ACTION OF *CLOSTRIDIUM PERFRINGENS* NEURAMINIDASE ON GANGLIOSIDES GM_1 AND GM_2 ABOVE AND BELOW THE CRITICAL MICELLE CONCENTRATION OF SUBSTRATE

Heikki RAUVALA

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17, Finland

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1. Introduction

Neuraminidase preparations from *Clostridium perfringens* and from other sources are generally employed for structural studies of glycolipids, glycoproteins and oligosaccharides. One problem in the study of neuraminic acid-containing glycolipids, the gangliosides, is that a proportion of sialic acid is often resistant to the action of neuraminidase. The sialic acid resistant to neuraminidase is suggested to have an α-stereoconfiguration [1,2], and the resistance to neuraminidase is assumed to be due to steric hindrance exerted by the terminal Gal-GalNAc and GalNAc residues [3] in gangliosides GM_1 and GM_2 respectively (the structures of these compounds are shown below). The neuraminic acid of these gangliosides becomes susceptible to neuraminidase after the Gal-GalNAc and GalNAc residues have been hydrolyzed from GM_1 and GM_2 respectively [4]. Accordingly, in the catabolism of these compounds, hydrolysis of sugar residues terminal from the central galactose is assumed to precede the hydrolysis of neuraminic acid [4].

Wenger and Wardell [5] have shown that the resistant neuraminic acid can be hydrolyzed by *Clostridium perfringens* neuraminidase in the presence of bile salt. It was recently shown that the enzyme preparations from *Clostridium perfringens* may contain endo-*N*-hexosaminidase activity [6]. If the hydrolysis of neuraminic acid from GM_1 and GM_2 by *Clostridium perfringens* neuraminidase is preceded by the hydrolysis of the hexosaminidic linkage, one would except that the neuraminic acid becomes susceptible to the enzymic attack. It is established in the present work that *Clostridium perfringens* neuraminidase hydrolyzes neuraminic acid from carbon 3 of galactose in GM_1 and GM_2 without prior hydrolysis of the hexosaminidic linkage and that the neuraminic acid can be hydrolyzed even without the addition of bile salt provided that the gangliosides are incubated below their critical micelle concentration.

Abbreviations: Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; AcNeu, *N*-acetylneuraminic acid; Cer, ceramide. The shorthand nomenclature of Svennerholm [7] is used for gangliosides.

2. Experimental

2.1. *Materials*

A mixture of human brain gangliosides and GM_2 from human brain (Tay-Sachs ganglioside) were made available by Professor E. Klenk (Köln, Germany). A mixture of pig brain gangliosides and GD_{1A} from pig brain were provided by Dr P. Maury (Helsinki, Finland), and the *N*-acetylneuraminic acid- and *N*-glycolylneuraminic acid-containing hematosides of bovine kidney were obtained from Dr K. Puro (Helsinki, Finland). βGal-(1→4)-βGal-(1→4)-Glc was a gift from Dr P. A. J. Gorin (Saskatoon, Saskatchewan, Canada) and βGal-(1→3)-βGlcNAc-(1→3)-βGal-(1→4)-Glc from Dr A. Gauhe (Heidelberg, Germany). Lactosylceramide was prepared from hematoside by neuraminidase treatment or by weak acid hydrolysis (0.1 M HCl, 100°C, 1 h). GM_1 was prepared from the

mixture of pig-brain gangliosides by treatment with *Vibrio Cholerae* neuraminidase (see below) followed by purification with preparative thin-layer chromatography.

2.2. *Analytical methods*

Total neuraminic acid was determined by the resorcinol method of Svennerholm [8], as modified by Miettinen and Takki-Luukkainen [9]. Free neuraminic acid was assayed by the thiobarbituric acid reaction of Warren [10]. Thin-layer chromatography was performed on 0.25 mm silica gel G plates. The solvents were chloroform/methanol/water, 60/35/8, and chloroform/methanol/2.5 M NH_4OH, 60/35/8 (v/v/v). Resorcinol-HCl [8] and $(NH_4)_2SO_4/(NH_4)$. HSO_4 [11] were used for detection. Gas-liquid chromatography was performed on a 1% OV-225 column (2 m × 3 mm, i.d.) operated isothermally at 180°C. Gas-liquid chromatography–mass spectrometry was performed with a Varian MAT CH-7 instrument equiped with a Varian Aerograph 1700 gas chromatograph and a SpectroSystem 100 MS data processing system. The ionization potential was 70 eV, and the ionization current was 300 μA.

2.3. *Neuraminidase treatment*

Clostridium perfringens neuraminidase was purchased from Sigma Chemical Co. (MO., USA; type VI neuraminidase, 0.58 units of enzyme/1 mg of solid). Ganglioside was hydrolyzed at 37°C for 20 h in the incubation mixture, which contained 1 mg of solid enzyme preparation per 1 ml of acetate buffer at pH 5.4 [5]. Sodium cholate was used in a concentration of 0.75 mg per 1 ml of the buffer [5]. The neutral glycolipid formed in the neuraminidase treatment was isolated with a Dowex 1 × 8 column and analyzed by methylation. In the experiments without cholate, glycolipids were partitioned into the lower Folch phase by using 0.1 M $CaCl_2$ at pH 2.2 instead of distilled water [12,13] and analyzed by methylation.

