Minor Influence of Sialic Acid on Conformation of a Membrane-Bound Oligosaccharide Recognition Site[†]

David H. Jones, Kathryn R. Barber, and Chris W. M. Grant*

Department of Biochemistry, University of Western Ontario, London, Canada, N6A 5C1

Received December 15, 1995; Revised Manuscript Received February 1, 1996[⊗]

ABSTRACT: Wideline ^{2}H NMR spectroscopy was used to assess the conformational and orientational effects of *N*-acetylneuraminic acid (NeuAc) (sialic acid) as a component of a particular oligosaccharide chain at a bilayer membrane surface. For this purpose, three glycosphingolipids, sharing a neutral core tetrasaccharide and differing only in the number of sialic acid residues, were compared. The starting compound was GD_{1A}, which has terminal sialic acid attached to the second and fourth sugars of its neutral tetrasaccharide core. GD_{1A} was probe-labeled in a nonperturbing fashion on both of these sialic acid residues and on its single GalNAc residue by replacement of -$COCH_3$ with -$COCD_3$, giving [(d_3NeuAc)$_2$,d_3-GalNAc]GD_{1A}. This represents the most complex glycolipid to have been studied by ^{2}H NMR spectroscopy at a bilayer membrane surface. The sialic acid residue on the fourth sugar from the membrane was subsequently removed to produce the glycolipid [d_3NeuAc,d_3GalNAc]GM_1, deuterated at the two remaining amino sugars. The neutral glycolipid [d_3GalNAc]asialo-GM_1 was then generated by removal of the second sialic acid residue, leaving an uncharged species deuterated at one (internal) oligosaccharide chain site (GalNAc). The effect of sialic acid was further examined by selective deuteration of GM_1 and asialo-GM_1 at C_6 of the terminal Gal residue, giving [d_2Gal]GM_1 and [d_2Gal]asialo-GM_1. Spectra of the three glycosphingolipids were compared at 7.7 mol % in unsonicated fluid bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine containing 23 mol % cholesterol. For liposomes suspended in buffered salt solutions with 2 mM Ca^{2+}, ^{2}H NMR spectra demonstrated the presence of a well defined average conformation for each oligosaccharide chain. This preferred average conformation persisted over a wide temperature range, consistent with there being a single major oligosaccharide conformer in each case. Spectral features arising from both deuterated amino sugars of GM_1 were present, essentially unchanged, in spectra of GD_{1A}. Features associated with the single deuterated amino sugar (GalNAc) of asialo-GM_1 could be identified, little changed, in spectra of GM_1 and GD_{1A}. Similarly, deuterons in the terminal Gal residue of asialo-GM_1 produced the same spectrum seen for this residue in GM_1. Our findings indicate that certain major conformational and orientational features of this complex oligosaccharide recognition site are preserved, within a maximum angular deviation of ±5° or less, upon addition or removal of a sialic acid residue.

Carbohydrate chains at the surface of eukaryotic cells are generally considered to play key roles as recognition sites. In this regard, the presence of any given carbohydrate residue in an oligosaccharide chain can be critical in determining the chain's recognition function (Roseman, 1974; Shur, 1989; Feizi, 1991; Lasky, 1992). Thus a fundamental area of study in dissecting the molecular mechanism of membrane receptor operation involves testing the effects induced by carbohydrate modifications. However, it is widely accepted that oligosaccharide spatial arrangement, in addition to primary sequence, is a major determinant of specific binding (Strömberg et al., 1991). Hence it is important to develop a basis for judging the contribution of a particular primary structural change in isolation from secondary spatial alterations which might have resulted from that change. Very little information exists surrounding such considerations in membrane environments. A closely related issue, with important implications for signal transduction at the level of the membrane, is how perturbation of a recognition site might induce physical changes that would serve to initiate signaling or modulate signal propagation.

In the present work, we have focused on the physical effect of the (−)charged acidic sugar, sialic acid, using complex carbohydrate chains of glycosphingolipids as a study system. Sialic acid is a principal carbohydrate residue of eukaryote plasma membranes, most commonly in terminal sites. Its presence and location are critical determinants of cell interactions (Lloyd, 1975; Schauer, 1983; Wiegandt, 1985; Sweeley, 1993). There has been considerable interest in mechanism(s) whereby sialic acid might exert effects on receptor function [e.g., Shichijo and Alving (1986), Sweeley (1993), Powell et al. (1995), and Nemansky et al. (1995)]. Such investigations beg detailed information on receptor spatial arrangement. Although structural study of membrane glycoproteins is in its infancy, great strides have been made in application of high resolution NMR to solutions of glycolipids containing sialic acid (Sillerud et al., 1982;

[†] This research was supported by Grant No. MT 5441 from the Medical Research Council of Canada to C.W.M.G. NMR spectroscopy and molecular modeling were carried out in the R. S. McLaughlin Macromolecular Structure Facility, established with grants to the department from the R. Samuel McLaughlin Foundation, the London Life Insurance Company, the MRC Development Program, and the Academic Development Fund of the University.

* To whom correspondence should be addressed. E-mail: cgrant@voyager.biochem.uwo.ca.

[⊗] Abstract published in *Advance ACS Abstracts,* March 15, 1996.

0006-2960/96/0435-4803$12.00/0

Sabesan et al., 1983; Scarsdale et al., 1990; Aubin et al., 1993; Poppe et al., 1994, and references therein). These authors have provided detailed discussions of the associative forces to be considered among ganglioside headgroups [see also Sonnino et al. (1993)]. Recently, Prestegard and colleagues have demonstrated, using sialic acid attached to a hydrophobic chain, that NMR spectroscopy can furnish complete details of this sugar's behavior at a membrane surface. In our experiments, wideline ^{2}H NMR spectroscopy provided a technique immediately applicable *in situ* to more complex oligosaccharides. The method is nonperturbing and sensitive to details of molecular orientation and motion (Seelig, 1977; Mantsch et al., 1977; Davis, 1983, 1991; Smith, 1984). However it is not currently possible to selectively deuterate a sufficient number of sites to permit complete analysis in terms of complex chain conformation and motional characteristics using only ^{2}H NMR. Hence in the present work we concentrated on measuring the degree of alteration induced by sialic acid in a recognition site.

