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Polymorphic phases of galactocerebrosides: spectroscopic evidence of lamellar crystalline structures

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Abstract

Fourier transform infrared spectroscopy was applied to study the structural and thermal properties of bovine brain galactocerebroside (GalCer) containing amide linked non-hydroxylated or α -hydroxy fatty acids (NFA- and HFA-GalCer, respectively). Over the temperature range 0–90°C, both GalCer displayed complex thermal transitions, characteristic of polymorphic phase behavior. Upon heating, aqueous dispersions of NFA- and HFA-GalCer exhibited high order-disorder transition temperatures near 80 and 72°C, respectively. En route to the chain melting transition, the patterns of the amide I band of NFA-GalCer were indicative of two different lamellar crystalline phases, whereas those of HFA-GalCer were suggestive of lamellar gel and crystalline bilayers. Cooling from the liquid-crystalline phase resulted in the formation of another crystalline phase of NFA-GalCer and a gel phase of HFA-GalCer, with a phase transition near 62 and 66°C, respectively. Prolonged incubation of GalCer bilayers at 38°C revealed conversions among lamellar crystalline phases (NFA-GalCer) or between lamellar gel and crystalline bilayer structures (HFA-GalCer). Spectral changes indicated that the temperature and/or time induced formation of the lamellar crystalline structures of NFA- and HFA-GalCer was accompanied by partial dehydration and by rearrangements of the hydrogen bonding network and bilayer packing mode of GalCer. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Galactocerebroside; Thermotropic phase behavior; Lamellar crystalline phase; Fourier transform infrared spectroscopy

1. Introduction

Galactocerebroside (GalCer), together with its sulfate ester (or sulfatide), constitutes the major proportion of glycolipids from the myelin sheath [1], which may act as a multilayered insulator around axons to facilitate the transmission of nerve impulses [2]. This glycosphingolipid appears to be located exclusively in the outer leaflet of the membrane, and comprises

Abbreviations: DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; GalCer, galactocerebroside; HFA-GalCer, GalCer containing amide linked α -hydroxy fatty acids; L_cx, lamellar crystalline phase; NFA-GalCer, GalCer containing amide linked non-hydroxylated fatty acids; SGG, sulfogalactosylglycerolipid; T_m, chain melting transition temperature

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approx. 20 wt% of the total lipids of myelin [1]. GalCer contains both non-hydroxylated and α -hydroxy acyl chains, with the latter being 40–60% in distribution in vertebrate myelin [3]. The ceramide backbone of GalCer has been shown to consist mainly of 24:0, 24:1 and 18:0 fatty acids, linked to a sphingosine (18:1) moiety [4–6]. It has been suggested that GalCer and sulfatide play a role in the adhesion between the extracellular surfaces of myelin and in its stability and dynamic functions via cation [7,8] and non-cation [9] dependent carbohydrate–carbohydrate interactions. In addition, GalCer was shown to be an early differentiation marker of oligodendrocytes [10] and treatment of these cells with anti-GalCer antibodies inhibits their maturation [11], as well as in vitro [12] and in vivo [13] myelination. This indicates that GalCer is a signal transduction molecule essential for oligodendrocyte development and maturation [14]. Moreover, knockout mice deficient in the gene encoding UDP-galactose:ceramide galactosyltransferase, the enzyme catalyzing the final step in GalCer biosynthetic pathway [15], manifest unstable and abnormally functional myelin sheath [16–21], with an impaired saltatory conduction [18]. This result emphasizes the importance of GalCer for the functional integrity of myelin.

The complex phase behavior of ceramides [22,23], cerebroside [24–31], sulfatides [5,32,33] and other glycolipids [34] has been extensively studied by a variety of biophysical methods. The structural properties of GalCer were first explored by X-ray crystallography, discovering that the galactose head group of GalCer is parallel to the bilayer plane. This conformation involves extensive lateral interactions via hydrogen bonding between the amide groups and the hydroxy groups of the galactose head groups, thus imparting greater bilayer stability [35]. Pascher and colleagues [35,36] have suggested that the α -hydroxy fatty acids in GalCer increase hydrogen bonding and give rise to a locked/shovel conformation of the HFA-GalCer molecule. Differential scanning calorimetry (DSC) of GalCer has revealed conversions between metastable and stable bilayer structures, prior to their high temperature chain melting transition [25,27,31,37]. These bilayer structures were shown to be lamellar crystalline in nature by X-ray diffraction [25,27,31,38]. In addition, Saxena et al. [31] have confirmed that the polymorphic phase behavior and

properties of cerebroside are not altered by the precise isomeric structure of the linked sugar and neither by variations in the fatty acid chain length of their ceramide backbone. Sen et al. [39,40] and Mannock et al. [41] have shown that synthetic galactoglycerolipids and glucoglycerolipids exhibit structural and thermodynamic properties similar to cerebroside.

Fourier transform infrared spectroscopy (FTIR) spectroscopy is a powerful technique used for characterizing the organization and conformational order in lipid bilayers [23]. The patterns of the infrared absorption bands exhibited by GalCer, specifically the CH_2 and amide I $\text{C}=\text{O}$ stretching regions, reveal valuable information about the intermolecular interactions that occur at the hydrophobic core of the bilayer and head group region, respectively [42]. Although X-ray diffraction has revealed that GalCer adopts crystalline structures [25,27,31,38] en route to the liquid-crystalline phase, a diffraction pattern of the bilayer was not always obtainable at various temperatures. Therefore, the aim of the current FTIR study was to assess the phase behavior and to monitor the low temperature metastable polymorphism of both NFA- and HFA-GalCer, as a function of temperature and/or time. The contribution of chain packing and hydrogen bonding network to the structural forms adopted by GalCer was also examined. The significance and implications of these findings are discussed.

