Conformation of the Oligosaccharide Chain of G_{M1} Ganglioside in a Carbohydrate-Enriched Surface

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ABSTRACT The solution structure of ganglioside G_{M1} carbohydrate moiety at the surface of a 102-kDa lipid-modified-G_{M1} micelle is investigated by high-resolution ^{1}H-NMR in H_2O. The micellar surface can be considered a cluster-like lateral distribution of the gangliosides, each single monomer being anchored in a carbohydrate-enriched model membrane matrix. ^{1}H NOESY measurements at short mixing times reveal a rigid trisaccharide core -β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal- and a more flexible β-Gal-(1–3)-β-GalNAc- terminal glycosidic bond. In the lipid-modified G_{M1} ganglioside micellar system, there is no evidence that intermolecular side-by-side carbohydrate interactions modulate, or alter in any way, the head-group spatial arrangement. Possible intermonomer interactions at the level of the branched trisaccharide portion were further investigated on mixed micelles of natural *N*-glycolyl- and *N*-acetylneuraminic acid containing G_{M1} in D_2O, taking advantage of the different NMR features of *N*-glycolyl- and *N*-acetylneuraminic acids, which allow discrimination between sialic acid ring proton signals. Measurements of the water/ganglioside-OH proton chemical exchange rates suggest hydroxyl group involvement at position 8 of sialic acid in strong intramolecular interaction processes.

INTRODUCTION

It is now widely accepted that many cellular functions are modulated by information transfer processes at the external surface of plasma membrane, such processes being mediated by specific interaction events. Thus investigations into the molecular basis of such interactions could lead to a better understanding of the role of the receptor primary structure, the receptor conformation, and dynamics at the membrane surface. There is evidence that the properties of the receptor membrane environment influence the determination and regulation of information transfer at a given recognition site (Hakomori, 1986; Curatolo, 1987). In fact, the amount of "conformational information" carried by a particular recognition site in the crowded layer of a physiological membrane might reflect the presence and/or alteration of intermolecular interactions involving the molecular species in the receptor environment.

GSLs contain a number of recognition sites, and it is known that their carbohydrate chains protruding from the cell surface play a key role in cell adhesion and growth regulation processes (Hakomori, 1981, 1984, 1993; Fishman, 1982). NMR techniques have been widely applied in structural and conformational studies on gangliosides. Monomeric gangliosides have been studied in detail by high-resolution ^{1}H-^{13}C NMR in DMSO solvent, where they do not aggregate (G_{M3}, Siebert et al., 1992; G_{M2}, Levery, 1991; G_{M1}, Scarsdale et al., 1990, and Acquotti et al., 1990; G_{D1b}, Acquotti et al., 1991; G_{D1a} and GalNAc-G_{D1a}, Acquotti et al., 1994). The main conformational features determined in the organic solvent for the ganglioside oligosaccharides were observed for a single ganglioside monomer inserted in a small DPC micelle in water solution (G_{M3}, Siebert et al., 1992; G_{M1}, Acquotti et al., 1990; G_{D1a} Poppe et al., 1994; G_{M1}(Nue5Gc), Brocca et al., 1996). The DPC micelle model takes into account the influence of water and a phospholipid surface environment on the ganglioside head-group spatial arrangement.

In the last decade several ^{13}C- and ^{2}H-NMR studies have investigated the behavior of gangliosides and other GSLs at the surface of oriented lipid bilayers (Aubin and Prestegard, 1993; Aubin et al., 1993; Skarjune and Oldfield, 1979, 1982; Jarrel et al., 1987a,b, 1992; Hamilton et al., 1994; Singh et al., 1995; Jones et al., 1996). ^{13}C-NMR can provide complete details on the behavior of the ^{13}C-marked sugars, but ^{13}C enrichment in different locations is a synthetic challenge that is unsuitable for complex oligosaccharides (Aubin and Prestegard, 1993).

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Abbreviations used: Ganglioside nomenclature is in accordance with Svennerholm (1980), and the IUPAC-IUB recommendations (1977, 1982). GSL, glycosphingolipid; Cer, ceramide; G_{M1}, II^{3}Neu5AcGgOse$_4$Cer, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; G_{M1}(Neu5Gc), G_{M1} containing Neu5Gc; G_{M1}-acetyl, G_{M1} containing an acetyl group as acyl moiety; deacetyl-deacyl-G_{M1}, G_{M1} lacking the sialic acid acetyl group and the fatty acyl chain; G_{M3}, II^{3}Neu5AcLacCer, α-Neu5Ac-(2–3)-β-Gal-(1–4)-β-Glc-(1–1)-Cer; G_{D1a}, IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$Cer, α-Neu5Ac-(2–3)-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; GalNAc-G_{D1a}, IV^{4}GalNAcIV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$Cer, β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; DMPC, dimyristoylphopsphatidylcholine; CHAPSO, 3-[(cholamidopropyl)dimethylamino]-2,2-dihydroxy-1–1-propanesulfonate; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; DPC, dodecylphosphocholine; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.

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The G_{M3} ganglioside, ^{13}C-marked on the sialic acid moiety, was studied in DMPC/CHAPSO magnetically oriented membrane, and the results were in accordance with a well-extended backbone structure with quite low motional average effect (Aubin et al., 1993).

