale neuraminidase overduidelijk betrokken is bij de lysosomale afbraak van siaalzuur bevattende oligosacchariden en glycoconjugaten, is de precieze functie van de andere neuraminidases niet bekend. In tegenstelling tot bacteriële en virale neuraminidases is de kennis van de zoogdier enzymen beperkt. Als gevolg van hun labiele en soms membraan gebonden karakter, alsmede de geringe hoeveelheid waarin het enzym in de cel aanwezig is, zijn zoogdier neuraminidases nauwelijks in pure vorm gezuiverd, waardoor ook de corresponderende genen nog niet zijn gecloneerd (hoofdstuk 2).

Medisch gezien is het lysosomale neuraminidase het meest interessante zoogdier neuraminidase omdat het betrokken is bij bepaalde ziekten. Het lysosoom vormt de belangrijkste locatie voor de intracellulaire afbraak van macromoleculen in hun oorspronkelijke bouwstenen welke, eenmaal naar het cytosol getransporteerd, opnieuw kunnen worden gebruikt of verder worden afgebroken. Voor dit doel is het lysosoom uitgerust met een groot aantal substraat specifieke hydrolasen, activator eiwitten en transport eiwitten. Een defect in een dergelijk lysosomaal eiwit resulteert in stapeling van niet afbreekbare substraten of niet transporteerbare reactie producten. Er zijn twee lysosomale stapelingsziekten bekend waarbij tengevolge van een beschadigd lysosomaal neuraminidase grote hoeveelheden siaalzuur bevattende oligosacchariden in de cel worden opgehoopt en in de urine worden uitgescheiden. In sialidosis is uitsluitend de neuraminidase activiteit aangedaan doch de precieze moleculaire aard van het defect is nog niet aangetoond. In galactosialidosis, klinisch sterk lijkend op sialidosis, wordt een gecombineerde deficiëntie van lysosomaal neuraminidase en ß-galactosidase gevonden als gevolg van een defect "protective protein". Dit laatste eiwit is een cathepsine A-achtige carboxypeptidase dat onafhankelijk van zijn protease functie ß-galactosidase beschermt tegen versnelde intralysosomale afbraak door met laatstgenoemde te multimerizeren in een groot complex. Ook dient het als subunit voor neuraminidase door te binden met een neuraminidase polypeptide, dat anders katalytisch

inactief zou zijn. Voor een oplosbare vorm van lysosomaal neuraminidase is aangetoond dat het aanwezig is in een groot complex met ß-galactosidase en zijn "protective protein" en als zodanig met hoge specifieke activiteit kan worden gezuiverd op een ß-galactosidase specifieke affiniteits kolom (hoofdstuk 2 en 4).

Omdat in de natuur tenminste 23 verschillende siaalzuren bestaan en siaalzuur wordt gevonden in verschillende soorten moleculen (bijv. oligosacchariden, glycoproteïnen, glycolipiden) worden neuraminidases geconfronteerd met een grote verscheidenheid aan potentiële substraten. De substraat specificiteit van virale en bacteriële neuraminidases is uitvoerig gedocumenteerd en met de clonering van de betrokken genen is een begin gemaakt met de opheldering van de structuur van het katalytisch centrum. In het geval van influenza A virus neuraminidase is zelfs een model voor het reactie mechanisme opgesteld (hoofdstuk 3). De substraat specificiteit van zoogdier neuraminidases is nog niet goed onderzocht.

Het experimentele werk, besproken in hoofdstuk 4 van dit proefschrift, richt zich op de identificatie van het zoogdier lysosomale neuraminidase eiwit en de betrokkenheid van andere eiwitten bij de neuraminidase activiteit, stabiliteit en substraat specificiteit.

