Structural Basis for the Resistance of Tay-Sachs Ganglioside GM2 to Enzymatic Degradation*

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To understand the reason why, in the absence of GM2 activator protein, the GalNAc and the NeuAc in GM2 (GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–1’Cer) are refractory to β-hexosaminidase A and sialidase, respectively, we have recently synthesized a linkage analogue of GM2 named 6’GM2 (GalNAcβ1–6(NeuAcα2–3)Galβ1–4Glcβ1–1’Cer). While GM2 has GalNAcβ1–4Gal linkage, 6’GM2 has GalNAcβ1–6Gal linkage (Ishida, H., Ito, Y., Tanahashi, E., Li, Y-T., Kiso, M., and Hasegawa, A. (1997) Carbohydr. Res. 302, 223–227). We have studied the enzymatic susceptibilities of GM2 and 6’GM2, as well as that of the oligosaccharides derived from GM2, asialo-GM2 (GalNAcβ1–4Galβ1–4Glcβ1–1’Cer) and 6’GM2. In addition, the conformational properties of both GM2 and 6’GM2 were analyzed using NMR spectroscopy and molecular mechanics computation. In sharp contrast to GM2, the GalNAc and the Neu5Ac of 6’GM2 were readily hydrolyzed by β-hexosaminidase A and sialidase, respectively, without GM2 activator. Among the oligosaccharides derived from GM2, asialo-GM2, and 6’GM2, only the oligosaccharide from GM2 was resistant to β-hexosaminidase A. Conformational analyses revealed that while GM2 has a compact and rigid oligosaccharide head group, 6’GM2 has an open spatial arrangement of the sugar units, with the GalNAc and the Neu5Ac freely accessible to external interactions. These results strongly indicate that the resistance of GM2 to enzymatic hydrolysis is because of the specific rigid conformation of the GM2 oligosaccharide.

Tay-Sachs disease is caused by the impaired catabolism of ganglioside GM2† (GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1’Cer). It has been shown that the terminal GalNAc in GM2 is resistant to β-hexosaminidase A (Hex A), and that a specific protein cofactor, GM2 activator, is required to assist the hydrolysis (1–4). The disease, therefore, can be caused by the deficiency of either Hex A (5–7) or GM2 activator protein (8–10). The reason for the resistance of GM2 to Hex A is still an enigma.

We have shown that, in addition to the GalNAc, the enzymatic hydrolysis of the Neu5Ac in GM2 also requires GM2 activator protein (11). In GM2, both the GalNAc and the Neu5Ac are linked to the Gal to form GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1’Cer, a branched trisaccharide (GM2-epitope). We have rationalized that the resistance of the GalNAc and the Neu5Ac in GM2 to enzymatic hydrolysis may be because of the specific rigid structural conformation of the GM2-epitope and that GM2 activator may interact with the GM2-epitope to make the GalNAc and the Neu5Ac accessible to Hex A and sialidase, respectively (11). To show that this is the case, we have recently synthesized a GM2 analog, 6’GM2 (GalNAcβ1–6(Neu5Acα2–3)Galβ1–4Glcβ1–1’Cer), in which the GalNAc is linked β1–6 to the Gal (12). Here, we report that, in sharp contrast to GM2, the GalNAc and the Neu5Ac in 6’GM2 were readily hydrolyzed by Hex A and clostripidial sialidase, respectively, independent of GM2 activator protein. NMR analysis and molecular mechanics computation revealed that the trisaccharide head group of 6’GM2 is much more flexible than that of GM2. Our results provide the structural basis for the resistance of the GalNAc and the Neu5Ac in GM2 to enzymatic degradation.

EXPERIMENTAL PROCEDURES

**Materials—GM2 was isolated from the brain of a Tay-Sachs patient according to the published method (13). Asialo-GM2 (GA2) was prepared from GM2 by mild acid hydrolysis (14). Hex A (33.3 units/mg) from human liver was prepared according to our previous report (1). 6’GM2 was chemically synthesized (12). The oligosaccharides OM2, 6’OM2, and OA2 were prepared by the removal of ceramide moieties from GM2, 6’GM2, and GA2 using ceramide glycanase (15). The following were obtained from commercial sources: precoated silica gel-60 TLC plates, Merck (Darmstadt, Germany); GM3, Matreya; sialyllactose, lactose, type X clostripidial sialidase, (CD₃)₅SO, and D₂O, Sigma; and Chelex-100 resin, Bio-Rad.

