Glycoconjugate J (1987) 4:291-295

Substrate Specificities of a Bacterial Sialidase and Rat Liver Ganglioside G_{M3} Sialyltransferase

D KLEIN, G POHLENTZ, G SCHWARZMANN and K SANDHOFF*

Institute für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, D-5300 Bonn 1, Federal Republic of Germany

Received May 19, 1987.

Key words: G_{D3} synthase, bacterial sialidase, ganglioside G_{M3} derivatives, substrate recognition

Sialidases cleave off sialic acid residues from the oligosaccharide chain of gangliosides in their catabolic pathway while sialyltransferases transfer sialic acid to the growing oligosaccharide moiety in ganglioside biosynthesis. Ganglioside G_{M3} is a common substrate for both types of enzymes, for sialidase acting on ganglioside G_{M3} as well as for ganglioside G_{D3} synthase. Therefore, it is possible that both enzymes recognize similar structural features of the sialic acid moiety of their common substrate, ganglioside G_{M3}. Based on this idea we used a variety of G_{M3} derivatives as glycolipid substrates for a bacterial sialidase (*Clostridium perfringens*) and for G_{D3} synthase (of rat liver Golgi vesicles). This study revealed that those G_{M3} derivatives that were poorly degraded by sialidase also were hardly recognized by sialyltransferase (G_{D3} synthase). This may indicate similarities in the substrate binding sites of these enzymes.

Gangliosides are glycosphingolipids that contain one or more sialic acid residue. They are synthesized in the Golgi apparatus in a stepwise manner by the sequential addition of individual sugars to the growing glycolipid. The enzymes involved are membrane-bound glycosyl- and sialyltransferases (for review see [1]). The lysosomal degradation of gangliosides is accomplished by the sequential action of water soluble glycosidases and sialidases. An inherited deficiency of one of these lysosomal enzymes results in fatal ganglioside storage disorders [2].

Ganglioside G_{M3} (monosialolactosylceramide) plays a remarkable role in ganglioside metabolism. It can act as glycolipid substrate for three enzymes, namely G_{M2} synthase (UDP-*N*-acetylgalactosamine:*N*-acetylneuraminyl-D-galactosyl-D-glucosylceramide β(1-4)-*N*-acetylgalactosaminyltransferase, EC 2.4.1.92), G_{D3} synthase (CMP-*N*-acetylneuraminate:*N*-acetylneuraminyl-D-galactosyl-D-glucosylceramide α(2-8)-sialyltransferase, EC 2.4.99.8), and sialidase (*N*-acetylneuraminylhydrolase, EC 3.2.1.18). Moreover, G_{D3} synthase and sialidase both act on the neuraminic acid residue of the G_{M3} molecule. While G_{D3} synthase transfers another sialic acid yielding G_{D3} (disialolactosylceramide),

*Author for correspondence.

sialidase hydrolyzes G_{M3} to LacCer (lactosylceramide). Using various G_{M3} analogues as glycolipid substrates in appropriate enzyme assays for a bacterial sialidase and for G_{D3} synthase in rat liver Golgi vesicles we found that even these two evolutionary quite distant enzymes recognize quite similar structural aspects of the sialic acid moiety of G_{M3}.

Materials and Methods

Synthesis of Lyso-G_{M3} Derivatives

Lyso-G_{M3} (monosialolactosylsphingosine) was prepared as described before [3]. Briefly, ganglioside G_{M3} was deacylated and, after protection of the sphingoid amino group by the fluorenylmethoxycarbonyl group, the free amino group of the neuraminic acid residue was reacetylated with acetic anhydride. After removal of the protecting group in liquid ammonia, lyso-G_{M3} was obtained in an overall yield of 14%. Acylation of lyso-G_{M3} with acetic anhydride in 5% $NaHCO_3$ solution at room temperature followed by dialysis against water and silica gel chromatography in chloroform/methanol/water, 50/30/6 by vol, yielded 20% of *N*-acetyllyso-G_{M3} [4]. Reaction of lyso-G_{M3} with the *N*-succinimidyl ester of octanoic acid in tetrahydrofuran/25 mM $NaHCO_3$, 1/1 by vol, at room temperature yielded 30% of *N*-capryllyso-G_{M3} after silica gel chromatography in chloroform/methanol/water, 50/30/6 by vol, [4].

