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Rapid Report

Oligosaccharide order in a membrane-incorporated complex glycosphingolipid

Harold Jarrell [2], Dev Singh [1] and Chris W.M. Grant [1]

[1] *Department of Biochemistry, University of Western Ontario, London (Canada) and* [2] *Division for Biological Sciences, National Research Council of Canada, Ottawa (Canada)*

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Galactosylceramide (GalCer) and the ganglioside, GM_1, were ^2H-labelled at C-6 (the hydroxymethyl moiety) of their single terminal galactosyl residues. Each deuterated glycosphingolipid was incorporated at a biologically relevant low concentration into multibilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). ^2H-NMR spectra of aqueous dispersions of GalCer-POPC in the liquid crystal phase were characteristic of restricted headgroup motion (ordering) with effective axial symmetry. The degree of headgroup ordering was analogous to that of GalCer in pure aqueous multibilayers (Skarjune, R. and Oldfield, E. (1979) Biochim. Biophys. Acta 556, 208–218). In the case of GM_1, ^2H-labelled in the terminal galactose residue of the pentasaccharide headgroup, the ^2H-NMR spectra were remarkably like those of the simple glycolipid, GalCer. This suggests substantial restriction of motion about the glycosidic and sugar-ceramide bonds of the complex GM_1 headgroup, and that both lipids have comparable degrees of orientational averaging (fluctuation) about the bilayer normal. The result is the first direct demonstration that headgroup orientational order can exist for a complex glycolipid incorporated into 'fluid' bilayer membranes. Such behaviour argues for the possibility of modulation of membrane receptor properties through surface effects on average headgroup orientation and conformation.

As a result of their role as specific recognition sites, considerable attention has been focused on glycosphingolipid oligosaccharide headgroup conformation (and internal motion). Recent studies employing two-dimensional high resolution nuclear magnetic resonance (NMR) in combination with conformational energy calculations have provided valuable information regarding headgroup arrangement for intact gangliosides [1,2] and globoside [3,4] in isotropic solution and in detergent micelles (see also Refs. 5 and 6 and references therein). It seems a reasonable expectation that, in the complex environment generated by a membrane, oligosaccharide conformation will be sensitive not only to intramolecular interactions, but also to intermolecular effects resulting from close proximity to the membrane surface. Indeed considerable evidence exists that the nature and composition of the membrane significantly modulate glycosphingolipid receptor function [7–12]. Solid state ^2H-NMR has been used to begin to elucidate the properties of glycolipid headgroups in bilayer environments, with the expectation that insight will be provided into the molecular bases of such effects [13–15]. Since the initial studies on galactosylceramide (GalCer) [16] and glucosylceramide (GlcCer) [17], ^2H-NMR has been employed to investigate the bilayer orientational and motional characteristics of a number of glycerolipids containing neutral mono- [18,19] and disaccharide [20,21] headgroups. In this article we describe the first attempt to extend wide line ^2H-NMR studies to a complex glycosphingolipid, GM_1. In order to mimic surface constraints imposed by a natural membrane surface, the glycolipids were as-

Abbreviations: GSL, glycosphingolipid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; GalCer, Galβ1 → 1ceramide; GM_1, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glcβ1 → 1ceramide.

Correspondence: C.W.M. Grant, Department of Biochemistry, University of Western Ontario, London, Canada, N6A 5C1; H. Jarrell, Division for Biological Sciences, National Research Council of Canada, Ottawa, Canada, K1A 0R6.

assembled at low concentration into bilayers of the mono-unsaturated phospholipid, 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC).