**Enzymatic Hydrolysis—**For enzymatic hydrolysis of the GalNAc from glycolipids or from the oligosaccharides derived from glycolipids, each reaction mixture contained 8 nmol of the substrate and the specified amount of Hex A, as indicated in each figure, in 100 µl of 10 mM sodium acetate buffer, pH 4.6. Incubations were carried out at 37 °C for the preset time. For hydrolysis of the Neu5Ac from the glycolipids, 8 nmol of GM2 or 6’GM2 was incubated with 10 units of clostripidial sialidase in the presence or absence of 5 µg of GM2 activator protein (11) in 100 µl of 20 mM sodium acetate buffer, pH 5.5. Incubation was carried out at 37 °C for 16 h.

**TLC Analysis—**After incubation, each reaction mixture was evaporated to dryness and analyzed by silica gel-60 thin layer chromatography. The solvent system used for developing the glycolipids was chloroform/methanol/water (60:35:8) and that for the oligosaccharides was n-butyl alcohol/acetic acid/water (2:1:1). The plate was sprayed with the
Enzymatic Hydrolysis of GM2

**TABLE I**

Hydrolysis of GM2 and 6′GM2 by human Hex A in the presence of GM2 activator

| Incubation time | % Hydrolysis of GM2* | % Hydrolysis of 6′GM2* |
|-----------------|----------------------|------------------------|
| min             | −Activator | +Activator | −Activator | +Activator |
| 5               | 13.8       | 24.9       | 23.0       | 49.3       |
| 10              | 21.0       | 46.1       | 49.3       | 68.3       |
| 20              | 42.1       | 58.4       | 68.3       | 68.3       |
| 30              | 80.9       | 59.4       | 68.3       | 68.3       |

* It was necessary to use the reduced amount of Hex A for the hydrolysis of 6′GM2 since 6′GM2 was much more readily hydrolyzed than GM2.

**FIG. 1.** Thin layer chromatograms showing the susceptibility of the GalNAc and the NeuAc in GM2 and 6′GM2 to enzymatic hydrolysis. A, hydrolysis of the GalNAc from GM2 and 6′GM2. Hex, Hex A; h, hour; m, minute; 6′M2, 6′GM2. For the hydrolysis of GM2, 0.25 unit of Hex A was used. For the hydrolysis of 6′GM2, only 0.06 unit of Hex A was used because this ganglioside was completely hydrolyzed by 0.25 unit of Hex A in less than 5 min. B, hydrolysis of the NeuAc from GM2 and 6′GM2. CS, clostralid sialidase; Act, GM2 activator protein. Detailed conditions are described under “Experimental Procedures.”

**FIG. 2.** Thin layer chromatogram showing the susceptibility of the GalNAc in OM2, OA2, and 6′OM2 to enzymatic hydrolysis. OM2, OA2, and 6′OM2 are the oligosaccharides derived from GM2, GA2, and 6′GM2, respectively. For each incubation, 0.1 unit of Hex A was used. SL, sialyllactose; Hex, Hex A; h, hour; Lac, lactose. Detailed conditions are described under “Experimental Procedures.”

The computational models—All calculations were performed using the MacroModel/Batchmin package (20) (version 5.5) on an O2 SGI workstation and the AMBER* sugar force field (21) augmented by modified neglect of diatomic differential overlap (MINDO)-derived parameters for the anomic torsion of sialic acid (22). To simplify the computational problem, all calculations on 6′GM2 were run on the methyl derivatives. The calculations were carried out using the generalized born/surface area (GB/SA) water solvation model (23) of MacroModel. This model treats the solvent as an analytical continuum starting near the van der Waals surface of the solute and uses a dielectric constant of 78 for the bulk water and 1 for the molecule. Extended nonbonded cut-off distances were used. Thus, all calculations were run with a van der Waals cut-off of 8.0 Å and an electrostatic cut-off of 20.0 Å.

The conformational searches were carried out using the pseudo system variant (SUMM) (24) of the Monte Carlo (MC) energy minimization procedure (25). The search proceeded by a pseudo systematical

diphosphoryl reagent (16) and heated at 110 °C for 15 min to reveal oligosaccharides and glycoconjugates. Quantitative results on the % hydrolysis of substrates were obtained from the scanning of the TLC plates using ScanJet 2C/ADF scanner (Hewlett Packard) and the NIH Image 1.41 program.