Grant and co-workers applied ^{2}H-NMR to deuterated gangliosides dispersed as minor components in model membranes of POPC (Jarrell et al., 1992; Barber et al., 1994; Singh et al., 1995; Jones et al., 1996) and demonstrated that ganglioside backbone displacements have, with respect to the membrane surface, a preferred average conformation, similar for gangliosides from G_{M3} to the more complex G_{D1a}. It appears that both the conformation and the motional order of the backbone are unaffected by the lipidic composition of the hydrophobic portion, the presence of cholesterol in the membrane (decreasing membrane fluidity), and temperature variation. Not even the presence of the charged *N*-acetylneuraminic acid residue seems to influence the backbone spatial arrangement. Thus it is difficult to relate any of these physical factors to ganglioside crypticity, i.e., to the degree of the receptor availability to interactions with extracellular ligands (Singh et al., 1995). Gangliosides segregate into clusters at the cell membrane surface, and it has been shown that they become immunoactive only when they form part of high-density ganglioside microdomains (Nores et al., 1987). One hypothesis is that modifications of physical factors such as the lipidic composition, the membrane fluidity, temperature variation, etc., could induce a modification of the lateral distribution of the membrane receptor species (Mehlhorn et al., 1988) and that lateral distribution could alter receptor site accessibility. Great interest has been shown in "side-by-side" and "head-to-head" carbohydrate-carbohydrate interactions that could be the basic biophysical mechanism regulating some cell functions (Bovin, 1996). The segregation process itself could be influenced by intermonomer interactions, possibly related to some conformational change in the molecules.

This paper reports studies on the behavior of ganglioside carbohydrate head groups forced to stay in a highly enriched lateral distribution. The proposed experimental model consists of micelles uniformly composed of either natural G_{M1} or modified G_{M1} ganglioside [β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer]. High-resolution ^{1}H-NMR studies of the micelles show few interresidual dipole-dipole interactions for the CH protons of the oligosaccharide chain; thus studies are also needed on hydroxyl proton interactions. To detect the OH signals in a micellar system, the temperature must be decreased to prevent fast exchange with water proton. However, natural G_{M1} micelles of 480 kDa are too large for high resolution at low temperature, and it was for this reason that a single-chain ganglioside derivative was introduced: G_{M1}-acetyl, a modified G_{M1} ganglioside with only one lipid chain, as the hydrophobic moiety (Sonnino et al., 1990). G_{M1}-acetyl aggregates in water as small, almost spherical micelles of 102 kDa, i.e., about one-fifth the molecular weight of natural G_{M1} micelles (Corti et al., 1980), that allow high-resolution spectra to be obtained (Brocca et al., 1995).

OH assignment is presented, and through-space NOE interactions for the head group are analyzed. The intramolecular and carbohydrate-carbohydrate intermolecular interactions are discussed. The relevant features of the head-group conformation derived from NOESY measurements are highlighted by applying molecular modeling (MM) energy minimization, with NOE distances introduced as restraints. The interactions between the solvent molecules and the carbohydrate head group are discussed, and the chemical exchange rates of some OH protons are measured.

To take into account any possible effects on the oligosaccharide conformation of an irreversible micellar transition induced by temperature previously observed by Corti and co-workers (Cantù et al., 1996), parallel experiments were performed on two samples, one prepared at 12°C and the other heated at 65°C before analysis at 12°C.

MATERIALS AND METHODS

Gangliosides

Ganglioside G_{M1} was purified to over 99% (Acquotti et al., 1994) from a ganglioside mixture extracted from bovine brain (Tettamanti et al., 1973). G_{M1} containing *N*-glycolyl neuraminic acid, G_{M1}(Neu5Gc), was prepared by a semisynthetic procedure from G_{M1} (Sonnino et al., 1988). Both G_{M1} and G_{M1}(Neu5Gc) ceramide moieties contained stearic acid as the main acyl chain, this corresponding to ~90% of the total fatty acid content, as determined by gas chromatography (Casellato et al., 1995). G_{M1}-acetyl was derived from natural G_{M1} following a procedure that involved alkaline hydrolysis to yield deacetyl-deacyl-G_{M1} and a subsequent *N*-acetylation reaction (Sonnino et al., 1985, 1990). The ganglioside micelle aggregation parameters were determined by static and dynamic light scattering (Corti et al., 1980; Sonnino et al., 1988, 1990). Ganglioside-bound sialic acid was determined by the resorcinol/HCl method (Svennerholm, 1957; Miettinen and Takki-Lukkainen, 1959), using Neu5Ac and Neu5Gc as the reference standards (Sigma Chemical Co.).

Sample preparation

Two 15 mM G_{M1}-acetyl samples were prepared at 12°C in H_2O/5% D_2O solution corrected to pH 7.4 by the addition of HCl; one sample was maintained and analyzed at 12°C, the other was heated to 65°C, kept at this temperature for 2 h, and then analyzed at 12°C. Samples of G_{M1} and a mixture of G_{M1}/G_{M1}(Neu5Gc) at a 1:1 molar ratio were prepared at 15 mM total concentration in D_2O solvent. To completely screen the micelle surface charge, sodium chloride was added to all of the samples to obtain 25 mM solutions (Cantù et al., 1986), the micelle properties and NMR spectral parameters being in no way affected.

Conformational study

NMR experiments were performed on Bruker AM 500, DRX 500, and DRX 600 spectrometers, the last two being upgraded with a pulsed field gradient system. Data were processed and analyzed with Bruker XWIN-NMR software. All of the reported chemical shifts in water are referenced to the typical sphingosine olefinic H4 resonance at 5.45 ppm, whose resonating frequency in water is not affected by the elimination of the N-linked fatty acid or by temperature variation. G_{M1}-acetyl full assignment of nonlabile protons and carbons in D_2O has been published elsewhere (Brocca et al., 1995). Labile proton assignments were obtained through