Synthesis of G_{M3} Derivatives with Modifications in the Acetamide Group of the Sialic Acid Moiety

NeuGc-containing G_{M3} (NeuGc = *N*-glycolylneuraminic acid) was isolated from horse blood as described before [4]. Neu-LacCer (Neu = neuraminic acid) was prepared by deacetylation of G_{M3} in 0.5 M KOH at 100°C for 24 h. After dialysis and silica gel chromatography in chloroform/methanol/water, 65/25/4 by vol, the yield was 58% [4]. NeuBu-LacCer (NeuBu = *N*-butyrylneuraminic acid) was prepared from Neu-LacCer and butyric acid *N*-succinimidylester in dimethylformamide. After dialysis and preparative TLC in chloroform/methanol/0.2% $CaCl_2$, 60/35/8 by vol, the yield was 26% [4]. NeuBio-LacCer (NeuBio = *N*-biotinylneuraminic acid) was prepared as described in [5].

Synthesis of G_{M3} Derivatives with Modifications in the Carboxyl Group of the Sialic Acid Moiety

G_{M3}-amide and G_{M3}-methylamide were prepared from G_{M3}-methylester by reaction with ammonia and methylamine, respectively, as described in [4, 6].

G_{D3} Synthase Assay

Isolation of Golgi vesicles from rat liver and assay conditions for G_{D3} synthase were as described before [7]. G_{D3} synthase assay contained 50 μg of Golgi protein, unlabeled glycolipid acceptor (150 μM), CMP-[^{14}C]NeuAc (cytidine-5'-monophospho-*N*-acetylneuraminic acid; 1 mM, 5 000 cpm/nmol; Amersham, Braunschweig, W. Germany), and Triton CF-54 (0.15%, w/v) in a total volume of 50 μl. Assays were incubated at 37°C for 30

min. Enzyme reaction was linear with time up to 45 min of incubation and linear with protein in the range of 0.25 - 1.0 mg/ml. TLC analysis, fluorography, and product quantification were carried out as described previously [4].

Sialidase Assay

In a total volume of 50 μl, sialidase assays contained: Glycolipid (200 μM), Nonidet P 40 (50 μg; Fluka, Buchs, Switzerland), sodium acetate buffer (50 mM, pH 5), ovalbumin (1 μg; Sigma, Deisenhofen, W. Germany), and sialidase (0.2 mU, *C. perfringens*, 19 mU/μg; Sigma). Chloroform/methanol, 2/1 by vol, solutions of glycolipids and detergent were first evaporated to complete dryness under a stream of N_2; buffer was added and the mixtures were sonicated for 30 sec in a water-cooled cup-horn sonicator (Branson, Danburg, CT, USA) at 100 W. Sialidase was added at 0°C. The mixtures were briefly vortexed and incubated for 30 min at 37°C. Appropriate blanks (1, without glycolipid; 2, without sialidase) were run for each enzyme determination. The reactions were stopped with 1 ml chloroform/methanol, 2/1 by vol. Under these conditions the reaction was linear with time up to 30 min and linear with protein up to 0.2 μg. The samples were applied to Sephadex G-25 superfine columns and eluted as described for G_{D3} synthase assays [7]. Column eluates were evaporated to dryness under N_2, redissolved in 100 μl of chloroform/methanol, 2/1 by vol, and applied to precoated silica gel 60 plates (0.25 mm; Merck, Darmstadt, W. Germany). The reaction products were chromatographed in chloroform/methanol/0.2% $CaCl_2$, 60/35/8 by vol. Glycolipid spots were then visualized by spraying the plates with acetic acid (glacial)/sulphuric acid (conc.)/anisaldehyde, 50/1/0.3 by vol, [8] and heating in a closed chamber for 5 min at 150°C. Product quantification was done by scanning the glycolipid spots in a UV TLC scanner (Shimadzu, Japan) at 580 nm. The validity of this method was established by comparison with sialidase degradation of [^{3}H]G_{M3}, labeled in its ceramide moiety [9]. The radio-labeled spots were scraped from the TLC-plates, mixed with 12.5 ml Pico-Fluor 30 (Packard, Downers Grove, IL, USA), and counted in a liquid scintillation counter (Packard, USA) (data not shown).