2. Materials and methods

Two molecular species of bovine brain GalCer, type I (with 98% α -hydroxy fatty acids) (HFA-GalCer), type II (with 98% non-hydroxylated fatty acids) (NFA-GalCer), and tris[hydroxymethyl]aminomethane (Tris) hydrochloride were purchased from Sigma (St. Louis, MO, USA). $^2\text{H}_2\text{O}$ (99.9 atom%) was purchased from Merck Sharp and Dohme/Isotopes (Pointe-Claire, PQ, Canada). The preparation of GalCer bilayers proceeded as follows. Multilamellar bilayers of NFA- and HFA-GalCer were generated by resuspending the dried lipids (2 mg) in 38 μl of $^2\text{H}_2\text{O}$ buffered with 50 mM Tris-HCl (p^2H 7.05). The lipid dispersions (5 wt%) were then subjected to four freeze-thaw cycles to obtain a homogeneous, uniform distribution of buffer solutes

across the bilayers [43,44]. During each thawing cycle, the lipid dispersion was brought to 90°C and vortex-mixed prior to subsequent freezing, which was achieved by immersing the lipid suspension in liquid nitrogen (−215°C). After the final thawing, the dispersion was equilibrated to room temperature (23°C).

FTIR measurements were conducted on a Digilab FTS-40A spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector. Aqueous dispersions (10 µl) of GalCer were placed between two CaF₂ windows separated by a 6 µm spacer in a thermostated cell mount. Temperatures were varied at 1°C/min with a water circulation bath. Heating and cooling mode FTIR spectra were recorded between 0 and 90°C, in increments of 3°C. The spectra were derived from 128 scans collected at a spectral resolution of 2 cm^{−1}, signal-averaged, Fourier transformed and ratioed against a background interferogram. Each spectrum was recorded in approx. 5 min, after a waiting period of 7 min. For the time varied studies, NFA- and HFA-GalCer bilayers were either heated or cooled from liquid-crystalline temperatures (≥ 80°C) to 38°C, and maintained at that temperature for 96 h. For NFA-GalCer, additional incubation experiments were conducted at 65°C following cooling from liquid-crystalline temperatures (89°C), and at 38°C for prolonged time periods after cooling from the latter temperature. Dehydrated GalCer bilayers were obtained by leaving open the transmission cell in the sample compartment of the infrared spectrophotometer for 96 h after the addition of the appropriate amount of 5 wt% dispersion of GalCer in ²H₂O (p²H 7.05). The instrument was under continuous dry air purge to eliminate atmospheric water vapor.

The hydrocarbon chain packing and conformation of GalCer were monitored by following changes of the CH₂ symmetric stretching band (2846–2854 cm^{−1}); below the *T_m*, most of the hydrocarbon chains are in the all-*trans* configuration, whereas at the *T_m*, the progressive *trans-gauche* isomerization results in lateral area expansion of the bilayer and in a shift to higher frequencies [45]. The amide I C=O stretching vibration (1580–1680 cm^{−1}) was analyzed, as a function of temperature, in order to assess the degree of hydrogen bonding and the polymorphic bilayer structures of GalCer. Data process-

ing was performed using GplotC and RAMOP programs, developed at the National Research Council, Ottawa, ON, Canada [46]. Band frequencies were determined from third order derivative spectra with a breakpoint of 0.3 and 0.95 for the CH₂ and amide I C=O stretching bands, respectively [47].

3. Results

3.1. Packing of NFA-GalCer bilayer

Plots of the temperature dependence of the CH₂ symmetric stretching band are shown in Fig. 1, displaying the phase behavior of NFA-GalCer upon heating (●) and subsequent cooling (○). NFA-GalCer bilayers underwent a main phase transition at approx. 80°C, in good agreement with previously published DSC results [25–28,30,31]. The abrupt increase in the frequency of the CH₂ band at 80°C (approx. 4 cm^{−1}) represented the onset of the *trans-gauche* isomerization and indicated an increase in the conformational disorder of the hydrocarbon chains of NFA-GalCer, typical of chain melting in the liquid-crystalline phase (Fig. 1, ●). The temperature profile also exhibited another, small increase in the CH₂ symmetric stretching frequency at approx. 50°C. Curatolo [26] and Saxena et al. [31] have demonstrated that GalCer interconverts between metastable and stable bilayer structures, below their high temperature phase transition. Moreover, a structural analogue of the sulfate ester of GalCer

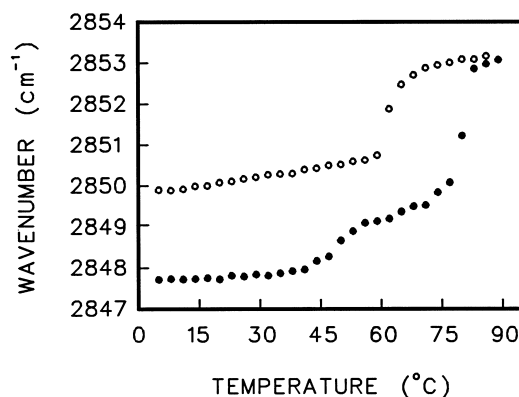


Fig. 1. Thermotropic response of the CH₂ symmetric stretching frequency of hydrated NFA-GalCer upon heating (●) and cooling (○). The sample was dispersed in ²H₂O buffered with 50 mM Tris-HCl (p²H 7.05).

(i.e., sulfogalactosylglycerolipid, SGG) exhibits a polymorphic behavior, including two crystalline structures, before its main phase transition [48]. The transition of NFA-GalCer observed at 80°C presumably represented the lamellar crystalline to liquid-crystalline phase transition, as discussed below. Upon cooling from 90°C, the hydrated NFA-GalCer bilayers exhibited significant hysteresis, clearly indicating a slow equilibration process, which resulted in a sharp transition centered at 62°C (Fig. 1, ○). This hysteresis behavior has been observed for ceramides [22] and GalCer [25–28, 30, 31]. The thermotropic event observed at 50°C in the heating run of NFA-GalCer was not observed during the cooling process and the frequency of the CH₂ symmetric stretching band decreased gradually below the main transition. The higher frequency obtained for NFA-GalCer upon cooling indicated that its hydrocarbon chains were more disordered than at the beginning of the heating scan (Fig. 1).