2D-TOCSY (Bax and Davis, 1985a). 2D NOESY (Jeener et al., 1979) experiments were performed in light water with the WATERGATE solvent suppression technique (Piotto et al., 1992; Sklenár et al., 1993). Two series of 2D spectra were acquired on the two G_{M1}-acetyl samples at 12°C, one with mixing times of 15, 25, and 35 ms and the other with 15, 20, and 30 ms. To suppress remaining high-order coherence, a strong gradient pulse (10 G cm^{-1} for 1 ms) was applied at the beginning of the mixing time, and a low-power gradient pulse (1 G cm^{-1}) was applied during the rest of the mixing to avoid radiation damping effects. Cross-peak volumes were normalized by diagonal peak volumes, when strong signal overlapping did not preclude such correction. The initial slopes of the build-up curves were then measured by a linear fit procedure. Initial slopes were then transformed into proton-proton distances by using internal calibration on Glc-H1/H2, Gal(II)-H1/H2, GalNAc-H1/H2, Neu5Ac-H3eq/H3ax distances. The average distances in the two series were considered. 2D ROESYs (Bax and Davis, 1985b) were acquired in D_2O with a spin-lock strength of 2.6 kHz. The spin-lock pulse was applied at one end of the spectrum by using a pulse sequence described elsewhere (Farmer and Brown, 1987; Bax, 1988; Acquotti et al., 1990). The spin-lock duration ranged from 80 to 140 ms. 1D selective ROESY (Bothner By et al., 1984) spectra were recorded at 60°C in D_2O with selective excitation (Sklenár and Feigon, 1990) of Gal(IV)-H1 based on DANTE pulse trains (Morris and Freeman, 1978). The spin-lock radio frequency (rf), at 2.5 kHz power, was shifted by 1.2 kHz from the excited signal.

Molecular modeling

The minimum energy conformation of the oligosaccharide moiety was computed with an INSIGHT/DISCOVER 2.2.1 package (MSI/Biosym). This was accomplished by the use of restrained molecular mechanics, with the application of an AMBER* force field (Homans, 1990) with a dielectric constant of 80. NOE constraints were inserted as harmonic potential. In the simulation the lipidic portion of the molecule was replaced by a methyl group.

Hydration study

The interaction of water protons with the protons of the ganglioside sugar head group was studied by means of ^{1}H-NMR pulse sequences based on the WEX II sequence (Mori et al., 1996). The pulse scheme started with a selective excitation on the water resonance, followed by a spin-echo filter aimed at eliminating magnetization from signals under or near the water frequency, based on their difference in transverse relaxation. The spin-echo filter was adjusted for complete T_2 relaxation of the anomeric protons (total 28 ms spin echo duration), and low-gradient amplitude was applied to prevent excessive water signal loss due to the spatial diffusion affecting complete refocalization. A mixing period followed, during which the magnetization was transferred from water protons to other protons through dipole-dipole interactions or chemical exchange. Two series of experiments were performed. For the first series, a NOESY-type mixing period was used, where the magnetization evolved through longitudinal dipolar relaxation. The second series was a ROESY version of the WEX II sequence, where the mixing period consisted of a strong adiabatic off-resonance rf field (Desvaux et al., 1994, 1995). The mean angle between the static field and the effective field experienced by the nuclei was chosen to be 35.3°. At this angle the major contribution to cross-relaxation arose from chemical exchange, the dipolar relaxation terms being negligible. The chemical exchange rates were then measured for the few nonoverlapped OH signals, namely Gal(II)-OH2, Neu5Ac-OH4, and Neu5Ac-OH8. The NOESY series consisted of 14 spectra with the following mixing times: 1, 2, 3, 4, 5, 7, 10, 15, 25, 37, 50, 70, 90, and 120 ms. For the off-resonance ROESY version of WEX II sequence, 12 experiments were performed, the mixing times being 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, and 120 ms.

OH-signal enhancement was analyzed as a unique chemical exchange process, and peak intensities were fitted according to

$$M(t) = M_0 + A[1 - \exp(-t/t_m)]$$

where M is the magnetization at time t, M_0 is the initial magnetization, A is a scale factor, and t_m is the exchange time. The fits were optimized by eliminating the last one or two points when necessary. These points might be affected by a magnetization loss through dipolar mechanisms. The measurements were repeated at different temperatures to give the OH chemical exchange rates at 3°C, 7°C, and 12°C.

RESULTS

Labile proton assignment

The small, quite spherical G_{M1}-acetyl micelle, showing a hydrodynamic radius of 34 Å and an aggregation number of 76 (Sonnino et al., 1990), gives well-resolved NMR spectra, but at temperatures higher than 27°C the water/ganglioside-OH proton chemical exchange is extremely efficient, resulting in a collapse of the OH proton signals at the water frequency. Recently, the full assignment of nonlabile protons of G_{M1}-acetyl micelles in water was published (Brocca et al., 1995). While studying the molecular spatial arrangement by the analysis of dipolar NOE interactions, we detected a low number of interresidual interactions for nonexchangeable protons that led us to find experimental conditions where at least the most informative OH resonances could be followed. Fig. 1 shows that by decreasing the temperature to 12°C and adjusting the pH to 7.4, the Gal(II)-OH2, Neu5Ac-OH4, Neu5Ac-OH8, Neu5Ac-OH7, and Glc-OH3 resonances appear at 6.51, 6.34, 6.24, 5.86, and 5.84 ppm, respectively. Their assignment was derived by TOCSY and NOESY maps and is in agreement with the one given for the G_{D1a}/DPC system (Poppe et al., 1994).

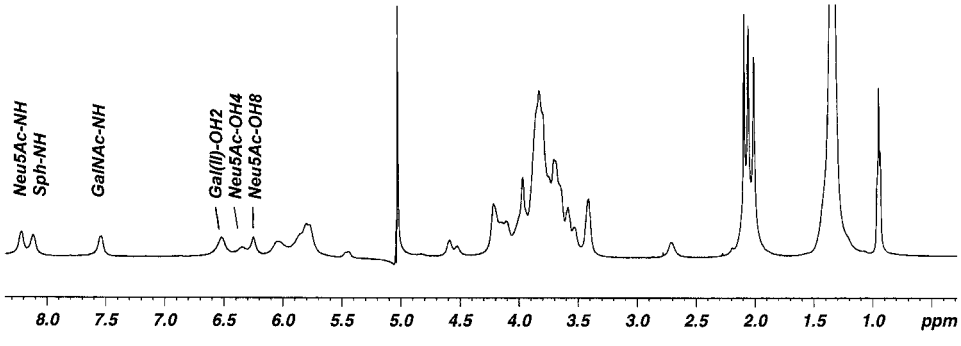

FIGURE 1 ^{1}H-NMR spectrum (500 MHz) of G_{M1}-acetyl micelle in H_2O/5% D_2O at pH 7.4 and at 12°C. Water is suppressed by the WATERGATE sequence. The detectable OH and NH protons were assigned by means of 2D TOCSY and NOESY experiments.