The glycolipids studied were chosen as important examples of species found in plasma membranes of higher animal cells. Gangliosides—the sialic acid bearing sphingolipids—and their asialo- derivatives, are key antigens and tumor markers that have been widely implicated in cell recognition, adhesion, and growth regulation (Wiegandt, 1985; Curatolo, 1987; Hakomori, 1989). GD_{1A},[1] the parent compound for our experiments, is the predominant ganglioside of beef neural tissue. It possesses two sialic acid residues, both in terminal locations. These charged residues are attached to the galactose (Gal) residues of the neutral tetrasaccharide which defines the glycolipid, asialo-GM_1 (Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1ceramide). GM_1, another primary acidic glycolipid of the central nervous system, is derived from GD_{1A} by removal of sialic acid from the Gal residue farthest from the ceramide portion. Since these three glycolipids are related to one another by the addition or removal of sialic acid residues from a common chain, they provided a mechanism for testing the influence of this ((−) charged) group in the absence of other factors. The primary difficulty involved was replacement of selected ^{1}H nuclei in the carbohydrate chains by deuterium. Sites that could be successfully dealt with were sialic acid (NeuAc) and *N*-acetylgalactosamine (GalNAc) (by replacing -$COCH_3$ with -$COCD_3$) and the hydroxymethyl group of terminal galactose (Gal) (by replacing -CH_2OH with -CD_2OH).

Deuterated glycolipids to be compared were dispersed as minor components in bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) containing cholesterol. Ca^{2+} was included in the buffer system at the 2 mM concentration found in extracellular fluid. Spectra were recorded at temperatures above the solidus curve of the POPC/cholesterol binary phase diagram (Thewalt & Bloom, 1992), the intent being that the host matrix should be uniform and of intermediate fluidity.

MATERIALS AND METHODS

POPC was obtained from Avanti Polar Lipids (Birmingham, AL), and cholesterol was from Sigma (St. Louis, MO); both were used without further purification. GD_{1A} was isolated from beef brain gangliosides (Folch et al., 1957; Kanfer, 1969) and further purified on an Iatrobead silicic acid column (Iatron, Japan) (Momoi et al., 1976) eluting with a gradient of $CHCl_3/CH_3OH/H_2O$. Typically 50 (to 100) mg of crude extract was loaded onto a 1.7 × 90 (to 1.7 × 120) cm column. Identification was by comparison with standards obtained from Supelco (Bellefonte, PA). GM_1 was obtained as a separate fraction from the same procedure or by treatment with neuraminidase according to Cestaro et al. (1980) and Thompson et al. (1985). Asialo-GM_1 was obtained from either of the above products by treatment with formic acid according to Mansson (1973). The reaction mixture was subsequently dialyzed against distilled water at 4 °C with frequent changes of the water, followed by purification on an Iatrobead column. Natural and deuterated glycolipids comigrated with commercially obtained standards.

The -$COCD_3$ derivative of GD_{1A} was made by partial synthesis, the natural fatty acid composition being preserved, following a method outlined by Higashi and Basu (1982) for incorporating ^{14}C radiotracers into amino sugars. Dry glycolipid was dissolved in anhydrous hydrazine, sealed in glass ampoules, and heated at 105 °C for 8 h. Degree of hydrazinolysis was followed by silicic acid thin layer chromatography (TLC), eluting with 55:45:10 $CHCl_3/CH_3OH/0.2\%\ CaCl_2$ or 55:40:8:2 $CHCl_3/CH_3OH/H_2O/NH_4OH$. Hydrazinolysates were dried down and left under vacuum for 6 h. They were subsequently dissolved in methanol by gentle warming (and, where necessary, with the addition of small amounts of benzene) and reacetylated using d_6-acetic anhydride (MSD isotopes) in benzene. This material was purified on an Iatrobead column eluted with linear gradients of chloroform/methanol/water (Momoi et al., 1976) and identified by TLC. High resolution ^{1}H NMR in 98% [d_6]DMSO/2% [d_2]H_2O) demonstrated that the sialic acid acetate groups of GD_{1A}, whose resonances overlap at 1.89 ppm (relative to TMS) (Koerner et al., 1983), had been virtually completely replaced, while the acetate group of GalNAc at 1.76 ppm, was only 45% replaced [see also Neuenhofer et al. (1985)].

Deuterium labeling in the exocyclic hydroxymethyl group of the terminal Gal residue in GM_1 and asialo-GM_1 was performed using galactose oxidase and $NaBD_4$ as previously described (Jarrell et al., 1992). It typically produced a yield of 75−85%, as demonstrated by ^{1}H NMR in 98% [d_6]DMSO/2% [d_2]H_2O). Two "rounds" of oxidation and subsequent reduction were employed to optimise replacement of the C_6 protons with deuterons.

Lipid samples were multilamellar vesicles (MLV) prepared by vortexing at 50 °C. Hydration was with 30 mM HEPES buffer, pH 7.4, containing 20 mM NaCl and 2 mM Ca^{2+}. Following repeated lyophilization from deuterium-depleted water, samples were rehydrated with deuterium depleted water and freeze-thawed 3−4 times. The amount of labeled lipid used in each sample varied from 5 to 16 μmol, with a 9:3:1 composition (POPC/cholesterol/glycolipid). Sample volumes were 200 μL. Wideline ^{2}H NMR spectra were acquired at 76.7 MHz on a Varian Unity spectrometer with Doty 5 mm solenoid probe. The $\pi/2$ pulse width was 5.2 μs. Pulse spacing in the quad echo sequence was 30 μs, with a repetition time of 100−200 ms. DePaking was performed by a noniterative method utilizing a nonnegative least-squares algorithm (Whittall et al., 1989). Spectra were

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[1] Abbreviations: PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-PC; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid (sialic acid); GD_{1A}, NeuAcα2→3Galβ1→3GalNAcβ1→4Gal-(3←2αNeuAc)β1→4Glcβ1→1ceramide; GM_1, Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1ceramide; asialo-GM_1, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1ceramide.