Results and Discussion

Table 1 compares the relative activities of G_{D3} synthase with the relative activities of sialidase when G_{M3} and G_{M3} derivatives were used as glycolipid substrates. The lyso-G_{M3} derivatives were degraded to the coresponding LacCer derivatives almost as fast as the authentic substrate G_{M3} to LacCer. Only lyso-G_{M3} seemed to be a poorer substrate than G_{M3} and other lyso-G_{M3} derivatives. This was in parallel with the observation that the lyso-G_{M3} derivatives were as good glycolipid acceptors for G_{M3} synthase as was G_{M3} [4] again with the exception of lyso-G_{M3} which was a slightly poorer substrate than the other derivatives. Due to protonation of the free sphingoid amino group lyso-G_{M3} carries a positive charge which may disturb its interaction with both enzymes.

When G_{M3} derivatives with a modified neuraminic acid moiety were used as substrates in G_{D3} synthase and sialidase assays we found an amazing correspondence of G_{D3} synthase activity with sialidase activity (Table 1). All G_{M3} derivatives which were not degraded by sialidase were at the same time poor glycolipid acceptors for G_{D3} synthase. Apparently both enzymes recognize the negative charge of the carboxylic group and the acyl residue in amide bond to the neuraminyl group. Loss of the negative charge implies

Table 1. Relative activities of G_{D3} synthase and sialidase on G_{M3} and G_{M3} derivatives.

Substrate	Relative activities (%)	
	G_{D3} synthase[a]	sialidase[b]
G_{M3}	100[c]	100[d]
lyso-G_{M3}	70	16
N-acetyllyso-G_{M3}	120	72
N-capryllyso-G_{M3}	101	69
Neu-LacCer	1	0
NeuGc-LacCer	103	21
NeuBu-LacCer	48	0
NeuBio-LacCer	6	0
G_{M3}-amide	11	0
G_{M3}-methylamide	3	0

[a] Under the assay conditions for G_{D3} synthase 7.5 nmol of unlabeled glycolipid acceptor, 50 nmol [^{14}C]NeuAc and 50 µg of Golgi protein (derived from rat liver) were incubated for 30 min at 37°C as described in Materials and Methods. Product analysis and quantification were carried out as described in [4].

[b] Sialidase assay contained 10 nmol of glycolipid, 50 µg Nonidet P 40, 1 µg ovalbumin and 0.2 mU of sialidase (*C.perfringens*). Incubation was carried out by TLC and scanning of glycolipid spots. For exact procedure see Materials and Methods.

[c] 100% = 20 nmol/mg/h.

[d] 100% = 30 nmol/mU/h.

that the glycolipid is not accepted as substate by either enzyme. That loss of negative charge (at least the terminal sialic acid residue in G_{D1a} derivatives) rendered them unsusceptible to sialidase has been shown by Nakamura and Handa [6]. We also found that when the acetyl residue in the neuraminic acid was lacking neither sialidase nor sialyltransferase would accept these G_{M3} derivatives as substrates. Here again a positive charge on the free amino group may be the reason to this phenomenon (see lyso-G_{M3} above). The chain length of this acyl residue also appears to be important. G_{M3} derivatives with acyl residues longer than acetyl were hardly accepted as substrate by either enzyme.

In this study two enzymes, rather distant in evolution, a mammalian sialyltransferase and a bacterial sialidase, were compared. From the results presented here we conclude that the substrate recognition may be similar for both enzymes due to similarities in the structures of their binding sites. That the two enzymes recognize the same features of their common substrate seems to be beyond coincidence. The major criterion that "the key fits the lock" appears to be the proper recognition of the sialic acid moiety, where the negative charge of the carboxyl group and the unmodified acetamide group are apparently crucial recognition sites for both enzymes.

Acknowledgements

This work was financially supported by Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen.

References

1 Schachter H, Roseman S (1980) in The Biochemistry of Glycoproteins and Proteoglycans, ed. Lennarz W J, Plenum Press, New York, p 85-160.
2 Sandhoff K, Conzelmann E (1984) Neuropediatrics 15:85-92.
3 Neuenhofer S, Schwarzmann G, Egge H, Sandhoff K (1985) Biochemistry 24:525-32.
4 Klein D, Pohlentz G, Schwarzmann G, Sandhoff K (1987) Eur J Biochem, in press.
5 Schwarzmann G, Sandhoff K (1987) in Methods Enzymol 138:319-41.
6 Nakamura K, Handa S (1986) J Biochem 99:219-26.
7 Yusuf HKM, Pohlentz G, Schwarzmann G, Sandhoff K (1983) Eur J Biochem 134: 47-54.
8 Jatzkewitz H, Sandhoff K (1963) Biochim Biophys Acta 70:354-56.
9 Schwarzmann G (1978) Biochim Biophys Acta 529:106-14.