Conformational study of the headgroup

The averaged area available for a single monomer at the hydrophobic-hydrophilic interface of G_{M1}-acetyl micelles has been evaluated as 64.8 $Å^2$ (Sonnino et al., 1990). This means that a single monomer could be close to its neighbor at the level of their head groups. A priori, intermonomer carbohydrate-carbohydrate interactions could compete or participate with intramonomer interactions in yielding the ganglioside preferred conformation in the micellar system.

Lowering the temperature to detect OH signals of the G_{M1}-acetyl micelles obviously decreases the T_2 relaxation times, which fall between 5 and 10 ms at 12°C. This not only affects the spectral line resolution, but also prevents the use of ROE measurements where transverse relaxation competes with the cross-relaxation mechanism. Therefore, as only longitudinal dipolar relaxation could be followed, the experimental set-up and data analysis were aimed primarily at getting rid of spin diffusion cross-relaxation effects. In fact, the large dimension and low mobility of the system cause very effective spin diffusion to be observed. The choice of very short mixing times (15–35 ms) in NOESY experiments allowed us to fulfill the condition of linear dependence of the NOE versus time, thus ensuring that important spin diffusion effects are avoided.

Nevertheless, the most effective spin-diffusion patterns were detected by performing 2D-ROESY at higher temperatures, 30°C and 33°C, where T_2 relaxation did not impede signal detection. In a ROESY experiment, a combined ROE-ROE cross-correlation (three-spin effect) took up the opposite phase with respect to direct ROE (Bax and Davis, 1985b). It was possible to establish that the main interresidual spin-diffusion patterns for the carbohydrate moiety were across the glycosidic linkages -GalNAc-Gal- and Neu5Ac-Gal-. The former was determined by the opposite phase Neu5Ac-H3eq/Gal(II)-H3 contact mediated by the Neu5Ac-H3eq/Neu5Ac-H3ax interaction, the latter by the observation of the GalNAc-H1/Gal(II)-H3 and Gal(II)-H2 contacts mediated by the GalNAc-H1/Gal(II)-H4 strong NOE. This would suggest a longer correlation time for the trisaccharide -GalNAc-(Neu5Ac-)Gal- moiety than for the glucose and the external galactose, thus indicating a more restricted mobility for the trisaccharide.

On the basis of ROESY results, the NOESY build-up series of experiments was analyzed. The data interpretation was simplified by the finding that the detected interactions were in complete agreement with those expected for a ganglioside head group that maintained the same conformational features already observed for a monomer in DMSO (Acquotti et al., 1990, 1994) and for a single ganglioside molecule per DPC micelle in water (Acquotti et al., 1990; Poppe et al., 1994; Brocca et al., 1995). On comparing the results of these authors with the NOE interaction of G_{M1}-acetyl micelle, it can be seen that, qualitatively, there were no new contacts and no missing contacts at the level of the saccharidic portion of the G_{M1}-acetyl micelle (Table 1, column a) with respect to the other mentioned systems

TABLE 1 Main experimental interresidual proton-proton distances (Å) for the G_{M1} pentasaccharide and for the same pentasaccharide belonging to other gangliosides

	(a) G_{M1}-acetyl micelle in H_2O	(b) G_{M1} monomer in DMSO	(c) G_{D1a} monomer in DMSO	(d) $GalNAcG_{D1a}$ monomer in DMSO	(e) G_{M1}(Neu5Gc)/DPC monomer in D_2O	(f) G_{D1a}/DPC micelle in H_2O
Gal(IV)-H1/GalNAc-H2	3.0	3.5	n.p.	3.3	3.5	d
Gal(IV)-H1/GalNAc-H3	n.e.	2.5	2.5	2.4	2.2	d
Gal(IV)-H1/GalNAc-H4	n.e.		n.p.	3.5	2.7[§]	d
Gal(IV)-H1/GalNAc-NH	3.0	3.5	3.8	3.6		
Neu5Ac-OH8/H6	2.2	2.4				d
Neu5Ac-H8/GalNAc-H1	n.p.	3.1	3.3	2.4	2.7[§]	
Neu5Ac-OH8/GalNAc-H1	2.9	2.6	2.8	2.7		
Neu5Ac-OH8/GalNAc-NH*	3.6					d
Neu5Ac-H3eq/Gal(II)-H3[#]	Spin diffusion					Spin diffusion
Neu5Ac-H3ax/Gal(II)-H3	2.4		2.3	2.3	2.6	d
Neu5Ac-H3ax/Gal(II)-OH2	3.4	3.2				
GalNAc-NH/H1	2.5	2.6				
GalNAc-NH/Gal(II)-H2	3.4	3.6	3.6	4.0		
GalNAc-H1/Gal(II)-H4	2.4	2.2	2.3	2.4	2.3	d

(a) G_{M1}-acetyl micelle in H_2O/5% D_2O solution measured by short mixing time NOESY build-up.
(b) G_{M1} ROESY-derived distances from Acquotti et al. (1990).
(c) and (d) G_{D1a} and $GalNAcG_{D1a}$ ROESY-derived distances from Acquotti et al. (1994).
(e) G_{M1} (Neu5Gc) ROESY-derived distances from Brocca et al. (1996).
(f) G_{D1a} NOE interactions from Poppe et al. (1994).
n.e., Distances not evaluated because of overlap.
n.p., Interaction not proved because of overlap.
d, Detected interaction. NOE data were discussed qualitatively.
*The Neu5Ac-OH8/GalNAc NH contact was proved to originate from dipolar interaction and was not affected by chemical exchange.
[#]The Neu5Ac-H3eq/Gal(II)-H3 contact arises from a three-spin effect involving Neu5Ac-H3ax.
[§]Distance was underestimated because of overlapping.