3.2. The amide I C=O stretching band of NFA-GalCer

Fig. 2A and B show the amide I band of the original FTIR spectra of NFA-GalCer obtained upon heating and cooling, respectively. From 5 to 44°C, there appeared to be a major peak near 1629 cm⁻¹, with an apparent shoulder extending from 1580 to 1620 cm⁻¹. Between 47 and 71°C, the relatively broad shoulder became more convex, forming two discernable peaks at approx. 1613 and 1602 cm⁻¹ (Fig. 2A). At 74 and up to 80°C, the amide I band consisted mainly of two components near 1629 and 1613 cm⁻¹, with the latter peak shifting to 1614 cm⁻¹ at 80°C. These FTIR spectral patterns of a sharp peak(s) and a visible shoulder(s) in the amide I stretching region were indicative of the presence of a lamellar crystalline phase(s) prior to the high temperature phase transition, similar to previously reported FTIR results on diacyl phospholipids [49] and SGG [48], and in agreement with the crystal structure revealed by X-ray diffraction of GalCer [25,27,31] and structurally related glycolipids [40]. Close examination of the amide I band revealed that NFA-GalCer adopted two lamellar crystalline phases en route to the chain melted state: the first (termed L_c1) existing between 5 and 44°C, and the

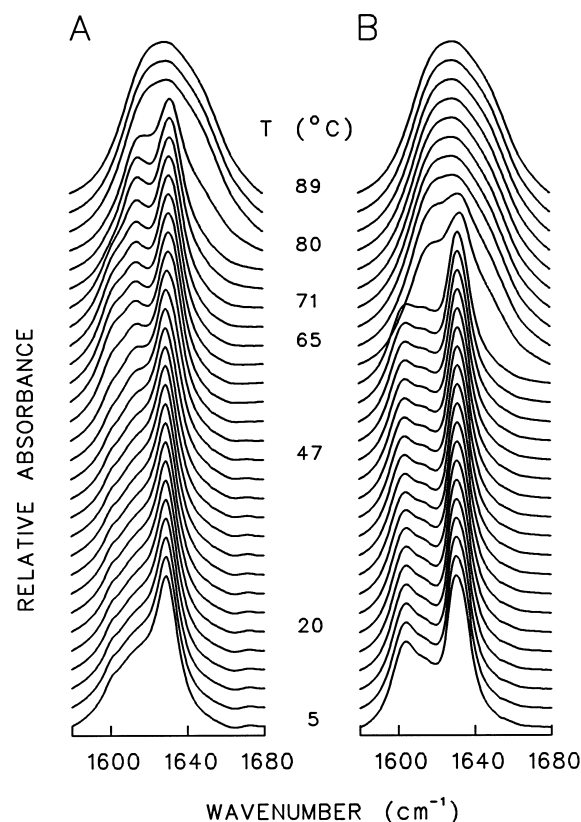


Fig. 2. Amide I region of the infrared spectra of hydrated NFA-GalCer, obtained at the temperatures indicated, upon heating (A) and following subsequent cooling (B). The sample was dispersed in ²H₂O buffered with 50 mM Tris-HCl (p²H 7.05).

second (termed L_c2) between 74 and 80°C (Fig. 2A). The slow transformation of L_c1 into L_c2 started at 47°C, with both crystalline phases coexisting between 47 and 71°C. This transformation was completed at 74°C, at which point the spectral features of a different lamellar crystalline phase (termed L_c2) were established. As the hydrocarbon chain conformational disorder increased, the patterns of the amide I band of NFA-GalCer changed significantly above 80°C (Fig. 2A): a substantially broader band was observed (Fig. 2A), indicative of a liquid-crystalline phase. The temperatures where significant changes of the amide I band of NFA-GalCer took place paralleled those at which chain packing rearrangements and bilayer organization occurred (Fig. 2 vs. Fig. 1).

When NFA-GalCer bilayers were cooled from the chain melted state (89°C) (Fig. 2B), the broad amide I band was maintained until 65°C, at which point a peak at approx. 1629 cm⁻¹ and an adjacent down-

stream shoulder at approx. 1620 cm^{-1} became apparent. This low frequency shoulder presumably represented hydrogen bonded species of NFA-GalCer [50–52]. At 65 and 62°C , the spectral patterns of the amide I band were analogous to those in the L_c2 phase. However, the components of the amide I band were not as narrow as L_c2 . Therefore, we concluded that the NFA-GalCer bilayers were presumably in an intermediate state corresponding to a biphasic mixture (liquid-crystalline+ L_c2). From 59 to 5°C , the shoulder became more resolvable from the 1629 cm^{-1} component band and comprised two overlapping peaks near 1614 and 1603 cm^{-1} . This suggested a strengthened hydrogen bonding network of NFA-GalCer (Fig. 2B), and the observed crystalline phase was termed L_c3 (due to its difference from L_c1 and L_c2). Although L_c3 was somewhat similar to the L_c1+L_c2 mixture between 47 and 71°C , the lamellar crystalline features of L_c3 were more evident (i.e., the overlapping peaks were more separated from each other) (Fig. 2A,B).