For the preparation of GM_1 about 5 mg of pig-brain gangliosides was dissolved in 5 ml of 0.01 M Tris-acetate buffer at pH 6.8 [14], and 250 μl of *Vibrio cholerae* neuraminidase was added (Calbiochem. Calif., USA; 500 units of enzyme/1 ml). The mixture was incubated at 37°C for 20 h, after which 250 μl of enzyme solution was again added and the incubation was continued for another 20 h. The main reaction product was GM_1, which was purified by preparative thin-layer chromatography to give a homogeneous ganglioside.

2.4. *Methylation studies*

Methylation was carried out with methyl iodide in dimethylsulfoxide in the presence of dimethylsulfinyl-carbanion [15]. The permethylated glycolipid or oligosaccharide was degraded, reduced and acetylated as described earlier [16]. Partially methylated alditol acetates were analyzed by gas-liquid chromatography [17]. The positions of methyl groups in the alditol chain were confirmed by mass spectrometry [18]. The partially methylated hexose derivatives obtained from glycolipids and oligosaccharides (see above) were used as standards.

3. Results

When the mixture of brain gangliosides or GD_{1A} was incubated with *Clostridium perfringens* neuraminidase without cholate at micellar concentration of substrate (above 10^{-4} M), the end product of the reaction was GM_1, as judged by analysis on thin-layer chromatography. When cholate was added to the incubation mixture, thin-layer chromatography revealed that GM_1 also lost its neuraminic acid, which is in agreement with the results of Wenger and Wardell [5]. The same reaction was observed even without cholate, when the concentration of ganglioside was lowered below 10^{-4} M. It was also shown by thin-layer chromatography that GM_2 was resistant to *Clostridium perfringens* neuraminidase at micellar concentration, but was readily hydrolyzed when cholate was added to the incubation mixture or when the concentration of ganglioside was lowered below 10^{-4} M.

The percentage of neuraminic acid hydrolyzed from GM_1 at various concentrations of substrate, with and without cholate, is shown in fig.1. The hydrolysis of neuraminic acid was almost complete at substrate concentrations up to 5×10^{-4} in the presence of cholate. Above 5×10^{-4} M concentration the percentage neuraminic acid hydrolyzed gradually decreased and was about 30% at 10^{-3} M concentration. In the absence of cholate at substrate concentra-

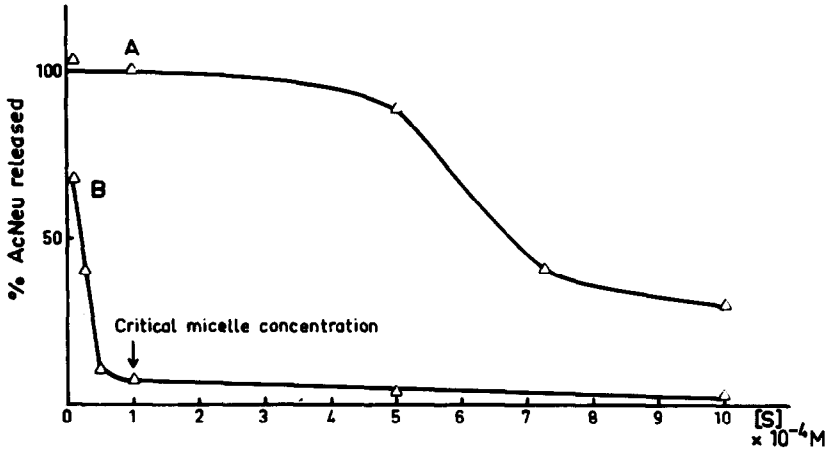

Fig.1. Percentage of neuraminic acid released from GM_1 after incubation with *Clostridium perfringens* neuraminidase at 37°C for 20 h. A, in the presence of sodium cholate; B, without cholate; [5], concentration of substrate.

tion above 10^{-4} M only traces of neuraminic acid were released. Below 10^{-4} M, the hydrolysis of neuraminic acid increased gradually and was about 70% at 10^{-5} M concentration. Probably even more than 70% of neuraminic acid was released below 10^{-5} M concentration, but it was technically difficult to measure neuraminic acid from such a dilute solution, and therefore exact numbers cannot be given.