**NMR Sample Preparation**—Five mg of 6′GM2 were dried under vacuum and were dissolved in 0.5 ml of (CD3)2SO or (CD3)2SO2D (20:1) under a stream of nitrogen. To overcome signal broadening of OH groups, such as the Neu5Ac OH5, Gal OH2, and GalNAc OH6, the sample was passed through a Chelex-100 column (0.5 × 2.5 cm, pH 6) before analysis. This procedure allowed the detection of the Neu5Ac OH5 proton as a typical sharp signal at low fields (6.15 ppm), but caused the loss of other OH signals. To complete the assignment, about 4 mg of 6′GM2 in 2 ml of water was dialyzed against 2 mM EDTA, followed by water, and then passed through a Chelex-100 column at pH 7.0. This treatment furnished the GalNAc OH6 and Gal OH2 resonances at 5.16 and 4.35 ppm, respectively, while it caused the loss of the previously assigned OH2 of Glc.

**NMR Spectroscopy**—1H-NMR and 13C-NMR spectra were obtained at 500 and 125 MHz, respectively, on a Bruker AM500 spectrometer and analyzed on a X32 Bruker satellite station equipped with standard Bruker UXNMR software. 1H-1H and 1H-13C two-dimensional spectra were acquired as 2048 × 512 and 2048 × 556 matrices, respectively, with 64 scans per t1 point and processed after zero filling in F1 dimension and appropriate window function multiplication. Proton inter- and intra-residual contacts were evaluated by NOE experiments in the rotating frame ROESY (17) with a spin lock pulse strength of 2.6 kHz applied at one end of the spectrum to avoid scalar transfer (18, 19). Temperature was varied in the range of 305–323 K and mixing time between 100–200 ms. The experiments were conducted on both the D2O-exchanged and not-exchanged samples as well as the samples before and after the treatments. Cross-peak volumes were transformed into proton-proton distances r, under the hypothesis of the 1/2π NOE dependence and using internal calibration on Glc, Gal, GalNAc H1/H3, and H1/H5 distances, and on Glc H1/H2 when accessible. Distances from four experiments were averaged to give the reported values. To achieve reliable comparisons between the oligosaccharide structure of 6′GM2 and GM2, parallel NMR analyses were conducted on GM2 and 6′GM2.

**Computational Methods**—All calculations were performed using the MacroModel/Batchmin package (20) (version 5.5) on an O2 SGI workstation and the AMBER* sugar force field (21) augmented by modified neglect of diatomic differential overlap (MINDO)-derived parameters for the anomic torsion of sialic acid (22). To simplify the computational problem, all calculations on 6′GM2 were run on the methyl derivatives. The calculations were carried out using the generalized born/surface area (GB/SA) water solvation model (23) of MacroModel. This model treats the solvent as an analytical continuum starting near the van der Waals surface of the solute and uses a dielectric constant of 78 for the bulk water and 1 for the molecule. Extended nonbonded cut-off distances were used. Thus, all calculations were run with a van der Waals cut-off of 8.0 Å and an electrostatic cut-off of 20.0 Å.

The conformational searches were carried out using the pseudo system variant (SUMM) (24) of the Monte Carlo (MC) energy minimization procedure (25). The search proceeded by a pseudo systematical
Energy minimization was performed using the Truncated Newton Conjugate Gradient (TNCG) procedure (26) and was terminated either after 200 iterations or when the energy gradient RMS fell below 0.1 kJ/mol A. All conformers that differed from the global minimum energy conformation by no more than 50 kJ/mol were saved, and comparison was made only on the heavy atoms to avoid duplicate conformations. Thus, only the global minimum for each conformer was retained, independent of the OH conformations. After addition of explicit H atoms on the sugars, they were further subjected to energy minimization to reduce the energy gradient RMS to 0.01 kJ/mol A. Fifty four conformers with minimum energy that differed from the global minimum energy conformation by no more than 10 kJ/mol were found. Among them, 15 were contained within 4 kJ/mol and 37 within 8 kJ/mol from the global minimum.

RESULTS AND DISCUSSION

Hydrolysis of GM2 and 6'GM2 by Hex A and Clostridial Sialidase—The enzymatic susceptibilities of GM2 and 6'GM2 were examined, and the results are shown in Fig. 1. While the GalNAcβ1→4(Neu5Acα2→3)Galβ- in GM2 is resistant to Hex A (Fig. 1A, a), the GalNAcβ1→6(Neu5Acα2→3)Galβ- in 6'GM2 is readily hydrolyzed by Hex A without the assistance of GM2 activator protein (Fig. 1A, b). We subsequently examined the susceptibility of the Neu5Ac in 6'GM2 to clostridial sialidase, and the result is shown in Fig. 1B. The hydrolysis of the Neu5Ac in GM2 by clostridial sialidase required the assistance of GM2 activator, whereas the Neu5Ac in 6'GM2 could be readily hydrolyzed by clostridial sialidase in the absence of GM2 activator protein. These results clearly indicate that the mutation of the GalNAc linkage in GM2 from GalNAcβ1→4Gal to GalNAcβ1→6Gal affects not only the enzymatic susceptibility of the GalNAc but also that of the Neu5Ac which is linked to C3 of the Gal in both gangliosides.