The thermal treatment of NFA-GalCer significantly influenced its polymorphic phase behavior. When NFA-GalCer was heated to a temperature below its chain melting transition ($\leq 80^\circ\text{C}$), conversions between metastable (L_c1) and stable (L_c2) bilayer structures were observed, as revealed by the patterns of the amide I band. Upon cooling from 80°C , L_c2 was stable even at 5°C , a temperature favoring the formation of L_c1 (results not shown). On the other hand, heating NFA-GalCer above 80°C and subsequent cooling resulted in the formation of L_c3 following a transition from an intermediate biphasic mixture (liquid-crystalline+ L_c2) (Fig. 2B). These results indicated that, below the chain melted state, the most stable lamellar crystalline arrangement adopted by NFA-GalCer is L_c2 . However, once in the liquid-crystalline phase, conformational and orientational changes of NFA-GalCer may have occurred, leading to the generation of a stable L_c3 polymorph upon cooling.

The variations in the relative intensity of the 1614 and 1603 cm^{-1} amide I components of NFA-GalCer (in the L_c3 phase) reflected the presence of different populations of hydrogen bonded amide groups. Lewis et al. [34] studied glycolipid analogues by FTIR spectroscopy and concluded that the shift of the ester $\text{C}=\text{O}$ band to lower frequencies is possibly

due to a bond to a hydroxy group on the sugar residue. The authors also believed that, as a prerequisite of strong hydrogen bonds, partial dehydration of the polar/non-polar interfacial region of the bilayer should occur. Based on this notion, we presumed that L_c3 possessed a strong hydrogen bonding network, which may be attributed to a partial dehydration and/or localization of the amide groups of NFA-GalCer in less polar environments. Therefore, dehydration experiments were conducted to gain further insight into the lamellar crystalline structures of NFA-GalCer. The dehydrated bilayers exhibited no sign of a cooperative phase transition in the temperature range 0 – 90°C , as revealed by their CH_2 symmetric stretching band (results not shown). The spectral patterns of the amide I band of the dehydrated NFA-GalCer bilayers are presented in Fig. 3. Between 5 and 77°C , the amide I band was the sum of three overlapping components, peaked at approx. 1632 , 1614 and 1602 cm^{-1} . The two low frequency components at 1614 and 1602 cm^{-1} presumably rep-

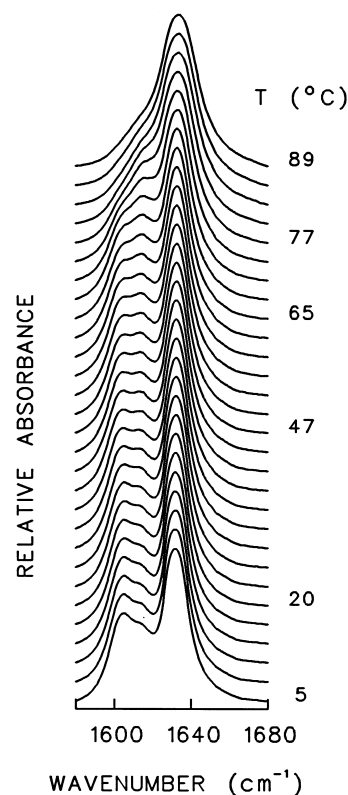


Fig. 3. Amide I region of the infrared spectra of dehydrated NFA-GalCer, obtained at the temperatures indicated. The sample was dispersed in $^2\text{H}_2\text{O}$ ($p^2\text{H}$ 7.05).

resented two populations of hydrogen bonded amide groups, which differ in the polarity of their surrounding environment and/or in the strength or degree of their hydrogen bonding interactions. While the 1632 and 1614 cm^{-1} components remained detectable up to 89°C, the 1602 cm^{-1} peak became unresolvable above 77°C, indicating that the hydrogen bonding network of dehydrated NFA-GalCer was weakened at high temperatures. However, hydrogen bonding was not totally disrupted since the 1614 cm^{-1} component band was detected even at 89°C. Upon cooling of dehydrated NFA-GalCer, the 1602 cm^{-1} component band was not recovered (results not shown), suggesting that heating of the dehydrated bilayers to high temperatures (89°C) induced irreversible rearrangements of the hydrogen bonding network of NFA-GalCer. Although the patterns of the amide I band (Fig. 3) in the dehydrated state were very homologous to those obtained in the L_c3 phase (Fig. 2B), the separation between the low frequency component bands was more evident in the dehydrated state. This resemblance indicated that L_c3 was poorly hydrated and that the dehydration degree was greater following cooling from the liquid-crystalline phase.

Since the temperature changes were done in a timely manner, it was questionable whether the polymorphic behavior of NFA-GalCer, especially in the lamellar crystalline phase, was temperature or time or both temperature and time dependent. To investigate this, in the first experiment NFA-GalCer was heated to 38°C to generate L_c1 , and the bilayers were held at that temperature for 96 h (Fig. 4A). After 45 min (time that would have been needed in the continuous heating experiment to increase the temperature from 38 to 47°C (Fig. 2A), where the transformation into L_c2 was apparent), the spectral patterns of L_c1 remained unchanged. Following incubation of the bilayers at 38°C for 24 h, a mixture consisting of L_c1+L_c2 was detected. The transformation of L_c1 into L_c2 was completed after 48 h, and L_c2 was stable up to 96 h at 38°C, a temperature favoring the formation of L_c1 (Fig. 4A). These results suggested that the formation of L_c1 and L_c2 was induced by either heat or time. Moreover, the slow transformation of L_c1 into L_c2 at approx. 47°C during the heating experiment of NFA-GalCer (Fig.