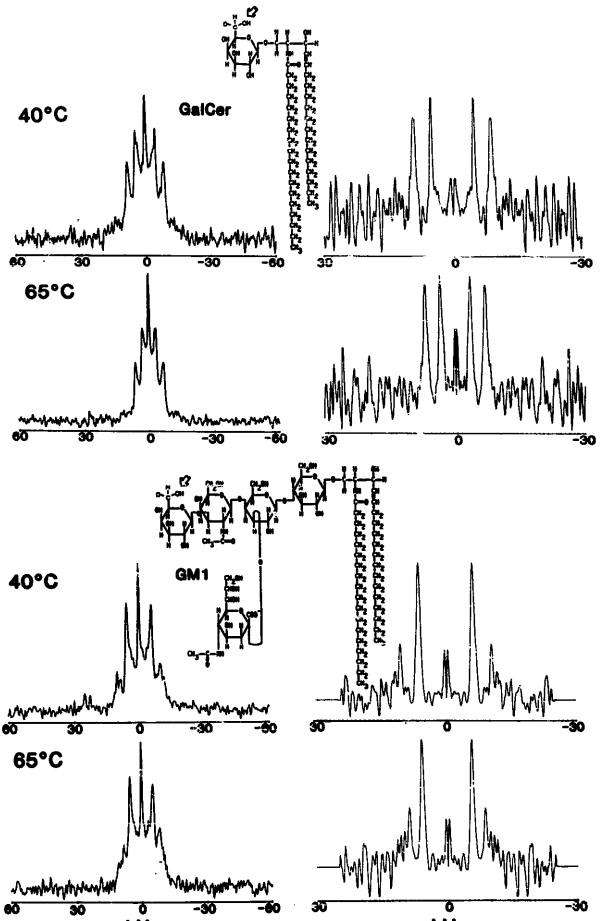
GM_1 from beef brain was a kind gift of Fidia Research Laboratories, Abano Terme, Italy. Beef brain galactosylceramide was obtained from Avanti Polar Lipids, Birmingham, AL. Glycolipids were ^2H-labelled at C-6 of the terminal galactose moiety using enzymatic oxidation following the procedure of Radin [21]. Lipid bilayer membranes were prepared by evaporation of chloroform/methanol (3:1, v/v) solution containing the lipid components in the desired ratio at 50°C (10 mol% glycolipid), followed by further drying in vacuo for 3 h at 22°C. Resultant lipid films were hydrated with deuterium-depleted water (MSD Isotopes, Montreal, Que., Canada) buffered with 10 mM phosphate at pH 7.4, lyophilized three times (rehydrating with 400 μl ^2H-depleted water), and subjected to eight freeze-thaw cycles following the final rehydration to 700 μl. Samples contained 14.5 mg (20.0 μmol) GalCer or 31.5 mg (20.5 μmol) GM_1. They were incubated at 65°C for 45 min prior to spectrum acquisition, and were subsequently cooled for the low temperature spectra. ^2H spectra were acquired at 30.7 MHz as described previously [18], and were not folded about the Larmor frequency.

Structures of deuterated GalCer and GM_1 are shown with their associated ^2H-NMR spectra in Fig. 1. It should be noted that the fatty acid composition of GalCer was uniform (100% 18:0, stearic acid), and that of GM_1 was the natural beef brain mixture (> 80% 18:0 [22]). Since the gel to liquid crystalline phase transition temperature of POPC is −3°C [23], the lipid matrix was in the disordered liquid crystalline phase at the temperatures used in this study. Inspection of Fig. 1 reveals that at 40°C and 65°C each glycolipid, ^2H-labelled at C-6 of the galactosyl residue, gives rise to a superposition of two quadrupolar powder ^2H spectra. In the case of GalCer, the two subspectra are of equal intensity – which may be seen more readily in the oriented-sample ('depaked' [24]) spectra, Fig. 1 (upper right). These two quadrupolar splittings have been shown previously to reflect the inequivalence of the two deuterons (pro-R and pro-S) on the hydroxymethyl group of the galactosyl residue. The residual quadrupolar splittings have values of 17.7 and 9.7 kHz at 40°C, and 13.8 and 7.0 kHz at 65°C. The values are comparable to those reported for pure GalCer bilayers at 90°C (13.5 and 9.5 kHz) [16]. The similarity in $\Delta\nu_Q$ values for GalCer and GalCer-POPC suggests that the average headgroup surface orientation and amplitude of fluctuations about this direction are similar for the two systems.