Het lysosomale neuraminidase polypeptide in gezuiverde zoogdier ß-galactosidase/ neuraminidase/"protective protein" complexen werd geïdentificeerd via twee verschillende benaderingen. **Publikatie 1** beschrijft de toepassing van een neuraminidase specifiek antilichaam voor de immunologische identificatie van het neuraminidase polypeptide in menselijke placenta. Zuivering van neuraminidase uit menselijke placentae wordt gekenmerkt doordat tijdens de isolatie procedure neuraminidase activiteit gegenereerd en gestabiliseerd dient te worden door middel van concentrering, repectievelijk verwarming (37 °C) van de glycoproteïne fractie. Imunoprecipitatie van een 66 kDa eiwit uit deze preparaten voorkwam de aanmaak van neuraminidase activiteit, hetgeen suggereert dat dit eiwit het neuraminidase polypeptide is dat door binding met het "protective protein" verantwoordelijk is voor katalytische activiteit. In een tweede benadering, beschreven in **publikatie 2**, werd een op het katalytisch centrum gericht neuraminidase specifiek merker molecuul ontwikkeld. Zulke stoffen waren tot dusver niet voorhanden. Introductie van een radioiodineerbare, fotoreactieve groep in de competitieve remmer NeuAc2en resulteerde in een fotoreactief merker molecuul dat neuraminidases herkent op basis van hun katalytische activiteit. De toepasbaarheid van dit molecuul voor de specifieke markering van neuraminidases werd aangetoond doordat neuraminidase van *Clostridium perfringens* radioactief kon worden gemaakt. Fragmentering van het radioactieve eiwit met behulp van CNBr liet zien dat ons merker molecuul zelfs gebruikt kan worden voor de identificatie van die gebieden binnen het eiwit die deel uitmaken van het katalytisch centrum van het enzym. In **publikatie 3** is het fotoreactieve merker molecuul gebruikt in combinatie met het rundertestis ß-galactosidase/neuraminidase/

"protective protein" complex. In dit preparaat werd een 55 kDa eiwit geïdentificeerd als neuraminidase polypeptide.

De betrokkenheid van het "protective protein" en ß-galactosidase bij de neuraminidase activiteit werd bestudeerd in **publikaties 1** en 4. Door middel van reconstitutie experimenten met glycoproteïne preparaten van menselijke placenta die het neuraminidase polypeptide of ß-galactosidase missen, of waarin het "protective protein" gedeeltelijk werd gereduceerd, is aangetoond dat alle drie componenten van het complex nodig zijn voor neuraminidase activiteit. Hoewel de reversibele aanmaak van labiele neuraminidase activiteit alleen afhangt van binding van het neuraminidase polypeptide met het "protective protein", is ß-galactosidase nodig voor stabilisering van deze activiteit. Het ontbreken van neuraminidase activiteit in galactosialidosis fibroblasten en de verlaagde stabiliteit van neuraminidase activiteit in ß-galactosidase deficiënte G_{M1}-gangliosidosis fibroblasten duiden op een vergelijkbare situatie *in vivo*. Onze experimenten suggereren ook dat het "protective protein" verschillende bindingsplaatsen bezit voor ß-galactosidase en het neuraminidase polypeptide. In tegenstelling tot menselijke placenta bevatten rundertestis homogenaten een stabiel ß-galactosidase/neuraminidase/"protective protein" complex met actief neuraminidase. Na uiteenvallen van het complex bij verhoogde pH, resulterend in een verlies van neuraminidase activiteit blijken de componenten van het rundertestis complex met betrekking tot aanmaak en stabilisering van neuraminidase activiteit een identiek gedrag te vertonen als de corresponderende eiwitten in human placenta. Het rundertestis neuraminidase polypeptide kan zelfs het menselijk eiwit vervangen in een hybride complex.

Publikatie 4 beschrijft ook de verdere zuivering van het rundertestis neuraminidase polypeptide uit het gezuiverde complex en de bepaling van aminozuur residuen, betrokken bij het katalytisch mechanisme van het enzym. In overeenstemming met onze experimenten met het fotoreactieve merker molecuul leverden aminozuur volgorde bepalingen van de overgebleven eiwitten in het gezuiverde preparaat een tot dusver onbekende sequentie voor het 55 kDa eiwit. Chemische modificatie van het rundertestis neuraminidase liet zien dat, analoog aan het influenza virus neuraminidase, histidine en zure aminozuren betrokken zijn bij het katalytisch mechanisme van het lysosomale neuraminidase.

De substraat specificiteit van menselijk lysosomaal neuraminidase met betrekking tot gangliosiden is beschreven in **publikatie 5**. In aanwezigheid van geschikte detergentia kan het lysosomale neuraminidase glycolipide substraten afbreken. Aangetoond wordt dat lysosomaal neuraminidase in afwezigheid van detergentia (zoals ook het geval in de intacte cel) het activator eiwit sap-B nodig heeft voor de hydrolyse van gangliosiden. In tegenstelling tot gangliosiden G_{M3}, G_{T1b} en G_{D1a} wordt ganglioside G_{D1b} nauwelijks afgebroken, hetgeen aangeeft dat de α(2-8) binding tussen twee siaalzuur moleculen de bottleneck vormt tijdens de afbraak van b-serie gangliosiden.