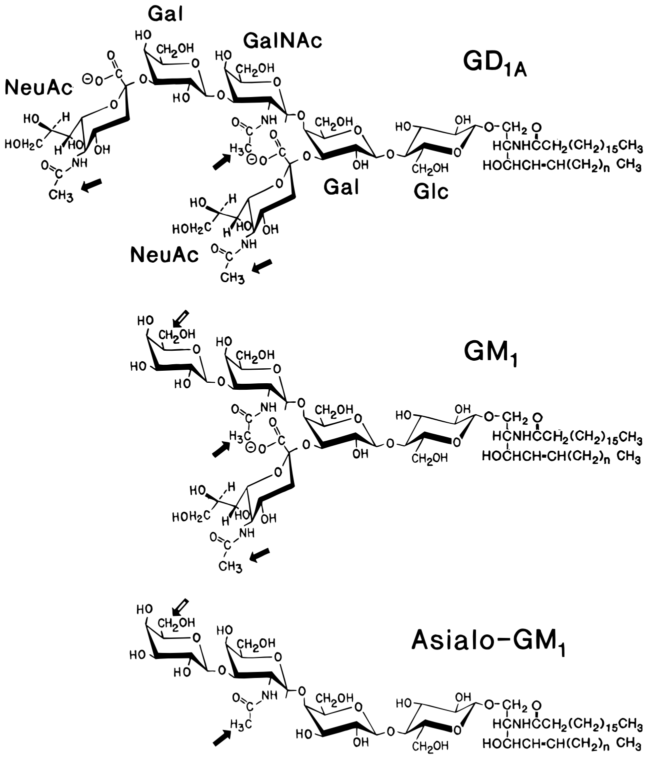

FIGURE 1: Structures for the glycosphingolipids labeled with deuterium probe nuclei. [d_3GalNAc]asialo-GM$_1$, [d_3NeuAc,d_3-GalNAc]GM$_1$, [(d_3NeuAc)$_2$,d_3GalNAc]GD$_{1A}$ (solid arrows indicate deuterated acetate groups in amino sugars), and [d_2Gal]asialo-GM$_1$, [d_2Gal]GM$_1$ (hollow arrows indicate deuterated hydroxymethyl groups in terminal galactose). The natural variability in the hydrophobic portions of the molecules is indicated, the sphingosine chain being 60−70% C18 and 30−40% C20 species ($n = 12$ or 14, respectively) (Corti & Degiorgio, 1980; Sonnino et al., 1985). Composition is reported as 90−96% sphingosine base (dihydrosphingosine comprising the remainder) (Abrahamsson et al., 1972; Thompson & Tillack, 1985). The natural fatty acid composition is primarily 18:0 (Corti & Degiorgio, 1980; Sonnino et al., 1985).

acquired from high to low temperature, after preequilibration in the spectrometer for 30 min. Molecular modeling was via molecular mechanics algorithms in BIOSYM (Biogen) using the default dielectric constant of 1.

RESULTS

Figure 1 displays structures and deuteration sites of the glycolipids studied in the present work. It is important to note that GD$_{1A}$ and GM$_1$ share the neutral tetrameric oligosaccharide chain, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→, which defines asialo-GM$_1$. Furthermore, all species studied, being derived from beef brain GD$_{1A}$, had identical ceramide composition (details in the figure caption). One set of experiments involved glycolipids in which acetate groups of all amino sugars were replaced with deuterated acetate. This permitted probing of the internal GalNAc of each species, in addition to the single sialic acid residue of GM$_1$ and the two sialic acid residues of GD$_{1A}$ (solid arrows in Figure 1). In another set of samples, hydroxymethyl protons of terminal Gal residues in GM$_1$ and asialo-GM$_1$ were enzymatically replaced with deuterium to afford an additional probe location (hollow arrows).

A source of ^{2}H NMR spectroscopy's considerable value in studies of fluid membranes is that, for axially symmetric molecular rotation, spectral quadrupolar splittings measured at the point corresponding to 90° field orientation obey the following simple quantitative relationship:

$$\Delta\nu_Q = (3/8)e^2Qq/hS_{\text{mol}}(3\cos^2\Theta_i - 1) \tag{1}$$

where e^2Qq/h is the nuclear quadrupole coupling constant [165−170 kHz for an aliphatic C−D bond (Seelig, 1977; Mantsch et al., 1977; Davis, 1983; Smith, 1984; Davis, 1991)], S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the lipid molecule relative to the bilayer normal, and Θ_i is the average orientation of each C−D bond relative to the molecular rotation axis.

For deuterated methyl groups, it is convenient to consider the projection of the C−D bond vector directed along the C−CD$_3$ bond attaching the methyl group: this can be dealt with in eq 1 by considering Θ_i to be the angle between the C−CD$_3$ bond and the molecular long axis, and introducing an additional factor of $^1/_3$. Independent motion of the rigid, planar, -NHCOCD$_3$ group about the C−N bond linking it to the sugar ring is highly restricted (Yadav & Luger, 1980; Acquotti et al., 1990; Poppe et al., 1990) and the C$_2$−N bond is approximately parallel to the C−CD$_3$ bond.

Analysis of ^{2}H NMR spectra for the glycolipids having probe nuclei in their amino sugars is addressed in Figure 2. These spectra correspond to asialo-GM$_1$, GM$_1$, and GD$_{1A}$, deuterated in the sugar acetate group: i.e., [d_3GalNAc]asialo-GM$_1$, [d_3NeuAc,d_3GalNAc]GM$_1$, and [(d_3NeuAc)$_2$,d_3GalNAc]-GD$_{1A}$. As described above, these contain one, two, and three deuterated acetates respectively. Powder spectra are shown to the left and dePaked spectra to the right. The unsplit central peaks of the powder spectra have been displayed with an expanded frequency axis in the boxed inserts. In each case the glycolipid involved was dispersed as a minor component in fluid bilayers of POPC/cholesterol at 37 °C.

For asialo-GM$_1$ labeled by deuteration of the acetate methyl, a single Pake doublet was expected, since asialo-GM$_1$ possesses only one amino sugar and the three deuterons within a given methyl group are spectrally equivalent. This was the observed result (Figure 2, lower left corner, thick solid arrows), the quadrupole splitting being 10.0 kHz (Table 1). DePaking isolates the zero degree field orientation component of powder spectrum Pake doublets, which in this case produces a single pair of peaks (Figure 2, lower right corner: solid arrow identifies right hand peak) (Bloom et al., 1981). The central region of the deuteroacetate powder spectrum is shown on an expanded frequency axis in the boxed insert. For asialo-GM$_1$ (and GM$_1$, see below) this central peak was composed of a sharp component common to spectra of deuterated lipids (indicated by *), which arises from highly curved and/or rapidly tumbling vesicles [e.g., Curatolo and Neuringer (1986)]; plus a lowfield shoulder attributable to residual traces of HOD (indicated by □). Note that the dePaking algorithm was not designed to deal with such unsplit and asymmetric features and leaves an oscillatory discontinuity in the center of the dePaked result. Figure 3 demonstrates that the powder spectrum of asialo-GM$_1$ shown in Figure 2 was maintained, with modest variation in quadrupolar splitting (Table 1), throughout a considerable temperature range. Measured spectral splittings are shown plotted in Figure 4A (filled triangles). There is a modest splitting decrease with temperature, consistent with thermal reduction in S_{mol} (eq 1).