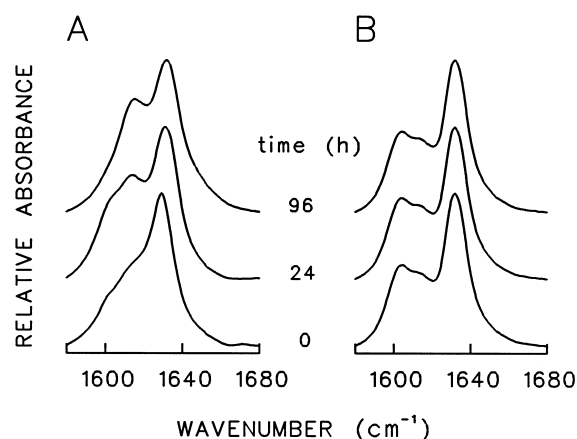


Fig. 4. Amide I region of the infrared spectra of hydrated NFA-GalCer, obtained after prolonged incubation at 38°C following initial heating of the sample (A) or subsequent cooling from 89°C (B). L_c2 and L_c3 were the most stable polymorphs upon heating and cooling, respectively. The sample was dispersed in $^2\text{H}_2\text{O}$ buffered with 50 mM Tris-HCl (pH 7.05).

2A) would probably have appeared at lower temperatures if the heating rate was slower, since L_c1 eventually transformed into L_c2 at 38°C (Fig. 4A).

In the second set of experiments we tested whether L_c3 , generated during the cooling experiment of NFA-GalCer from the liquid-crystalline phase, could be converted to L_c1/L_c2 or other crystalline polymorphs. Cooling of the bilayers from 89 to 38°C resulted in the formation of L_c3 , which was stable throughout the time course of the experiment (96 h) (Fig. 4B). However, when NFA-GalCer was cooled from 89 to 65°C, an intermediate mixture (liquid-crystalline+ L_c2) was observed. Further incubation at 65°C resulted in the formation of L_c3 after 30 min (results not shown), the time that was required for stepping down from 65 to 59°C (where L_c3 was first observed) in the cooling experiment (Fig. 2B). This indicated that the formation of L_c3 would have occurred at 65°C if the bilayers were cooled at a slower rate. Further cooling of the NFA-GalCer bilayers from 65 to 38°C revealed that once L_c3 was generated, it remained unchanged even after 96 h of incubation at 38°C. Although the bilayer packing of NFA-GalCer was more disordered in the L_c3 form (Fig. 1, ○), as evidenced by the higher frequencies of the methylene groups, its hydrogen bonding network was very stable.

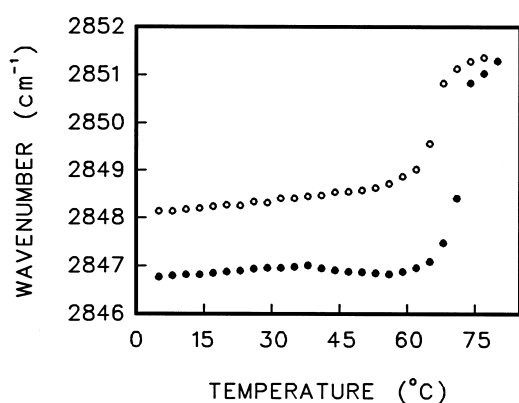


Fig. 5. Thermotropic response of the CH_2 symmetric stretching frequency of hydrated HFA-GalCer upon heating (●) and cooling (○). The sample was dispersed in $^2\text{H}_2\text{O}$ buffered with 50 mM Tris-HCl (pH 7.05).

3.3. Packing of HFA-GalCer bilayers

The thermotropic response of the hydrocarbon chains of HFA-GalCer upon heating (●) and cooling (○) is shown in Fig. 5. A cooperative phase transition occurred at 72°C and it was preceded by a small peak between 30 and 45°C . This peak possibly reflected the presence of a metastable low temperature form converting into a stable crystalline form (see Section 3.4) on heating (Fig. 5, ●). In comparison with NFA-GalCer, the organization of the hydrocarbon chains of HFA-GalCer was more ordered, as indicated by the lower frequencies of the CH_2 symmetric stretching band (Fig. 1 vs. Fig. 5). The α -hydroxy group increased the hydrogen bonding interactions with interfacial water and/or between neighboring HFA-GalCer molecules, thus leading to a tight chain packing. However, the lamellar crystalline to liquid-crystalline phase transition of HFA-GalCer occurred at 72°C , compared to 80°C for NFA-GalCer. Since HFA-GalCer contains primarily 24:0 [6], we believed that HFA-GalCer formed interdigitated crystalline bilayers [27], due to the hydrocarbon chain asymmetry, and to possess a strong hydrogen bonding network, which would favor a shovel conformation of the glycosphingolipid with respect to the hydrocarbon chain axis [35,36,53]. On the other hand, NFA-GalCer has a high percentage of 24:1, and it has been postulated that the *cis* double bond in 24:1 GalCer [54] and 24:1 sulfatide [55] may prevent the hydrocarbon chain interdigita-

tion, which was reported to induce a disordering effect for different phosphatidylcholine species [56,57]. Based on the above mentioned findings, hydrocarbon chain interdigitation and extensive hydrogen bonding of HFA-GalCer may destabilize the crystalline arrangement of the glycosphingolipid, resulting in a lower order-disorder T_m , relative to NFA-GalCer.

Cooling of HFA-GalCer from the chain melted state shifted the order-disorder T_m to 66°C , as well as the frequencies of the CH_2 stretching band to higher values, indicating an increase in the disorder of the hydrocarbon chains (Fig. 5, ○). The small peak seen at approx. 38°C in the heating profile was not observed in the cooling scan (Fig. 5), suggesting that HFA-GalCer formed a single bilayer state following the transition from the highly disordered liquid-crystalline phase.