Op grond van het werk beschreven in dit proefschrift is de verwachting dat in de nabije toekomst het cDNA en gen coderend voor het lysosomale neuraminidase polypeptide zal worden gecloneerd, hetgeen op zijn beurt een gedetaileerde analyse van de structuur en functie van het enzym mogelijk zal maken.

Dankwoord

Promoveren doe je nooit alleen! Vandaar dat ik een ieder wil bedanken die in meer of mindere mate heeft bijgedragen aan het tot stand komen van dit proefschrift.

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I also would like to express my gratitude to professor Conzellman and Dr. Fingerhut for the collaborative study on the ganglioside degrading capacity of lysosomal neuraminidase.

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Rita, wat jij voor mij betekent valt niet onder woorden te brengen. Samen staan we sterk!

Curriculum vitae

De schrijver van dit proefschrift werd geboren op 13 december 1956 te Rotterdam. Na het behalen van het diploma Atheneum B aan het St. Montfortcollege te Rotterdam werd in 1975 begonnen met de studie Biologie (B1') aan de Universiteit van Amsterdam. Het kandidaatsexamen werd afgelegd in oktober 1979. De doctoraalfase bestond uit een hoofdvak Celbiologie (Prof. Dr. H. Galjaard, Rotterdam) en bijvakken Biochemie (Prof. Dr. L.A. Grivell, Amsterdam) en Virologie (Prof. Dr. J. van de Noordaa, Amsterdam). Het doctoraalexamen werd in februari 1983 cum laude afgelegd.

Van april 1983 tot en met december 1985 was de schrijver als wetenschappelijk medewerker verbonden aan de afdeling Moleculaire Biologie (Prof. Dr. L.A. Grivell) van het Laboratorium voor Biochemie van de Universiteit van Amsterdam. In januari 1986 volgde een aanstelling als wetenschappelijk onderzoeker bij het instituut Celbiologie (Prof. Dr. H. Galjaard) van de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam, waar in 1987 een aanvang werd gemaakt met het in dit proefschrift beschreven onderzoek. Vanaf juni 1993 is de schrijver werkzaam bij het instituut Genetica (Prof. Dr. D. Bootsma) van de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam.

Stellingen behorende bij het proefschrift

IDENTIFICATION AND CHARACTERIZATION OF LYSOSOMAL NEURAMINIDASE

I

Dissociatie van het ß-galactosidase/neuraminidase/protective protein complex en reconstitutie van neuraminidase activiteit zijn noodzakelijke stappen in de zuiveringsprocedure voor het lysosomaal neuraminidase polypeptide.

Dit proefschrift

II

Het optimaal functioneren van lysosomaal neuraminidase vereist de continue samenwerking van vier verschillende lysosomale eiwitten.

Dit proefschrift

III

De bewering van Kuhn *et al.* dat de fotolabiele groep in een fotoreactieve probe voor glycosidases gelocaliseerd moet zijn in het aglycon gedeelte van een enzym-resistent glycoside is onjuist.

Kuhn et al. (1992) Bioconjugate Chem. 3:230-233

IV

Het feit dat de rol van Asp-boxen nog steeds niet systematisch is onderzocht door middel van *in vitro* mutagenese studies doet vermoeden dat deze sterk geconserveerde domains niet van belang worden geacht voor het functioneren van bacteriële neuraminidases.

V

Het gebruik van enzym-specifieke synthetische substraten is niet in alle gevallen toereikend voor de biochemische diagnose van lysosomale stapelingsziekten.

VI

Het lysosomale compartiment kan niet langer worden beschouwd als eindpunt van de intracellulaire tractus digestivus voor macromoleculen.

VII

Het is te verwachten dat met het vorderen van het "Human Genome Project" de reageerbuis in toenemende mate zal worden vervangen door de reageermuis.

VIII

De resultaten van *in vitro* DNA repair experimenten met SV40 minichromosomen, zoals beschreven door Sugasawa *et al.*, tonen aan dat conclusies over DNA excisie herstel van in chromatine verpakt DNA alleen kunnen worden getrokken wanneer de betrokkenheid van naakt DNA is uitgesloten.

Sugasawa et al. (1993) J. Biol. Chem. 268:9098-9104

IX

Het positieve effect van verhoogde *radio*activiteit in ruimten buiten het isotopenlab dient nog te worden aangetoond.

X

Als het aantal mensen met een gehoorprothese net zo hoog zou zijn als het aantal bril- of contactlensdragers, zou de term "gehoorgestoorden" waarschijnlijk al lang zijn uitgestorven.

Bert van der Horst
Rotterdam, 8 september 1993