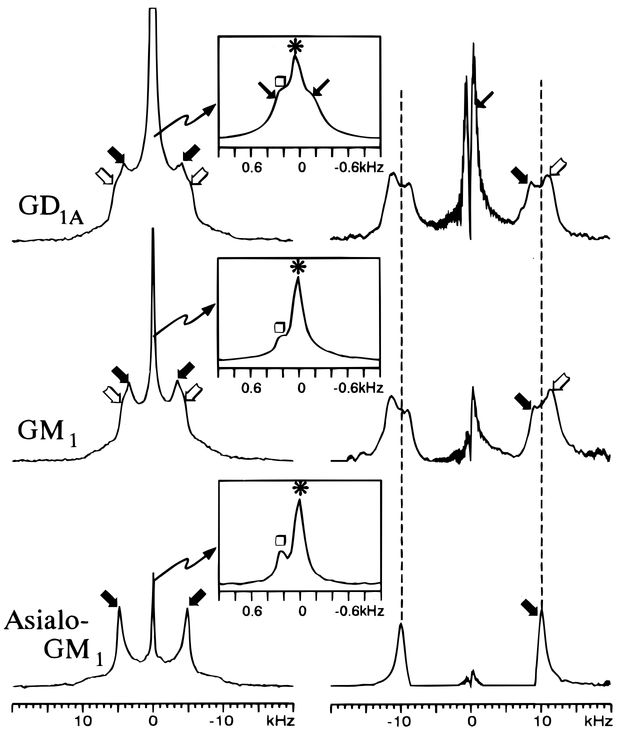

FIGURE 2: Comparison of ^{2}H NMR spectral features among asialo-GM$_1$, GM$_1$, and GD$_{1A}$, whose amino sugars were probe-labeled by deuteration of their acetate groups as in Figure 1. All glycolipids were dispersed at 7.7 mol % in bilayers of POPC/cholesterol at 37 °C (30 mM HEPES buffer, pH 7.4, containing 20 mM NaCl and 2 mM Ca^{2+}). Powder spectra have been stacked at the left, with corresponding dePaked spectra to the right. From bottom to top, [d_3GalNAc]asialo-GM$_1$, [d_3NeuAc,d_3GalNAc]GM$_1$, and [(d_3NeuAc)$_2$,d_3GalNAc]GD$_{1A}$. Since the deuterons of a given methyl group are spectrally equivalent, for asialo-GM$_1$ (which has only one amino sugar {GalNAc}), a single Pake doublet is expected (paired thick solid arrows in powder spectrum). The same style arrows have been used to show the assignment of the Pake doublet associated with the corresponding GalNAc deuteroacetate in GM$_1$ and GD$_{1A}$ powder spectra. For both GM$_1$ and GD$_{1A}$ an additional doublet is expected, attributable to the sialic acid residue attached to the second sugar from the membrane surface (paired thick open arrows). For GD$_{1A}$ only (uppermost spectrum), a third Pake doublet should be seen for the sialic acid residue attached to the fourth sugar from the surface; however, since the quadrupole splitting associated with this sugar is very small, the result appears only as additional intensity in the center of the spectrum. The latter assignment can be made from examination of the boxed inserts (but see also Figure 6): boxed inserts show frequency axis expansions of the central region of each powder spectrum. The sharp peak attributed to highly curved vesicles is indicated in each boxed insert by * and the HOD shoulder by □: paired thin solid arrows in the uppermost insert demonstrate underlying intensity not present in GM$_1$ or asialo-GM$_1$. Each spectrum represents 310 000 transients. Corresponding dePaked features have been indicated on the right-hand side of the dePaked spectra only, using single arrows. Broken vertical lines in the dePaked spectra mark the quadrupole splitting measured for the deuteroacetate group in asialo-GM$_1$ (note that dePaking calculates and displays the 0° orientated spectrum by convention and that the dePaking algorithm does not deal optimally with the shape of the unsplit central peaks).

Table 1: Quadrupolar Splittings ($\Delta\nu_Q$) for Acetate-Deuterated Asialo-GM$_1$, GM$_1$, and GD$_{1A}$ Dispersed in Fluid Bilayers of POPC/Cholesterol^a

deuterated glycolipid	$\Delta\nu_Q$ ($\pm$0.5 kHz)				
	5 °C	25 °C	37 °C	55 °C	75 °C
asialo-GM$_1$	**11.3**	**10.5**	**10.0**	**9.2**	**8.5**
GM$_1$	**10.4**	**9.4**	**8.9**	**8.2**	**7.5**
	12.0†	11.2†	11.3†	11.0†	10.6†
GD$_{1A}$	**11.4**	**10.6**	**8.8**	**7.9**	**7.2**
	12.8†	12.0†	11.4†	10.9†	10.6†
	0.7‡	0.5‡	0.4‡	0.6‡	0.7‡

^{a}All samples were buffered at pH 7.4 in the presence of 2 mM Ca^{2+}. Deuteration sites are shown in Figure 1. Bold numerals denote splittings measured from powder spectra for the doublets corresponding to thick solid arrows in Figure 2. Values marked by "†" correspond to the thick open arrows. Values marked by "‡" for GD$_{1A}$ are estimated widths at $^1/_8$ height for the intense unsplit peak (thin solid arrows in boxed insert) which appeared with addition of the sialic acid residue that distinguishes GD$_{1A}$ from GM$_1$.

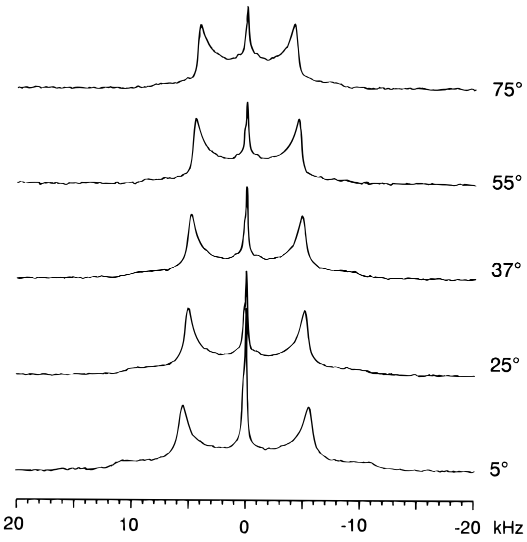

FIGURE 3: Temperature effects on ^{2}H NMR powder spectra of [d_3GalNAc]asialo-GM$_1$. Other sample descriptors are as in Figure 2. Spectra were run from high to low temperature after sample equilibration for 15 min 10 °C above the highest temperature studied. Hysteresis was not observed.

The powder spectrum shape seen for asialo-GM$_1$ (Figures 2 and 3) is characteristic of a single population of molecules undergoing rapid axially symmetric motion about their long molecular axes, as anticipated for amphipathic lipids in a fluid membrane. There is some evidence of partial bilayer orientation in the strong magnetic field, manifest by emphasis of the 90° edges of the powder spectra. This is least obvious at the highest temperatures. Partial orientation of bilayer samples by high magnetic fields has been recorded elsewhere (Seelig et al., 1985; Speyer et al., 1987; Reinl et al., 1992; Qui et al., 1993; Morrow et al., 1995).