(Table 1, columns b–f). Thus it appears unnecessary to assume any significant effect due to intermonomer interactions, allowing us to regard all of the contacts as belonging to a single monomer (see below). Even if NMR-invisible contact cannot be excluded, the results suggest that the carbohydrate-enriched surface does not influence the main conformational features of the single ganglioside oligosaccharide.

The typical contacts characterizing the Neu5Ac and the GalNAc interaction were detected and were as expected. In particular, Neu5Ac-OH8/GalNAc-H1, Neu5Ac-OH8/GalNAc-NH, and the contacts Neu5Ac-OH8/Gal-H4 (three-spin effect; Fig. 2), Neu5Ac-H3ax/Gal-H3, and Neu5Ac-H3ax/Gal-OH2 satisfy the proposed single fairly rigid spatial arrangement of the trisaccharide -GalNAc-(Neu5Ac-)Gal-.

The contemporary presence of the Gal(IV)-H1/GalNAc-H2 and Gal(IV)-H1/GalNAc-H4 contacts, which in a single conformer is not geometrically allowed, suggested a mobility of Gal(IV) around its glycosidic bond. The same interactions had already been detected for other systems (G_{M1} in DMSO, Acquotti et al., 1990; $GalNAcG_{D1a}$ in DMSO, Acquotti et al., 1994; G_{D1a} in H_2O, Poppe et al., 1994). Despite the strong overlap of the anomeric protons of Gal(IV) and Gal(II), the interaction of Gal(IV)-H1 with GalNAc-H2 was clearly accessible in NOESY experiments at 12°C, where the NOE build-up was fully analyzed. On the contrary, the interaction of the Gal(IV)-H1 with GalNAc-H4 was hidden in the region around 4.17 ppm, where the Gal(II)-H1/Gal(II)-H3 also falls. Fig. 3 shows that on raising the temperature to 60°C, because the two anomeric protons separate by 0.03 ppm, it was possible to selectively excite the Gal(IV)-H1 resonance and to perform a ROESY experiment proving its interaction with the GalNAc-H4. At high temperatures it is possible to obtain informative

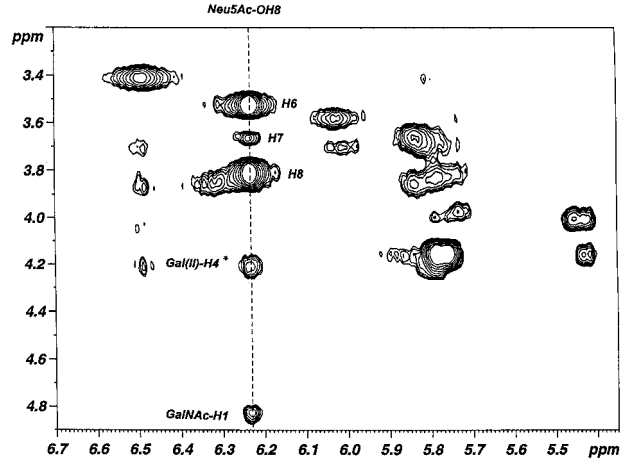

FIGURE 2 Portion of a 500-MHz 2D-NOESY spectrum of G_{M1}-acetyl micelle in $H_2O/5\%D_2O$ at pH 7.4 and at 12°C. The WATERGATE sequence is used for water suppression. Some cross-peaks concerning Neu5Ac-OH8 are shown, particularly the important interesidual interaction with GalNAc-H1. *The Neu5Ac-OH8/Gal(II)-H4 contact is not direct, but originates from a three-spin effect, probably involving GalNAc-H1, as inferred from the molecular model.

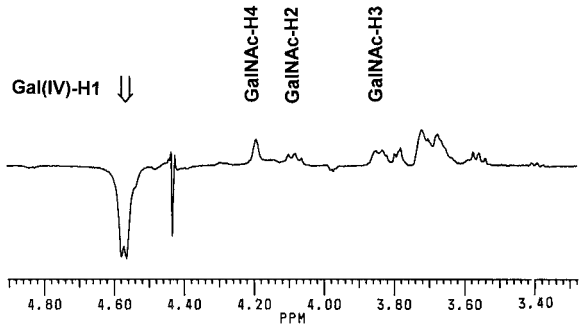

FIGURE 3 Selective 1D ROESY spectrum of G_{M1}-acetyl in D_2O for the Gal(IV)-H1 signal, acquired at 60°C with 120 ms mixing time. The ROEs with GalNAc-H4, -H3, and -H2 are highlighted. GalNAc-H4 and -H2 contacts cannot originate from a three-spin (RR) effect from -H3 because the peak sign is conserved; they are direct ROE. Strong Hartmann-Hahn effects are avoided by applying the spin-lock pulse 1.2 kHz off-resonance.

ROESY, also on the natural G_{M1} micelle for which the presence of all of the Gal(IV)-H1/GalNAc-H3, Gal(IV)-H1/GalNAc-H2, and Gal(IV)-1/GalNAc-H4 contacts was confirmed.

A further investigation of possible contacts between two monomers was carried out on natural G_{M1}/G_{M1}(Neu5Gc) mixed micelle in D_2O. The spectral parameters of the sialic acid ring protons for G_{M1} (Neu5Ac containing G_{M1}) and G_{M1}(Neu5Gc) are different, the protons concerned being those at positions 3, 4, 5, and 6 and those of the acyl group (Brocca et al., 1996). Those differences made it possible to check for any NOE originating from interresidual interactions between two sialic acid residues of distinct monomers. Note that the sialic acid belongs to the branched region of the oligosaccharide chain, possibly a favorable location for intermonomer interactions. No Neu5Ac/Neu5Gc NOE contact was detected.