In order to study the site from which neuraminic acid was detached, methylations were carried out before and after neuraminidase treatment. There was a clear difference in regard to partially methylated galactose derivatives before and after neuraminidase treatment (figs.2 and 3). The partially methylated hexose derivatives from GM_1 were identified as: 2,3,4,6-Me-Gal, 2,6-Me-Gal and 2,3,6-Me-Glc. After treatment with *Clostridium perfringens* neuraminidase in the presence of cholate or without cholate at submicellar concentration of the substrate (two sequential enzyme treatments to detach neuraminic acid completely), 2,3,4,6-Me-Gal, 2,3,6-Me-Gal and 2,3,6-Me-Glc were found in the molar ratio of 1:1:1 (fig.2). In methylation analysis of GM_2, 2,6-Me-Gal was also replaced by 2,3,6-Me-Gal, when neuraminic acid was released from GM_2 by the *Clostridium perfringens* enzyme prior to methylation (fig.3). These experiments establish that neuraminic acid was hydrolyzed from C-3 of the galactose residues in GM_1 and GM_2. Hydrolysis of hexosamine *plus* neuraminic acid would have produced 2,3,4,6-Me-Gal as the only methylated galactose derivate from GM_1 and GM_2. Further, hematoside would arise from the hydrolysis of the hexosaminidic linkage and lactosylceramide from the hydrolysis of hexosamine *plus* neuraminic acid. In

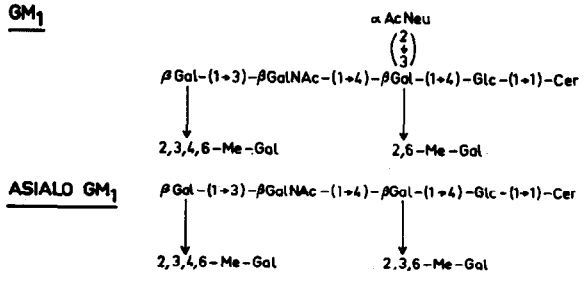

Fig.2. Partially methylated galactose derivatives obtained in methylation analysis from GM_1 and asialo GM_1.

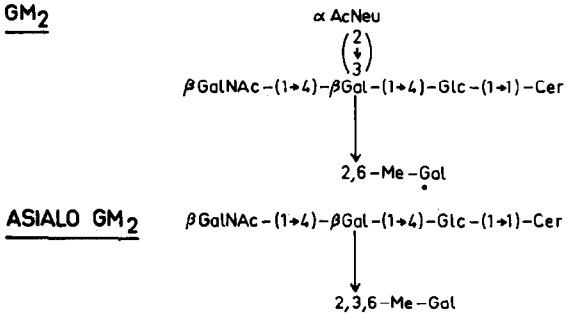

Fig.3. Partially methylated galactose derivatives from GM_2 and GM_2 treated *Clostridium perfringens* neuraminidase.

accordance with the results of methylation studies, neither of these two compounds was detected on thin-layer chromatography after digestion of GM_1 and GM_2 by *Clostridium perfringens* neuraminidase.

4. Discussion

The present work demonstrates that *Clostridium perfringens* neuraminidase hydrolyzes neuraminic acid from GM_1 and GM_2 without the prior hydrolysis of the Gal-GalNAc and GalNAc residues, which are thought to exert a steric hindrance to the action of neuraminidase on GM_1 and GM_2. Ganglioside exist in water solutions as micelles comprising of at least 200 monomers [19]. However, a striking change in the size of ganglioside aggregates occurs in dilute solutions, below the critical micelle concentration of ganglioside [19]. From measurements of surface tension and electrical conductivity of ganglioside solutions, the critical micelle concentration is 0.015 g/100 ml [19], which corresponds to about 10^{-4} M concentration of ganglioside. The value for the critical micelle concentration of ganglioside coincides well with the onset of marked hydrolysis of neuraminic acid (fig. 1), if cholate is not used in the neuraminidase incubation. When cholate is used, neuraminic acid is extensively hydrolyzed up to a substrate concentration of 5×10^{-4} M, above which the hydrolysis cannot be completed under the conditions used. This suggests that the action of bile salt in stimulating the release of neuraminic acid from GM_1 and GM_2 is due to a decrease of the size of ganglioside aggregates.

Neuraminidase treatment is an important analytical tool in structural studies on lipids containing neuraminic acid. In addition to brain, several extraneural tissues contain gangliosides, which are structurally related to GM_1 or GM_2 [20,21]. According to the present results, the treatment with *Clostridium perfringens* neuraminidase can be employed in the analysis of structures which have been regarded as resistant to neuraminidase. In such studies, however, the influence of ganglioside concentration on the hydrolysis of neuraminic acid must be taken into account.

In the catabolism of gangliosides, the hydrolysis of the terminal galactose and *N*-acetylgalactosamine residues is believed to precede the hydrolysis of the neuraminic acid attached to the central galactose [4]. However, a sialidase, which cleaves neuraminic acid from GM_2, from rat intestine has been described [22]. The concentration of substrate in the assays of the rat intestine sialidase was below 10^{-4} M. It remains to be elucidated if neuraminidases from other sources are able to cleave neuraminic acid from GM_1 or GM_2 below the critical micelle concentration of ganglioside.

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