3.4. The amide I $\text{C}=\text{O}$ stretching band of HFA-GalCer

The patterns of the amide I vibrational band of HFA-GalCer are shown in Fig. 6. Upon heating from 5 to 38°C , the patterns of the amide I band of HFA-GalCer were indicative of a hydrated lamellar gel phase [49]. This is in accordance with X-ray diffraction and DSC studies revealing that HFA-ceramide adopts a well-ordered gel phase at 20°C [22]. The contours of the amide I absorption band were asymmetric and leaning toward low frequencies (approx. 1628 cm^{-1}) (Fig. 6A), reflecting the prevalence of hydrogen bonded amide I $\text{C}=\text{O}$ groups presumably with interfacial water [51,52], due to the presence of the α -hydroxy group. From 44 to 71°C , two discernable peaks were detected at approx. 1635 and 1620 cm^{-1} (Fig. 6A), indicating the coexistence of non-hydrogen bonded and hydrogen bonded amide groups, respectively, at the bilayer surface. These two peaks showed band narrowing (Fig. 6A), indicative of an ordered lamellar crystalline structure (termed L_cI) [49,58]. Above 71°C (Fig. 6A), the two narrow amide I component bands disappeared: a broader band with its maximum at 1633 cm^{-1} was observed, suggesting that the HFA-GalCer bilayers were in the liquid-crystalline phase. The gel-crystalline conversions, prior to the chain melting transition, were believed to be influenced by chain packing and hydrogen bonding of HFA-GalCer, as suggested by Reed

and Shipley [27]. Cooling of the HFA-GalCer bilayers from 80°C resulted in the formation of a slightly broader amide I band, the patterns of which were representative of a lamellar gel phase (Fig. 6B). The gradual shift of the amide I band from approx. 1633 cm^{-1} in the liquid-crystalline phase to approx. 1619 cm^{-1} (Fig. 6B) in the gel phase suggested an increased hydrogen bonding potential of the amide groups of HFA-GalCer with neighboring molecules and/or with interfacial water, which was plausibly mediated by the α -hydroxy group of the glycosphingolipid. This would therefore stabilize the gel phase and prevent HFA-GalCer's polymorphism throughout the cooling process.

To further probe the polymorphic behavior of HFA-GalCer, as well as the nature of the lamellar crystalline phase observed on heating, dehydrated

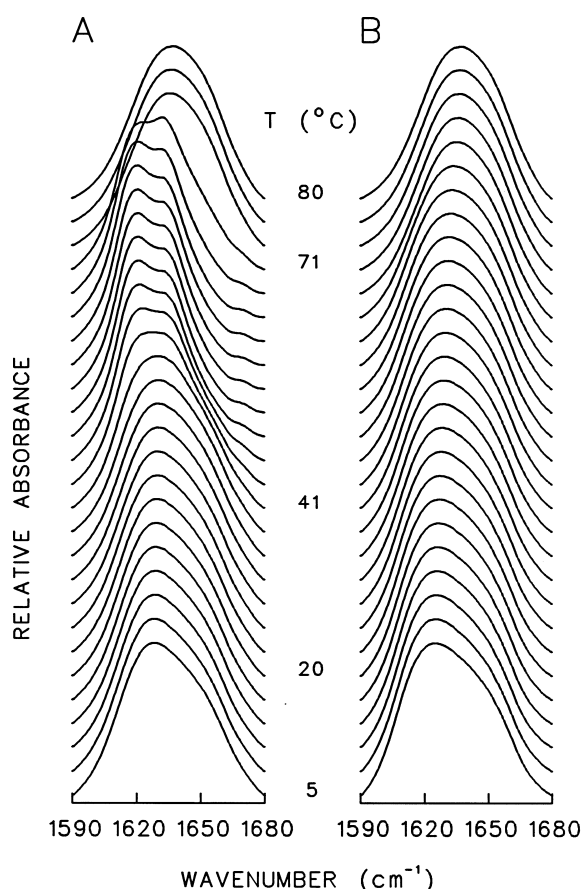


Fig. 6. Amide I region of the infrared spectra of hydrated HFA-GalCer, obtained at the temperatures indicated, upon heating (A) and following subsequent cooling (B). The sample was dispersed in $^2\text{H}_2\text{O}$ buffered with 50 mM Tris-HCl (p^2H 7.05).

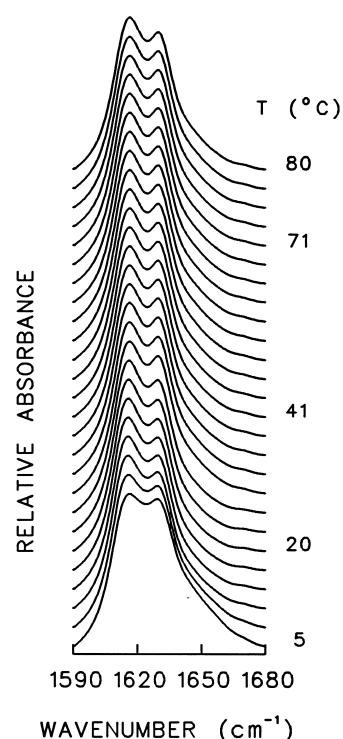


Fig. 7. Amide I region of the infrared spectra of dehydrated HFA-GalCer, obtained at the temperatures indicated. The sample was dispersed in $^2\text{H}_2\text{O}$ (p^2H 7.05).

bilayers were generated and studied as a function of temperature. The dehydrated bilayers did not exhibit a phase transition from 0 to 80°C, as revealed by their CH_2 symmetric stretching band (results not shown). The amide I band of HFA-GalCer consisted of a sharp peak and a narrow shoulder centered near 1628 and 1616 cm^{-1} , respectively, consistent with the existence of an ordered crystalline arrangement of the dehydrated bilayers (Fig. 7). Furthermore, the contours of the amide I absorption band of HFA-GalCer of L_cI were similar to those of the dehydrated form, suggesting that L_cI was partially dehydrated (Fig. 6A vs. Fig. 7). Interestingly, the amide I component bands of L_cI near 1635 and 1620 cm^{-1} shifted to 1628 and 1616 cm^{-1} , respectively, in the dehydrated form. This indicated that dehydration of HFA-GalCer changed the polarity of the environment surrounding the amide groups and/or increased the hydrogen bonding interactions between adjacent HFA-GalCer molecules.