It has been shown recently that the micelles of ganglioside present a bistable behavior between two stable states in which micelles show different chemical-physical properties, i.e., different size and aggregation number, triggered by temperature. It has also been suggested that this irreversible transition could be related to a monomer conformational modification (Cantù et al., 1996; see also Maggio et al., 1985). For this reason G_{M1}-acetyl micelles were prepared at both low and high temperatures before being analyzed at 12°C (see Materials and Methods). Overlapping results were obtained for the two preparations, suggesting that preparation temperature does not induce a detectable change in the oligosaccharide conformation.

Molecular modeling

The system under study presents many physical factors that are hardly accounted for during a simulation. Actually, the model accesible by MM calculation consists of the oligosaccharide portion of the ganglioside immersed in vacuum or in model water. However, the characteristic motions of a monomer in the micellar aggregate should be different from

those of a free pentasaccharide. In particular, the effects of motion restriction imposed by the surface matrix, of possible charge interactions between head groups, of possible protrusion motions of the ganglioside from the averaged hydrophobic-hydrophilic interface surface, and as mentioned, of NMR-undetectable interactions between monomers could influence the single pentasaccharide spatial arrangement and dynamics. The presented NOE measurements show that such factors seem to have no substantial effect on the NOE measured distances. Restrained MM calculation on the free oligosaccharide moiety of G_{M1} was intended to compare the NOE-derived structure of the pentasaccharide of G_{M1}-acetyl in a micellar carbohydrate-enriched surface, with structures already defined by different methods for monomeric ganglioside systems.

Fig. 4 shows a G_{M1}-acetyl oligosaccharide structure derived from restrained MM minimum energy calculations. NOE-derived distances were used as spatial constraints. The Gal-GalNAc glycosidic bond was found to sample different conformations for a number of gangliosides (G_{M1}, Acquotti et al., 1990; GalNAc-G_{D1a}, G_{D1a} Acquotti et al., 1994; G_{D1a}, Poppe et al., 1994; G_{M1}(Neu5Gc), Brocca et al., 1996). The presented NOE measurements for G_{M1}-acetyl confirm that the Gal-H1/GalNAc-H2 and Gal-H1/GalNAc-H4 are simultaneously sampled; thus motionally averaged distances are expected for those interactions as well as for that of Gal-H1/GalNAc-NH. In performing the MM calculation to obtain structure (1) in Fig. 4, we manually turned the external galactose residue to satisfy the detected Gal(IV)-H1/GalNAc-H2 NOE, and the Gal(IV)-H1/GalNAc-NH-averaged contact was introduced as a NOE restraint. The minimization procedure indicates a probable glycosidic bond torsion for the Gal-GalNAc- bond, satisfying the spatial arrangement where the Gal anomeric proton is directed toward the GalNAc-H2 position and the Gal(IV)-H1/GalNAc-NH distance is short. By imposing the NOE restraint that Gal(IV)-H1/GalNAc-H4 is less than 4 Å, another minimum is found with a long Gal(IV)-H1/GalNAc-NH distance (Fig. 4, *structure 2*).

For the core -GalNAc-(Neu5Ac-)Gal- portion, the derived glycosidic dihedral angles Φ and Ψ for -GalNAc-Gal- and Neu5Ac-Gal- were 36°, 8° and −162°, −29°, respectively. These values are in good agreement with those of the corresponding linkages in G_{M1} and G_{M1}(Neu5Gc) in DMSO (Scarsdale et al., 1990; Acquotti et al., 1990; Brocca et al., 1996), G_{M1}/DPC, and G_{M1}(Neu5Gc)/DPC micelles in water (Acquotti et al., 1990; Brocca et al., 1996), and G_{D1a}, GalNAc-G_{D1a} in DMSO (Acquotti et al., 1994). The slightly modified spatial arrangement of the disaccharide -GalNAc-Gal-, which in the other experimental ganglioside systems (gangliosides in DMSO or ganglioside/DPC micelles in water) showed Φ and Ψ glycosidic torsional angles in the range 30°-32°, 17°-24°, has very little effect on the whole head-group conformation: a very good overlap of the tridimensional structure with the previous ones was observed.