The quantitative comparison on the hydrolyses of GM2 and 6'GM2 by hex A in the presence or absence of GM2 activator protein is presented in Table I. Under our assay conditions, GM2 was not hydrolyzed by Hex A in the absence of GM2 activator. The resistance of GM2 to Hex A has been widely recognized. However, in the presence of the activator protein, GM2 became susceptible to Hex A. In contrast, 6'GM2 was readily hydrolyzed by Hex A without GM2 activator protein. To obtain a comparable level of the hydrolysis for both substrates, it was necessary to use only 0.06 units of Hex A for 6'GM2 hydrolysis and 0.25 units of the enzyme for GM2 hydrolysis (see Table I). This suggests that 6'GM2 is a better substrate for Hex A. Moreover, in the presence of the activator protein, the hydrolysis of 6'GM2 increased only 5–10%. These results indicate that GM2 activator protein does not stimulate the two substrates equally. The GM2 activator protein has a specific stimulatory effect on the hydrolysis of GM2 by Hex A.

Susceptibility of the Oligosaccharides Derived from GM2, 6'GM2, and GA2 to Hex A—To eliminate any possible influence by the ceramide moiety of the glycolipids on the enzymatic hydrolysis of the GalNAc residue, the ceramide residue was removed from the glycolipids, GM2, 6'GM2 and GA2, to yield the oligosaccharides OM2, 6'OM2 and OA2, respectively. As shown in Fig. 2, both 6'OM2 and OA2 were readily hydrolyzed by Hex A alone. In contrast, OM2 was completely resistant to Hex A. Similarly, the Neu5Ac of 6'OM2, but not that of OM2, was susceptible to clostridial sialidase (results not shown). Inclusion of GM2 activator protein in the reaction mixture did not alter the above results because this activator protein requires the lipid moiety of the substrate to exert its stimulatory activity. These results further support that the resistance of GM2 to Hex A and clostridial sialidase is because of the rigid carbohydrate structure of GM2-epitope.

Conformational Analysis of GM2 and 6'GM2—To understand the conformation/function relationship of GM2 and 6'GM2, we have also investigated the conformational properties of these two gangliosides by high resolution NMR spectroscopy and molecular mechanics computation, applying an MC conformational search. As shown in Fig. 3A, the pattern of interresidual NOE interactions detected on GM2 is consistent with a single prevalent spatial arrangement of the GalNAc-Gal glycosidic bond of GM2. The GalNAc anomeric torsion ϕ appears to be well defined around 45°, but the Gal C6-O6 bond is freely switching from the two conformations at ϕ ~ 90° (global minimum shown in Fig. 6B) and ϕ ~ 70° (1.2 kJ/mol above the global minimum shown in Fig. 6C). Another conformer of the Gal C6-O6 bond which has energy higher than 8 kJ/mol appears around ϕ ~ 180°.