To assess whether the low temperature polymorphism of HFA-GalCer could also be driven by pro-

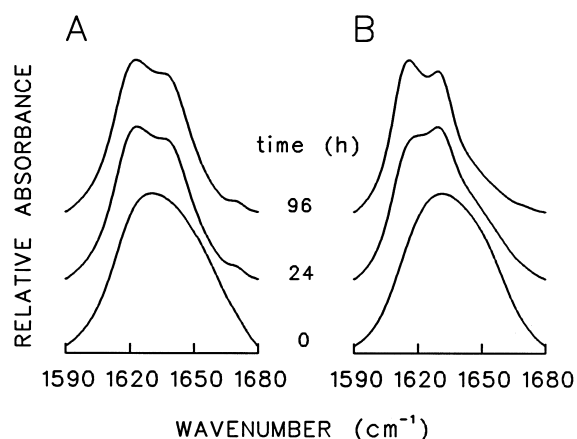


Fig. 8. Amide I region of the infrared spectra of hydrated HFA-GalCer, obtained after prolonged incubation at 38°C following initial heating of the sample (A) or subsequent cooling from 80°C (B). The sample was dispersed in $^2\text{H}_2\text{O}$ buffered with 50 mM Tris-HCl (pH 7.05).

longed incubation of the glycosphingolipid at specific temperatures, similar to NFA-GalCer, the behavior of hydrated HFA-GalCer bilayers was studied as a function of time (Fig. 8). In the first experiment, HFA-GalCer was heated to 38°C and incubated at that temperature for 96 h (Fig. 8A). Originally, the patterns of the amide I band reflected the attainment of a lamellar gel phase, as expected (see Fig. 6A), and remained unchanged after 45 min (time that would have been needed in the heating experiment to increase the temperature from 38 to 47°C (Fig. 6A), where the transformation into the $L_c\text{I}$ form was observed). However, this phase was metastable because additional incubation at 38°C resulted in its conversion into $L_c\text{I}$ after 24 h, and this crystalline arrangement was stable over the time course of the experiment (96 h) (Fig. 8A). These results suggested that the formation of $L_c\text{I}$ was induced by either heat or time. In the second experiment, HFA-GalCer was heated to 80°C (i.e., above its chain melting transition), cooled from that temperature and left at 38°C for 96 h (Fig. 8B). Initially, a metastable gel phase was observed, transforming after 24 h into an intermediate biphasic mixture (gel+ $L_c\text{I}$). Shortly afterwards (at the 26th h), this mixture converted into a crystalline phase whose amide I features were similar to those in the $L_c\text{I}$ form but more so to the dehydrated form (Fig. 7 vs. Fig. 8). Prolonged incubation of HFA-GalCer at 38°C, following cooling from the

chain melted state, was most likely accompanied by dehydration. This may have induced orientational and conformational changes of HFA-GalCer, leading to increased hydrogen bonding interactions and/or localization of one or more populations of amide groups in less polar environments.

4. Discussion

In this spectroscopic study, we compared the phase behavior of hydrated NFA- and HFA-GalCer bilayers, and investigated the nature of their polymorphic phases. Upon heating, chain melting of the lamellar crystalline phase occurred at 80 and 72°C for NFA- and HFA-GalCer, respectively. The long chain character of the fatty acids [6], combined with the extensive hydrogen bonding capacity of the galactosyl head group of GalCer, gave rise to the high order-disorder T_m . Interestingly, changing the *N*-acyl chain of GalCer from 16:0 to 18:0 or 24:0 has little effect on the main thermal phase transition [25,27,28,59]. The α -hydroxy group of HFA-GalCer presumably increased the hydrogen bonding interactions with interfacial water and/or between adjacent lipid molecules. This would allow HFA-GalCer to form a tighter chain packing than NFA-GalCer, as indicated by the lower frequencies of the methylene stretching mode. However, the higher degree of hydrogen bonding may ultimately lead to a disruption of chain packing, as well as the crystalline arrangement of HFA-GalCer. Cooling from the liquid-crystalline phase downshifted the disorder-order T_m of NFA- and HFA-GalCer to 62 and 66°C, respectively. The hysteresis effect exhibited by GalCer on cooling, which has been discussed in the literature [25–28,30,31], was indicative of the slow conversion of the melted chain bilayer to the lamellar crystalline (NFA-GalCer) or gel (HFA-GalCer) phase.

In terms of structure, both NFA- and HFA-GalCer exhibited metastable polymorphism, which was accompanied by a change in the hydrocarbon chain packing and hydrogen bonding network, and by a reduced bilayer hydration. The patterns of the amide I band revealed that NFA-GalCer exhibited metastable (L_c1) and stable (L_c2 , L_c3) lamellar crystalline structures, while HFA-GalCer displayed metastable gel and stable ($L_c\text{I}$) crystalline structures.

These conversions were consistent with a stronger hydrogen bonding network of the amide groups [50,51] in a presumably less polar environment in the lamellar crystalline phase. A summary comparing the characteristics of the metastable, stable and liquid-crystalline bilayer structures of NFA- and HFA-GalCer is shown in Fig. 9.

Heating of NFA-GalCer resulted in the formation of L_c1, which was metastable since it slowly converted to a stable L_c2 phase, in a temperature (47–74°C) and time dependent manner (24–48 h). Upon cooling from the chain melted state, an intermediate mixture (liquid-crystalline+L_c2) was initially detected, which subsequently converted into a stable lamellar crystalline L_c3 form (Fig. 9A). Furthermore, the contours of the amide I band of NFA-GalCer in L_c3 resembled those of the corresponding dehydrated form (Fig. 3). Based on the notion that an increase in the hydration of the interfacial region of diacyl phospholipids seems unfavorable during the formation of the lamellar crystalline phase [49], we suggested that partial dehydration of NFA-GalCer led to the formation of crystalline structures in which the amide groups were strongly hydrogen bonded to the hydroxy groups of the galactose residues. Therefore, we believed that L_c3 was poorly hydrated and stabilized by a strong hydrogen bonding. However, the chain packing lattice of L_c3 was less ordered than L_c1 and L_c2 (i.e., the frequencies of the methylene groups were observed at higher values, Fig. 1), perhaps due to modes of crystallization and/or solvent trapping, which would ultimately affect the conformation/tilt of the hydrocarbon chains. While the formation of L_c1 and L_c2 was both temperature and time dependent, the formation of L_c3 was only temperature driven following a transition from the liquid-crystalline phase.