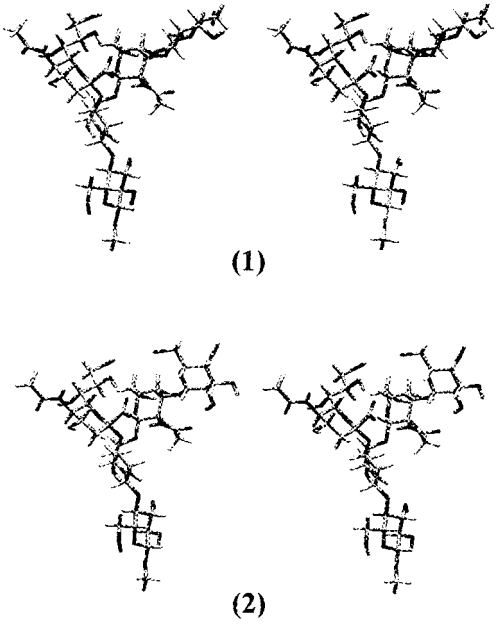

FIGURE 4 G_{M1} pentasaccharide structures derived from an MM minimum energy calculation, imposing NOE distance restraints measured for the G_{M1}-acetyl oligosaccharide moiety in micelles (Table 1, column a). The trisaccharide core -GalNAc-(Neu5Ac-)Gal- shows a single stable conformation corresponding to the minimum energy conformation already found for the same trisaccharide in a number of ganglioside systems (G_{M1}, Acquotti et al., 1990; G_{D1a} and GalNAc-G_{D1a}, Acquotti et al., 1994; G_{D1a}, Poppe et al., 1994; G_{M1}, Bernardi and Raimondi, 1995; G_{M1}(Neu5Gc), Brocca et al., 1996). The terminal Gal-GalNAc- glycosidic conformation shown in structure (1) satisfies the Gal-H1/GalNAc-H2 and Gal-H1/GalNAc-NH NOE averaged contacts, whereas structure (2) verifies the Gal-H1/GalNAc-H4 contact. It was previously found, using a distance mapping procedure, that this linkage samples several conformations (Acquotti et al., 1990). A two-state jump model, suggested for the same glycosidic bond in GalNAc-G_{D1a} and G_{D1a} (Acquotti et al., 1994), used partially unrestrained dynamics for this linkage. One of the two states displays a short Gal(IV)-H1/GalNAc-NH distance and a long Gal(VI)-H1/GalNAc-H4 distance, and the other has a long distance for Gal(IV)-H1/GalNAc-NH and a short distance for Gal(VI)-H1/GalNAc-H4. The solution structure of G_{M1}, obtained by applying the unrestrained Monte Carlo minimization procedure and using AMBER* in water solvent (Bernardi and Raimondi, 1995), showed the trisaccharide core to be essentially constrained to a single minimum energy conformation corresponding to that shown here, and found that the Gal-GalNAc- bond was more mobile, but did not sample two distinct conformations, contrary to what is suggested by NOE measurements.

Hydration

Based on the WEX II sequence (Mori et al., 1996), water interaction with the protons of the G_{M1} carbohydrate moiety was studied to confirm some aspects of the rigid conformation of the -GalNAc-(Neu5Ac-)Gal- trisaccharide core, with attention focused mainly on the study of the chemical exchange of the accessible hydroxyl protons.

By applying off-resonance ROESY (Desvaux et al., 1994, 1995), it was possible to distinguish between chemical exchange and dipolar relaxation, which give rise to signals of opposite sign. By setting the angle θ between the effective field and the external static field equal to 35.3°, the

dipolar cross-relaxation is made to vanish (Desvaux et al., 1994). Under such conditions, only chemical exchange is effective in determining magnetization transfer from water protons to ganglioside OH and NH. Thus it was possible to establish that water-ganglioside interaction is completely dominated by exchange in the low mixing time range, at the three considered temperatures of 12°C, 7°C, and 3°C at pH 7.4. The low temperatures, long correlation time, and aggregation state of the sample led to a water-ganglioside exchange in the time scale of 10 ms, an NMR-accessible time range. Absolute values for the exchange rates are expected to be extremely sensitive to sample parameters like exact pH, salt content, and the presence of minor contaminants (Brocca et al., 1993). Thus the most informative way to assess the results appears to be a comparison, for the same sample, of the exchange rates and temperature behavior of different hydroxyls. Changing the pH by 0.4 units in both directions causes an abrupt loss of resolution for the labile protons, making it infeasible to acquire the pH dependence of the same measurement.

Hydroxyl proton exchange was then followed in the evolution time range of 1–120 ms. The exchange rates were accurately calculated only for Gal(II)-OH2, Neu5Ac-OH4, and Neu5Ac-OH8, their resonances being quite isolated in the spectrum. Fig. 5 shows that on assuming a first-order mechanism for the exchange process, as described in Materials and Methods, the fits are very accurate. By comparing the exchange times of Gal(II)-OH2, Neu5Ac-OH4, and Neu5Ac-OH8, similar behavior can be seen for Gal(II)-OH2 and Neu5Ac-OH4, whereas the Neu5Ac-OH8 exchange time and its temperature dependence appear quite peculiar. In comparing the exchange times of Neu5Ac-OH4 and Gal(II)-OH2, the latter's constant higher exchange time can be attributed to a reduced solvent accessibility, according to the position depth of the group within the hydrophilic shell of the micelle. On the contrary, despite its relatively external position, Neu5Ac-OH8 exchanges less easily than Neu5Ac-OH4 and Gal(II)-OH2, and its exchange time shows a brusque increment at the lowest temperature studied, reaching 58 ms. This finding indicates that the activation energy for the Neu5Ac-OH8 proton exchange process is higher than for the Gal(II)-OH2 and Neu5Ac-OH4, suggesting that Neu5Ac-OH8 is involved in intramolecular interactions. This is in agreement with the observation that it belongs to the interaction region between the sialic acid lateral chain and GalNAc, which is probably less water accessible. Moreover, Neu5Ac-OH8 has been shown to have a hydrogen bond with the carboxylic group (note the strong Neu5Ac-OH8/Neu5Ac-H6 NOE contact; Table 1), thus making the sialic acid structure quite rigid (Acquotti et al., 1990, 1994).

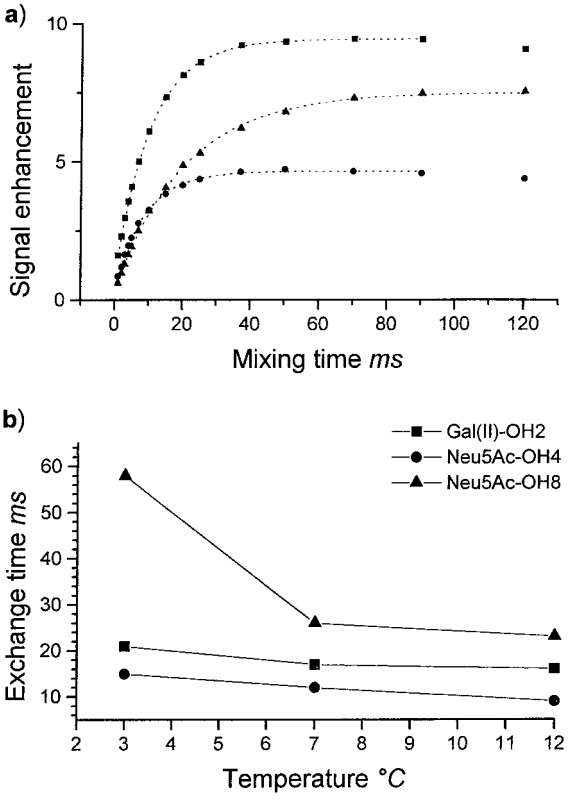

FIGURE 5 (*a*) Build-up curves representing the magnetization transfer via chemical exchange from water protons to OH. Dashed lines connecting points are obtained by fitting the data to an exponential function (see Materials and Methods) and interrupt at the last point used for the fit. (*b*) The chemical exchange half-life obtained by the fitting in *a* is reported as a function of temperature.