FIG. 4. The ϕ, ψ maps of the GalNAc-Gal glycosidic bond. The lowest energy conformations within 8 kJ/mol from the global minimum of the MC conformational searches performed on the methyl derivatives of the oligosaccharides of GM2 (GalNAcβ1→4(Neu5Acα2→3)Galβ1→4(GlcβO2Me) and 6'-GM2 (GalNAcβ1→6(Neu5Acα2→3)Galβ1→4(GlcβO2Me) are shown. Glycosidic angles ϕ, ψ maps of GM2 (C) and 6'-GM2 (D) are defined as follows: for GM2, ϕ = GalNAcH1-GalNAcC1-O1-GalC4-GalH4; for 6'-GM2, ϕ = GalNAcH1-GalNAcC1-O1-GalC6, ψ = GalNAcC1-O1-GalC6-GalC5. The GalNAc-Gal glycosidic bond of GM2 appears to populate a single well at ϕ, ψ ~ 30°, 20°, which is in agreement with the experimental data shown in Fig. 3. In contrast, a marked flexibility is observed around the GalNAc-Gal glycosidic bond of 6'-GM2. The GalNAc anomeric torsion ϕ appears to be well defined around 45°, but the Gal C6-O6 bond is freely switching from the two conformations at ϕ ~ 90° (global minimum shown in Fig. 6B) and ϕ ~ 70° (1.2 kJ/mol above the global minimum shown in Fig. 6C).
NOE interactions across the GalNAc β1–6Gal linkage of 6′GM2 consist uniquely of the contacts between the anomeric proton of the GalNAc and the two protons on the C6 of the Gal; the intensities of the two interactions are almost equal. Another important interresidual interaction detected on the GalNAc of 6′GM2 is between the GalNAc-NH and the Glc-OH3 (Fig. 3B). Fig. 5 shows that these three contacts cannot be achieved simultaneously in a single conformation. The MC analysis suggests that they arise from the average of two conformations of the GalNAcβ1–6Gal linkage at ψ, ψ 45°, −90°, and 45°, +70° (Fig. 4). Fig. 5 also shows how the ψ −90° conformers give rise to the GalNAcNH-GlcOH3 and GalNAcH1-GalH6′ contacts, whereas the ψ +70° conformers generate the GalNAcH1-GalH6 cross-peaks. The distances calculated by Boltzmann averaging (28) of the MC conformations in Fig. 5 agree well with the experimental data (see legend to Fig. 5). The interresidual interactions between the Neu5Ac and the Gal protons in 6′GM2 are centered on the side chain of the Neu5Ac, showing the contacts of Neu5Ac-H6 and -OH8 with Gal-H3 (Fig. 3B). These contacts are not detected in the trisaccharide GalNAcβ1–4Neu5Acα2–3Gal- of GM2, or in the same trisaccharide found in GM1, GD1a, and GalNAc-GD1a (17, 27, 29–31). The conformations of these gangliosides are highly restrained at the Neu5Ac-Gal glycosidic bond in an antiperiplanar conformation with the side chain of the Neu5Ac interacting with the GalNAc residue. This is not the case for 6′GM2. The interactions of Neu5Ac-H8 and -OH8 with GalH3 in 6′GM2 suggest that the most populated conformation of the Neu5Ac-Gal bond in this ganglioside is a gauche conformation. The MC calculations corroborate and strengthen the results of NMR analyses and also enable comparison of the lowest energy conformations of the oligosaccharide moieties of GM2 (Fig. 6A) and 6′GM2 (Fig. 6, B and C).

The conformational studies of 6′GM2 and GM2 reveal that the modification of the GalNAc linkage in GM2 from β1–4Gal to β1–6Gal dramatically alters the dynamics of the sugar chain. The overall effect of this modification is to give the oligosaccharide head group of 6′GM2 an “open” spatial arrangement (Fig. 6, B and C) in which the GalNAc and the Neu5Ac residues are freely accessible to external interactions. Indeed, it has been shown that a flexible ligand has a lower affinity to its receptor than a rigid ligand fitting perfectly to the binding site. However, a flexible ligand with a high degree of mobility can increase the rate of association (32).

The above results clearly show that the specific trisaccharide structure of GM2-epitope in which the GalNAcβ1–4 and the Neu5Acα2–3 are both linked to the penultimate Gal residue can render the GalNAc and the Neu5Ac resistant to enzymatic hydrolysis. The disruption of this rigid structure by mutating the GalNAcβ1–4Gal linkage to GalNAcβ1–6Gal makes the GalNAc susceptible to Hex A and the Neu5Ac to sialidase. A well known example where the linkage mobility is closely associated with the ganglioside-protein interactions is that of GD1a ganglioside. GD1a is a disialoganglioside whose struc-
ture can be regarded as having an extension of the sugar chain of GM2 by adding an extra Neu5Aco2→3Galβ- disaccharide to the C3 of the GalNAc. It is widely known that, of the two Neu5Aco2→3Galβ- linkages in GD1a, only the external one is susceptible to sialidase. The inner Neu5Ac in GD1a to sialidase is associated with the dynamics of limiting conformations (31). Thus, the susceptibility of the GalNAc residue is more flexible and fluctuates between two flexible conformations (31). Thus, the susceptibility of the Neu5Ac in GD1a to sialidase is associated with the dynamics of the Neu5Aco2→3Galβ- linkage.

Although complex carbohydrates have been recognized as information rich molecules, functions expressed by complex carbohydrate chains are still not readily decipherable as those of proteins and nucleic acids. The primary structure of a complex carbohydrate chain includes the sugar sequence, the anomeric configurations of the sugar units, and the linkages of the two adjacent sugar residues. It is not difficult to understand that the anomeric configuration of a sugar residue can profoundly affect physicochemical and biological properties of a sugar chain. However, the effect of a single sugar linkage on the overall physicochemical and biological properties of a sugar chain is still not well appreciated. Our results show that the biological properties of a sugar chain can be expressed through the specific linkage of the sugar chain. Our investigation through chemical mutation of sugar linkages provides an explanation for the resistance of GM2 to enzymatic degradation.

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