For the powder spectrum of GM$_1$ labeled by deuteration of acetate methyls, two Pake doublets were expected: that associated with the GalNAc residue as described above for asialo-GM$_1$, and a new doublet arising from the (single) sialic acid residue which distinguishes GM$_1$ from asialo-GM$_1$. The quadrupole splittings of these two Pake doublets in spectra of GM$_1$ proved to be very similar, particularly at low temperature (Table 1). Nevertheless, separate components could be (incompletely) resolved: thick solid arrows indicate the Pake doublet assigned to the GalNAc acetate (quadrupole splitting 8.9 kHz at 37 °C), and hollow arrows indicate that assigned to the sialic acid acetate in the powder spectrum of GM$_1$ (quadrupole splitting 11.3 kHz at 37 °C) (Figure 2). These features were more readily resolved from one another in spectra of samples at higher temperatures where the differences in quadrupole splittings were greater (e.g., Figure 5, 75 °C, arrows). This proved helpful in spectral interpretation. Individual features were also somewhat more apparent at any given temperature in the dePaked spectrum: e.g., in the Figure 2 dePaked spectrum of GM$_1$ an inner doublet of lesser intensity overlaps a more intense doublet with slightly

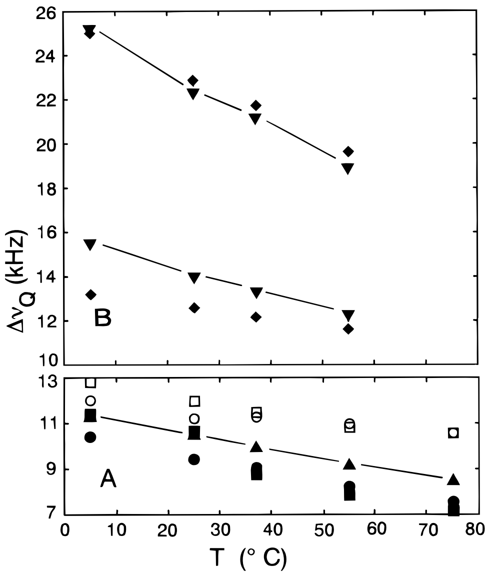

FIGURE 4: Quadrupole splittings plotted as a function of temperature for deuterated amino sugars (A, one value per sugar) and deuterated terminal galactose (B, two values per sugar) associated with oligosaccharide chains of the glycosphingolipids described in Figure 1. In each case, only points corresponding to asialo-GM$_1$ have been connected, as a visual reference. (A) Asialo-GM$_1$ (triangles), GM$_1$ (circles), and GD$_{1A}$ (squares) (filled symbols refer to GalNAC residues; open symbols refer to the sialic acid residue common to GM$_1$ and GD$_{1A}$; data for the sialic acid residue peculiar to GD$_{1A}$ have not been plotted but are estimated in Table 1). (B) Asialo-GM$_1$ (inverted triangles) and GM$_1$ (diamonds).

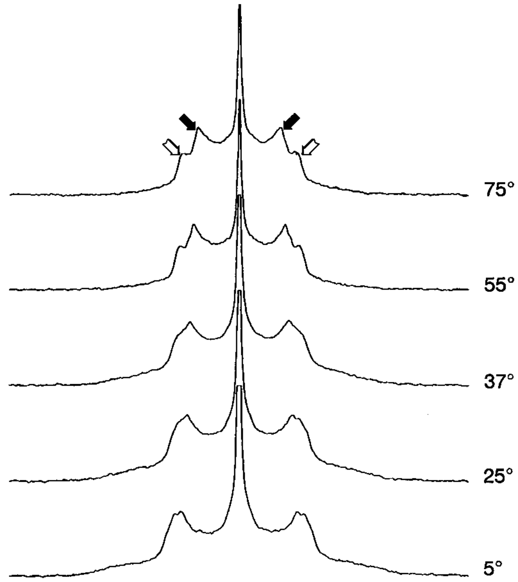

FIGURE 5: Temperature effects on ^{2}H NMR powder spectra of [d_3NeuAc,d_3GalNAc]GM$_1$, dispersed in bilayers of POPC/cholesterol. Other descriptors are as in Figure 2. Spectra were run from high to low temperature after sample equilibration for 15 min 10 °C above the highest temperature studied. Hysteresis was not observed.

larger splitting (the right-hand peaks of these doublets have been labeled with thick solid arrows and hollow arrows, respectively). The lesser intensity for the GalNAc peak in the dePaked spectrum (solid arrow) reflects reduced efficiency of acetate replacement in GalNAc compared to sialic acid [see Materials and Methods and Neuenhofer et al. (1985)]. Note once again that the dePaking algorithm does not deal effectively with the unsplit and asymmetric central peak. Spectral splittings for acetate-deuterated GM$_1$ are shown plotted as a function of temperature in Figure 4A (circles). The values track very closely those described above for asialo-GM$_1$.

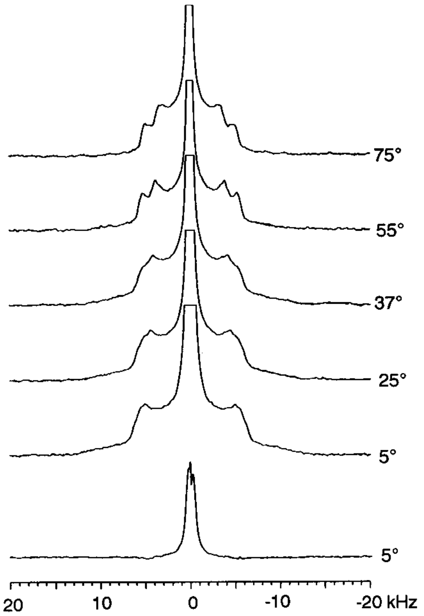

FIGURE 6: Temperature effects on ^{2}H NMR powder spectra of [(d_3NeuAc)$_2$,d_3GalNAc]GD$_{1A}$, dispersed in bilayers of POPC/cholesterol. The result of subtraction of the GM$_1$ spectrum at 5 °C from the GD$_{1A}$ spectrum at the same temperature is included at the bottom (shown at ×0.7 relative vertical scale), demonstrating the spectral location of the third deuteroacetate group. Other descriptors are as in Figure 2. Spectra were run from high to low temperature after sample equilibration for 15 min 10 °C above the highest temperature studied. Hysteresis was not observed.

The general appearance of the powder spectra for GM$_1$ (Figures 2 and 5) is, as noted for asialo-GM$_1$, characteristic of a single population of molecules undergoing rapid axially symmetric motion about their long molecular axes. The unsplit central peak (Figure 2, boxed insert) shows the features described above attributable to the presence of some highly curved vesicles and traces of HOD. There is, however, a significant increase in the intensity of the central peak at lower temperatures (Figure 5). This was not altered by increasing the spectral repetition time and thus does not appear to be a saturation phenomenon. Peaked nondoublet components are common features of spectra of deuterons undergoing axially asymmetric rotation in membranes (Huang et al., 1980; Meier et al., 1986; Siminovitch et al., 1988; Auger et al., 1990). Indeed, we have recently recorded this phenomenon for simpler deuterated glycosphingolipids in the same host matrix (Morrow et al., 1995). Thus this may reflect a measurable departure from strictly axially symmetric motion at low temperature. The prominence of the central region at lower temperature could also indicate that some aspect of the headgroup reorientation is occurring at an intermediate rate on the ^{2}H NMR time scale.