DISCUSSION

In the present study attention has been focused on how GSL lateral distribution could affect the conformational properties of the ganglioside carbohydrate moiety. The model used confines the ganglioside hydrophilic head group to a crowded ganglioside-rich physical environment. High-resolution ^{1}H-NMR on the surface of the G_{M1}-acetyl micelle and natural G_{M1}/G_{M1}(Neu5Gc) mixed micelle lets us look for direct carbohydrate-carbohydrate side-by-side interactions, utilizing information on labile and nonlabile protons in aqueous solution.

The model studied is consistent with the existence of a preferred spatial arrangement of the ganglioside carbohydrate moiety on the surface of model membrane, and is characterized by the stability of the core trisaccharide found in the free G_{M1} monomer. Despite the overall averaged orientations of the chain relative to the membrane surface being quite stable for different ganglioside carbohydrate compositions, the role of sialic acid seems to be important on a more local scale: it determines the conformation and the heterogeneous degree of flexibility of the chain through interaction with GalNAc. The determining role of this interaction is known. A comparison of the GalNAc-G_{D1a} and G_{D1a} conformations in organic solvent (Acquotti et al., 1994) showed that the presence of terminal GalNAc at the level of the second sialic acid branch confers motion restriction on the terminal GalNAc-(Neu5Ac-)Gal- of Gal-

NAc-G_{D1a} with respect to the terminal Neu5Ac-Gal- of G_{D1a}. On passing from G_{D1a} to GalNAc-G_{D1a}, the head-group volume is decreased, contradicting the fact that the number of sugars increases (Sonnino et al., 1994).

The role of sialic acid in determining ganglioside conformation has been studied by ^{2}H-NMR on G_{M1}, asialo-G_{M1}, and G_{D1a} in a multibilayer system of POPC (Jones et al., 1996). The ^{2}H spectrum of the terminal Neu5Ac of G_{D1a}, deuterium-labeled on the acetamide group, clearly showed that the degree of motional average for this residue is high, in contrast with the quite stable conformation of the rest of the chain. This result is in agreement with the observation that the lack of the Neu5Ac-GalNAc interaction determines an increased mobility for the external Neu5Ac-Gal(IV) bond. The authors found that for the terminal galactose marked on both protons at C6, there is no indication of any local higher degree of motional freedom of the Gal-GalNAc- glycosidic bond. Moreover, it was shown that the elimination of the sialic acid residue in asialo-G_{M1} does not affect Gal(IV)-GalNAc and GalNAc-Gal(II) glycosidic bond dynamics. Poppe and co-workers, applying ^{13}C-$T_{1\rho}$ relaxation, ^{13}C-^{1}H NOE, and ^{1}H-^{1}H ROE measurements, found a higher mobility of both terminal Neu5Ac and terminal Gal than in all of the other residues for the G_{D1a} saccharide moiety in the G_{D1a}/DPC micellar system (Poppe et al., 1994). The NOE measurements presented in this paper are in agreement with these results for what concerns the terminal Gal residue. There is, however, a partial contrast with ^{2}H-NMR results for G_{D1a} and G_{M1}, which, for the terminal Gal, indicate a higher restriction of mobility than for the terminal Neu5Ac and a restriction similar to that of the rest of the chain. The contrast might stem from the different degree of motional freedom of some residues in a multilayer pelletized system, with respect to a system of micelles in solution.

The fact that no NOE was observed between different monomers, together with the observation that the carbohydrate chain conformation was preserved when the composition of the head-group environment was ganglioside enriched, seems to exclude, for the micellar model, the presence of significant intermolecular side-by-side interactions capable of altering the "conformational information" carried by the single saccharidic chain of the ganglioside. However, it should be noted that the actual environment of each monomer at the membrane hydrophilic layer includes solvent. Water could mediate weak long-range interactions between monomers, and not be detected by the NMR experiments. These possibilities must be considered in the light of what appears to be the real mechanisms of biological recognition involving carbohydrate interactions. The "lock-and-key" mechanism, proposed in the case of proteins, seems not to be a suitable paradigm for carbohydrate-carbohydrate interactions. GSLs, and carbohydrates in general, are mostly involved in surface recognition events where large numbers of identical molecules cooperate in interacting with other surfaces (Bovin, 1996; Hakomori, 1994). A prominent work on Le^x (Eggens et al., 1989) described the properties of the very weak but cooperative carbohydrate-carbohydrate interactions. Pair interaction between molecules seems to be too low to be detected by the local probe provided by NMR. This was also the case for the Le^x-Ca^{2+}-Le^x system studied by ^{1}H-NMR in aqueous solution (Wormald et al., 1991), where no direct evidence of Le^x-Le^x interaction was detected.

CONCLUSIONS

^{1}H-NMR applied to large micelles of a single ganglioside component led to a detailed study of a surface system with high carbohydrate density, which could mimic the presence of phase-separated surface domains on cell membranes. The conformational properties of G_{M1} pentasaccharide, previously obtained in organic solvent and in ganglioside/detergent aqueous solution for a single monomer, are maintained. The presence of the charged *N*-acetylneuraminic acid seems to bear "conformational and dynamic information," yielding the core -β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal- rigidity by the interaction with GalNAc residue. The attempt to measure intermolecular side-by-side interaction among ganglioside head groups failed, confirming that intramonomer interactions are definitely dominant in determining single monomer properties.

Furthermore, the results obtained give little evidence that the hydrophobic portion composition could significantly influence head-group conformation.

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