Examination of the spectra of GM_1 in POPC bilayers (Fig. 1, lower) reveals a close correspondence with those for the GalCer-POPC system. Interestingly, the relative spectral intensities of the two overlapping powder spectra are not equal as was the case for the GalCer system. The most likely explanation of this is that, during reduction of the C_6 aldehyde produced by galactose oxidase, there is more stereoselectivity in the incorporation of deuterium than there was for the simple GalCer. As a result, for GM_1, the intensity of the powder spectrum associated with the pro-R deuteron is not the same as that of the pro-S deuteron. The values of the residual quadrupolar splittings for GM_1 are 21.2 and 12.4 kHz at 40°C and 17.7 and 11.6 kHz at 65°C. That the $\Delta\nu_Q$ values are larger than those of GalCer at the same temperature suggests that the orientational order of the galactose residue is not substantially less than that of galactose in the mono-glycosyl lipid. Previous studies have concluded that for the galactosyl residue in simple glycolipids there is rapid interconversion amongst rotamers (*gauche*(+), *gauche*(−) and *trans*) about the C-5–C-6 bond [17,20]. It is a likely possibility that the relative populations of the three rotamers differ between GM_1 and GalCer. As a result, even if the orientational fluctuations (order) of the two headgroups were identical, the $\Delta\nu_Q$ values for the pro-R and pro-S deuterons at C-6 would be expected to differ for the two systems [20]. It should also be noted that, at this time, a definitive assignment of each of the $\Delta\nu_Q$ values to a specific (pro-R or pro-S) deuteron is not possible. Thus detailed analysis of our results requires more information.

The present findings do permit an important definite conclusion. The degree of random reorientation (disorder) of the terminal galactosyl residue in the GM_1 molecules giving rise to the quadrupole splittings described here is of the same order as that present in GalCer, in which the headgroup is close to the membrane surface. Yet one would expect that for GM_1 anchored in a bilayer membrane the cumulative effects of motion about the various glycosidic bonds would

Fig. 1. ^2H-NMR spectra at 40°C and 65°C for headgroup-deuterated GalCer and GM_1 in fluid phospholipid bilayers. Deuteration was at C-6 of the galactose residue (arrows) – see inserted structures. In each case the glycolipid was dispersed at 10 mol% in unsonicated multilamellar vesicles of 1-palmitoyl-2-oleoylphosphatidylcholine. 90° oriented sample ('depaked') spectra were calculated from the powder spectra as described previously [24], and are displayed to the right of their corresponding powder spectra. Spectra were acquired at 30.7 MHz on a 'home-built' spectrometer operated by a Nicolet 1280 computer. The sample was enclosed in a glass dewar and the temperature was electronically regulated to within ±0.5 C°. Spectra were recorded using the quadrupolar echo pulse sequence [27] with full phase cycling [28] and quadrature detection. The $\pi/2$ pulse length varied from 5 to 6 μs (10 mm solenoid coil), the pulse spacing was 60 μs, and recycle time was 30 ms. The number of spectral accumulations for each spectrum ranged from 200 000 (GM_1) to 650 000 (GalCer).


40°C
GalCer
60
30
0
-30
-60
65°C
40°C
GM1
65°C
kHz
kHz

lead to greater orientational averaging (smaller $\Delta\nu_Q$ values) of the terminal residue than is present for the headgroup of GalCer. That this is not the case suggests that the membrane surface may impose constraints on overall headgroup motion, as well as possibly restricting motion about glycosidic bonds in complex glycolipids. High resolution NMR studies of GM_1 [1,2] and its oligosaccharide headgroup [29] in isotropic solution have indicated that there is a relatively well-defined structure to the carbohydrate portion. More definitive resolution of these issues for GM_1 in membranes will require additional labelling in other parts of the headgroup: this will be important in determining the time-averaged C-^{2}H bond orientation relative to the membrane surface. It will be interesting to apply the same approach to different complex GSLs in order to determine how general the preservation of conformational order at the membrane surface is, and what factors regulate it. Such studies are currently under way.

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