The outer portions of powder spectra for GD$_{1A}$ deuterated in the (three) sugar acetate groups demonstrated striking preservation of the features found for similarly labeled GM$_1$ (e.g., in Figure 2 the quadrupole splittings are 8.8 and 11.4 kHz for GD$_{1A}$ vs 8.9 and 11.3 kHz for GM$_1$). The dePaked spectra illustrate this more clearly: thick solid and thick open arrows mark the peaks on the right side of the dePaked spectrum in each case. This proved to be true for all temperatures (compare Figures 5 and 6; quadrupole splittings listed in Table 1 and plotted in 4A). However, for GD$_{1A}$ there was a new, intense central peak, with unresolved splitting. This is more readily apparent on an expanded frequency axis in the boxed inserts to Figure 2. As described above, for asialo-GM$_1$ and GM$_1$ this peak was composed of a sharp central component (indicated by *) arising from highly curved and/or rapidly tumbling vesicles plus a lowfield off-center shoulder attributable to residual traces of HOD (indicated by □). The difference in the case of GD$_{1A}$ is the appearance of an intense, broader additional central peak that underlies the latter two components (thin solid arrows in uppermost boxed insert), with the result that the (sharper) peak from highly curved vesicles now appears to have two shoulders instead of one. This feature of GD$_{1A}$ was always present, regardless of method of sample preparation and in separate batches of isolated material. When quantitated by integration, the intensity difference between spectra of GM$_1$ and GD$_{1A}$ was limited to the central region of the spectrum and corresponded to one deuteromethyl group. Since the only difference in the GD$_{1A}$ sample (compared to GM$_1$) is addition of the deuterated sialic acid residue on the fourth sugar from the membrane surface, and given the complete similarity of their spectra otherwise, the new central peak was readily assigned to this residue. Figure 6 displays GD$_{1A}$ spectra for a wide range of temperatures. The result of subtraction of the GM$_1$ spectrum from the GD$_{1A}$ spectrum at 5 °C is included at the bottom (shown at ×0.7 relative vertical scale). Quadrupole splittings are listed in Table 1 and shown plotted in Figure 4A (squares: values for the unresolved central peak were estimated from peak width at $^7/_8$ peak height but have not been plotted). As with GM$_1$ there was evidence of asymmetric and/or slowed rotation at temperatures below 37 °C as manifest by buildup of central intensity.

Interpretation of ^{2}H NMR spectra for the glycolipids having deuterium probe nuclei in the terminal galactose (Figure 1) is addressed in Figure 7 (sample conditions directly comparable to those of Figure 2). This probe location permitted consideration of sialic acid effects at a level four residues removed from the membrane surface. The spectra arise from experiments with asialo-GM$_1$ and GM$_1$ in which both protons at C$_6$ of the terminal galactose residue were replaced enzymatically with deuterons (powder spectra are shown to the left, dePaked spectra to the right). Each powder spectrum consisted of two Pake doublets and a central peak. The central peak was completely analogous to that described above in association with acetate-deuterated asialo-GM$_1$ and GM$_1$.

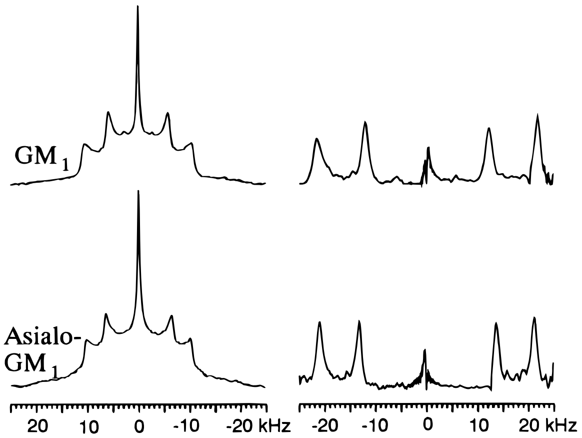

FIGURE 7: Comparison of ^{2}H NMR spectral features for GM$_1$ and asialo-GM$_1$, whose single terminal galactose residues were deuterated in the exocyclic hydroxymethyl group as shown in Figure 1: [d_2Gal]asialo-GM$_1$ and [d_2Gal]GM$_1$. Powder spectra are presented to the left, dePaked spectra to the right. Unlike the -CD$_3$ groups of Figure 2, the deuterons of -CD$_2$OH are spectrally inequivalent, so that a separate Pake doublet (arrows) is expected for each of the two hydroxymethyl deuterons in asialo-GM$_1$ and GM$_1$. Note that, while the dePaking algorithm cleanly isolates the spectral zero degree components from the powder spectrum Pake doublets, it cannot deal optimally with the unresolved central peaks and produces an oscillatory discontinuity in the centre of the dePaked result. Glycolipids were dispersed at 7.7 mol % in bilayers of POPC/cholesterol at 37 °C (30 mM HEPES buffer, pH 7.4, containing 20 mM NaCl and 2 mM Ca^{2+}). Each powder spectrum represents 300 000 transients.

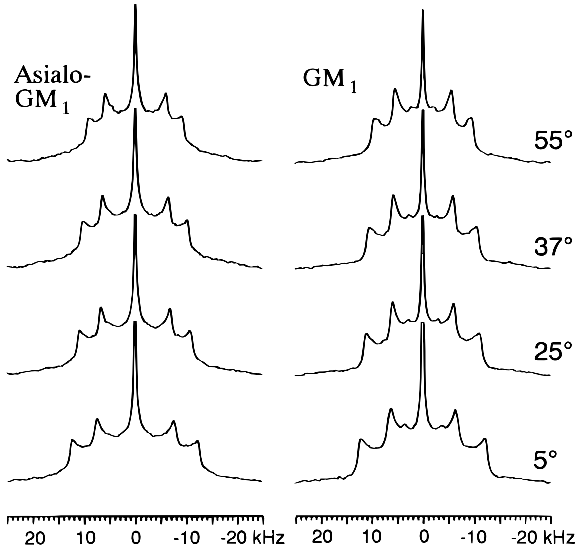

FIGURE 8: Temperature effects on ^{2}H NMR powder spectra of [d_2Gal]asialo-GM$_1$ and [d_2Gal]GM$_1$ dispersed in bilayers of POPC/cholesterol. Other descriptors are as in Figure 7. Spectra were run from high to low temperature after sample equilibration for 15 min 10 °C above the highest temperature studied. Hysteresis was not observed.