Paradoxically, HFA-GalCer adopted a gel phase upon heating and following cooling from the chain melted state (Fig. 9B). The α -hydroxy group of GalCer most likely increased hydrogen bonding with water, thus preventing partial dehydration of the bilayers and the immediate formation of crystalline polymorphs. However, the gel phase was metastable and transformed into a stable crystalline phase (L_cI) in a temperature (heating) and time (prolonged incubation at 38°C) dependent manner. This metastable-stable polymorphism of HFA-GalCer may be

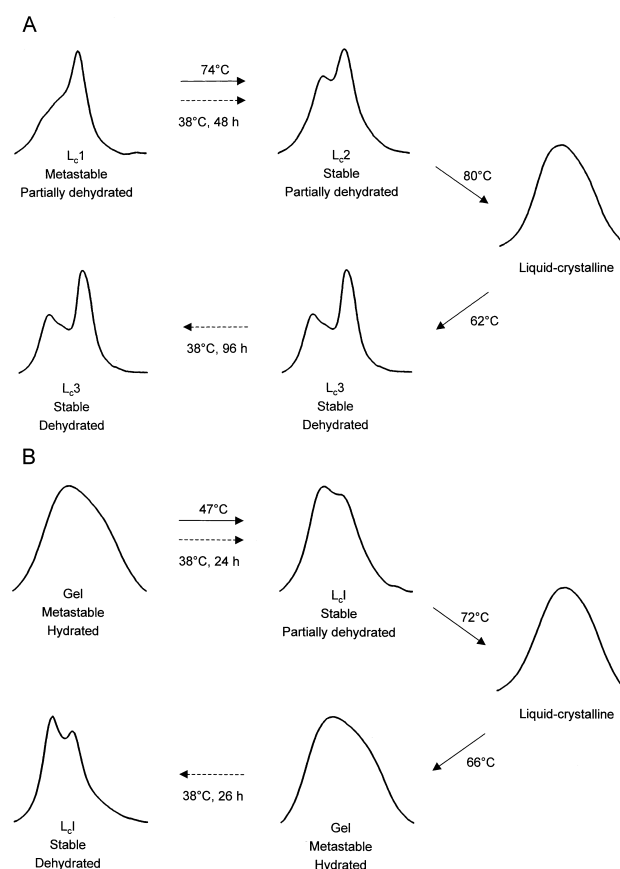


Fig. 9. Schematic representation of the temperature/time dependent polymorphic phases of NFA-GalCer (A) and HFA-GalCer (B), as assessed by the patterns of the amide I C=O stretching band.

mediated by dehydration, since the amide I spectral features of the stable L_cI polymorph were similar to those in the dehydrated state.

The pioneering work of Curatolo [26,59] and Shipley and colleagues [25,27,28,30,31] revealed that GalCer exhibits a complex phase behavior. DSC and X-ray diffraction studies on NFA- and HFA-GalCer, as well as on chain specific GalCer, showed evidence of conversions between metastable and stable bilayer phases, thought to be accompanied by cerebroside hydration [25,27]. Furthermore, Curatolo and Jungalwala [59] demonstrated that NFA- and HFA-GalCer exhibit metastable polymorphism. The authors concluded that the kinetic barriers to reaching the stable phase are greater for HFA-GalCer than for NFA-GalCer. To further investigate the structural and thermodynamic properties of GalCer, FTIR spectroscopy was employed in this study,

which provides direct characterization of chain packing, hydrogen bonding and hydration sites of GalCer. Our spectroscopic findings revealed that the polymorphism of GalCer reflected conformational and orientational changes of the glycosphingolipid that affect the polarity of the environment surrounding the amide groups. Moreover, these metastable conversions of GalCer were accompanied (and plausibly regulated) by chain packing, bilayer dehydration and interfacial hydrogen bonding interactions. While changes in chain packing remained important, the interfacial hydrogen bonding interactions between adjacent GalCer and/or with water presumably influenced significantly the formation of the lamellar crystalline phase. Although the α -hydroxy group of HFA-GalCer increased hydrogen bonding with interfacial water, which may explain the conclusions of Shipley and colleagues [25,27,28,30,31], it did not prevent GalCer's polymorphism and its tendency to adopt partially dehydrated lamellar crystalline structures.

In summary, the order-disorder T_m of NFA- and HFA-GalCer was extremely high, relative to body temperature. Interestingly, GalCer was shown to form bilayer cylinders, tubules and ribbon-like, helically twisted structures [54,60]. Our findings implicate GalCer's stabilization role in the maintenance of the curvature and cylindrical shape of the myelin sheath [60,61]. This would most likely reduce permeability to ions, allowing nerve fibers to effectively facilitate saltatory conduction [59,62]. In addition, the partially dehydrated lamellar crystalline structures adopted by NFA- and HFA-GalCer are presumably involved in the modulation of the local hydrophobicity and interfacial hydrogen bonding network of the bilayer, similar to gangliosides [63,64]. This high stability of GalCer, as well as its galactose head group, may mediate intercellular adhesion presumably through carbohydrate-carbohydrate/protein interactions [8,9], thus initiating subsequent signal transduction cascades in the myelin sheath [14].

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