As was noted for acetate-deuterated asialo-GM$_1$, the powder spectrum shape is characteristic of a single population of lipid amphiphiles undergoing rapid axially symmetric motion about their long molecular axes in fluid membranes. This was true at temperatures as low as 5 °C and showed little evidence of departure from axially symmetric behavior (Figure 8). The spectra in Figure 7 are highly reminiscent of ones described originally for a similarly deuterated galactose residue in the monoglycosyl glycolipid, galactosyl ceramide (Skarjune & Oldfield, 1979) [see also Singh et al. (1992) and Morrow et al. (1995)]. Apparently-related spectra have been recorded for the terminal galactose residue of lactosyl diglyceride (Renou et al., 1989). Skarjune and Oldfield (1979) demonstrated that the two Pake doublets were readily accounted for by spectral inequivalence of the *pro-R* and *pro-S* deuterons in the -CD$_2$OH group [see also Renou et al. (1989) and Morrow et al. (1995)]. On the basis of our observation that the first round of enzymatic oxidation and NaBD$_4$ reduction of both asialo-GM$_1$ and GM$_1$ produced greater intensity in the inner Pake doublet, while the second round increased the outer doublet [spectra not shown here; see also Singh et al. (1995)] and the observation by Maradufu et al. (1971) that this is the order of replacement of *pro-R* and *pro-S* protons by deuterium in galactose, it seems likely that the inner doublet corresponds to the *pro-R* stereoisomer in the present case.

Table 2: Quadrupolar Splittings ($\Delta\nu_Q$) for Hydroxymethyl-Deuterated Asialo-GM$_1$ and GM$_1$, Dispersed in Fluid Bilayers of POPC/Cholesterol[a]

deuterated glycolipid	$\Delta\nu_Q$ (±0.5 kHz)			
	5 °C	25 °C	37 °C	55 °C
asialo-GM$_1$	15.6	14.0	13.3	12.3
	25.2	22.3	21.1	18.9
GM$_1$	13.2	12.6	12.2	11.5
	25.0	22.9	21.8	19.7

[a] All samples were buffered at pH 7.4 in the presence of 2 mM Ca^{2+}. Deuteration sites are shown in Figure 1.

Figure 7 shows that the Pake doublets which characterise these two deuterons were well resolved from one another: e.g., quadrupole splittings of 13.4 and 21.0 kHz for [d_2Gal]-asialo-GM$_1$ (Table 2). Very similar values (12.5 and 21.6 kHz) were observed for [d_2Gal]GM$_1$. Analogous results were found for temperatures on either side of the physiological range (Figure 8 and Table 2, plotted quadrupole splittings in Figure 4B). The quadrupole splittings are comparable in magnitude to those seen for similarly labeled galactose and glucose residues in mono- and diglycosylglycolipids that have been studied previously, for which S_{mol} values of 0.3–0.5 have been recorded (Skarjune & Oldfield, 1982; Renou et al., 1989).

As noted for the glycolipids deuterated in amino sugar sites, the spectra contain some evidence of partial orientation in the strong magnetic field, more pronounced at lower temperatures. Once again, the most notable aspect of the temperature variation experiments is that the spectra were fundamentally preserved. There was a modest, linear reduction in quadrupole splitting at higher temperature, consistent with the anticipated increase in disorderly motion for molecules having increased thermal energy (Figure 8 and Table 2, plotted data shown in Figure 4B). The temperature variation in measured splittings for GM$_1$ tracked very closely that seen for asialo-GM$_1$ (Figure 4B).

DISCUSSION

A detailed literature exists on the use of ^{2}H NMR spectroscopy to define orientation and behavior of membrane-associated molecules [reviewed in Seelig (1977), Mantsch et al. (1977), Davis (1983, 1991), Smith (1984), Opella (1986), Smith and Peersen (1992), Ketchem et al. (1993), and Opella et al. (1994)]. Full spectral analysis for an inflexible molecule, providing absolute spatial orientation and motional order, would require up to five probe nuclei. Unfortunately, selective deuteration of oligosaccharides is an extremely difficult problem. As a result, complete ^{2}H NMR spectral analyses of membrane-associated sugars have been achieved only for relatively simple species containing one or two sugar residues (Skarjune & Oldfield, 1982; Renou et al., 1989; Winsborrow et al., 1992, and references therein). In the experiments described here, we probed a total of four distinct sites in a nonrigid oligosaccharide chain containing up to six sugar residues. Thus the information obtained is of necessity limited primarily to "extent of change" (induced by sialic acid).

One notable observation is that addition or removal of sialic acid had very modest effects on ^{2}H NMR spectra of nonperturbing probe nuclei located at key positions within the complex oligosaccharide chain. The implications of this result can be appreciated by examination of eq 1. The relationship dictates that a given deuterium quadrupole splitting is influenced both by C–D bond spatial orientation (Θ) and by motional order (S_{mol}). The largest splitting possible for a rapidly rotating -CD$_3$ group on a lipid in a fluid membrane is close to 42 kHz. The latter upper limit would occur only if S_{mol} were at its maximum value of 1.0 and the C–CD$_3$ bond were oriented perpendicular to the plane of the membrane. Hence, the 10 kHz range splittings seen for the deuterated acetate group of GalNAc (which is common to asialo-GM$_1$, GM$_1$, and GD$_{1A}$) (Table 1) indicate considerable preservation of orientational order ($S_{mol} \geq 0.25$) for the shared portions of their carbohydrate chains up to and including GalNAc. Moreover, the fact that the splitting measured for this residue was the same, within ±0.5 kHz, for each of the three glycolipids at any given temperature strongly suggests that these portions of their oligosaccharide chains share very much the same average orientation, conformation, and motional order. GalNAc occupies an internal position as the third carbohydrate residue from the membrane surface in each of the recognition sites studied. Its orientation must reflect cumulative effects associated with residues closer to the ceramide portion.

The above findings at the level of GalNAc are reinforced by our spectral observations related to the sialic acid residue common to GM$_1$ and GD$_{1A}$. For this residue the quadrupole splittings were 11–13 kHz (Table 1). Following the reasoning already described, this would imply that the order parameter for the sialic acid residue involved cannot be less than 0.28. Indeed, Aubin et al. (1993) have calculated an order parameter, S_{mol}, of 0.73 for the sialic acid residue of GM$_3$ in bilayer-like micelles. Since GM$_3$ has the same oligosaccharide structure as GM$_1$ (with the GalNAc and terminal Gal residues of the latter removed), it seems reasonable that a similar S_{mol} value would apply for the sialic acid residue common to GM$_1$ and GD$_{1A}$. Also, as noted for the GalNAc residue, at any given temperature, the values measured for the GM$_1$ sialic acid were within ±0.5 kHz of those found for the corresponding residue in GD$_{1A}$ and within ±0.2 kHz above 25 °C. Like GalNAc, this sugar is again the third residue from the membrane surface (although it occupies a terminal location); hence its spectroscopic characteristics are a reflection of cumulative differences along the chains of GM$_1$ and GD$_{1A}$.

The concepts that the shared portions of the oligosaccharide chains studied here must possess considerable conformational stability, and that they assume similar average conformations at a membrane surface, are further supported by the spectra observed for -CD$_2$OH on terminal galactose. Gal in a terminal location is the fourth residue from the ceramide portion of asialo-GM$_1$ and GM$_1$. The magnitudes of the quadrupole splittings for these deuterons were found to be in the same range as those reported for similarly deuterated terminal Gal residues in galactosyl ceramide and lactosyl diglyceride (Skarjune & Oldfield, 1979; Renou et al., 1989). For this residue in lactosyl diglyceride, an S_{mol} value of 0.51 was calculated (Renou et al., 1989). Once again, in spite of the fact that the terminal Gal orientation and behavior must reflect the summation of all differences along the chain, for a given temperature the splittings measured for asialo-GM$_1$ were remarkably close to those for GM$_1$ (within ±0.5 kHz, with the exception of the inner splittings at 5 °C which were 15.6 and 13.2 kHz, respectively).

If one considers the above findings, presuming even the minimum order parameters (0.25−0.28), eq 1 dictates that average spatial orientations of the probed residues were maintained within a maximum angular deviation of some ±5°, and quite possibly within a considerably tighter range. This value reflects summative characteristics involving residues closer to the membrane surface. It is interesting that asialo-GM$_1$ has been observed to be more antigenic than most gangliosides (Sillerud & Yu, 1983). It would appear from the present work that the latter effect does not arise from intrinsically different conformation or order.

By the same reasoning, the small (unresolved) spectral splitting which was observed for the sialic acid residue that is only present on GD$_{1A}$ (Figure 1) suggests that either S_{mol} for this residue is small and/or that the sugar is oriented such that its C−CD$_3$ bond makes an angle approaching 54.7° (or 125.3°) to the molecular long axis (eq 1). We did not find the latter orientations to be consistent with molecular modeling when GD$_{1A}$ was examined using oligosaccharide conformation data from Acquotti et al. (1990) and Scarsdale et al. (1990) superimposed on a membrane, employing the glucose orientation of glucosyl ceramide [Skarjune & Oldfield, 1982; see Nyholm and Pascher (1993)]. It seems quite possible that more disordered motion exists for this residue, as suggested by Poppe et al. (1994) on the basis of high resolution studies of GD$_{1A}$ in detergent solution.

One might have anticipated that systematic removal of sialic acid residues from GD$_{1A}$ would have had a larger effect on oligosaccharide backbone conformation and order than was observed here. The sugar in question is charged in addition to being a possible source of steric and polar interactions. Important X-ray studies of simple glycosphingolipids demonstrated early-on the potential for conformational effects of hydrogen bonding in single crystals (Pascher & Sundell, 1977; Nyholm et al., 1990). However, it was recognized that the magnitudes of polar interactions may be different for molecules dispersed in hydrated fluid membranes [e.g., Bunow and Levin (1980) and Curatolo (1987)]. Scarsdale et al. (1990) used high resolution 2-D NMR spectroscopy to compare GM$_1$, GM$_{1B}$, and GD$_{1A}$ oligosaccharide conformation in DMSO/2% H$_2$O solution. They remarked on the possibility of significant conformational effects related to placement of the sialic acid residue. However, they also demonstrated that the lowest energy conformers for the core oligosaccharide of GM$_1$ and GD$_{1A}$ were very similar: indeed the differences shown are consistent with our findings. It is certainly possible that at a fully hydrated bilayer surface factors related to surface constraint can override subtle differences based on primary sequence changes. The latter possibility has been indicated by Nyholm and Pascher (1993): that “in a bilayer arrangement the range of possible conformations for the saccharide-lipid linkage is considerably reduced” [see also Winsborrow et al. (1992) and Aubin and Prestegard (1993)]. It should also be noted that Aubin et al. (1993) found no conformational effect of lectin binding on the conformation of the GM$_3$ headgroup at a model surface.

It is noteworthy that the key observations described in the present work remained the same over a wide range of temperatures (from 5 °C to as high as 75 °C). In each case, spectral features associated with probe nuclei displayed a modest, essentially linear, decrease in quadrupole splitting as temperature was raised (Figure 4). This is readily accounted for by the greater motional disorder (lower S_{mol}) expected at higher temperatures (eq 1). In agreement with our conclusions that the three oligosaccharide chains studied had similar conformations and orientational behavior, the temperature effects at a given probe location were virtually identical within experimental error (Figure 4), i.e., for a given probe location, the curves tracked one another closely. Our results provide little evidence for the existence of more than one major low energy conformer, since in such a case one would expect preferential shift to this conformer at lower temperatures, with resultant more striking alteration in spectral splittings.

There was some evidence of axially asymmetric motion associated with sialic acid residues in the present work. This was evident in GM$_1$ and GD$_{1A}$ having deuterium probe nuclei on the acetate groups of sialic acid, below the physiological temperature range. It was manifest as a low-temperature-induced increase in intensity toward the central region of the spectrum. Since this effect was not as evident in the case of deuterons located on GalNAc or terminal Gal, it seems quite likely that it reflects an asymmetric component of motion peculiar to the sialic acid residues. It is interesting therefore that Aubin and Prestegard (1993) noted anisotropy associated with the motion of a single sialic acid residue attached to a hydrocarbon chain at a model membrane surface. They suggest the possibility of sialic acid interaction with a membrane surface. Raju and Davidson (1994) have recently reported an apparent contribution of sialic acid residues to viscosity in solutions of mucin glycoproteins.

CONCLUSIONS

There exists in the literature the important question of how modification of a cell surface receptor may alter its conformation and orientation, as a mechanism of controlling recognition events or of signaling. In the present work we addressed a specific aspect of this question by measuring the effect of sialic acid in a membrane-localized oligosaccharide recognition site. Wideline NMR spectroscopy was used to study three complex glycosphingolipids differing only in the presence of this acidic sugar. Deuterium probe nuclei in a GalNAc residue permitted direct comparison amongst all three chains at an internal location three residues from the membrane surface. Information was also provided by probe nuclei in sialic acid of GM$_1$ and GD$_{1A}$ at a terminal location three residues from the membrane, and by deuterons in a terminal galactose four residues from the surface which further linked the GM$_1$ data to asialo-GM$_1$. Highly preferred average conformations were found to exist for the carbohydrate portions of asialo-GM$_1$, GM$_1$, and GD$_{1A}$ at bilayer surfaces mimicking important plasma membrane characteristics. The average conformation and orientation of the complex oligosaccharide were preserved, as monitored at the probe sites, within an angular range of ≤10° after addition or removal of sialic acid. Our observations argue that the conformations of oligosaccharide recognition sites at membrane surfaces can be surprisingly stable to perturbation. The greatest limitation to the approach used was the difficulty of selectively deuterating multiple sites in oligosaccharides, which importantly restricted our ability to define absolute structure and motional order. Moreover, although the deuteration sites selected were critical ones for probing the structure as a whole, in general it would be desirable to obtain separate information on each portion of a flexible receptor, in this case on each individual carbohydrate residue.

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BI952964M