Characterization of the Interaction of Ca^{2+} with Hydroxy and Non-hydroxy Fatty Acid Species of Cerebroside Sulfate by Fourier Transform Infrared Spectroscopy and Molecular Modeling[†]

Abdellah Menikh,[‡] Per-Georg Nyholm,[§] and Joan M. Boggs*,[‡,||]

The Research Institute, Hospital for Sick Children, Toronto, Canada M5G 1X8, Department of Molecular and Medical Genetics, University of Toronto, Toronto, Canada M5S 1A8, and Department of Clinical Biochemistry, University of Toronto, Toronto, Canada M5G 1L5

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ABSTRACT: Ca^{2+}-mediated interactions between the carbohydrate groups of glycolipids, including that of cerebroside sulfate (galactosylceramide I^3-sulfate), have recently been implicated as a basis of cell recognition and adhesion. Hydroxylation of the fatty acid of this lipid has an effect on these interactions. Therefore, FT-IR spectroscopy was used to study the interaction of Ca^{2+} with semisynthetic hydroxy (HFA) and non-hydroxy fatty acid (NFA) species of cerebroside sulfate (CBS). Ca^{2+} caused partial dehydration of the sulfate group and reduced hydrogen bonding of the sugar hydroxyls of both species. The amide I and II bands of the lipids in the absence of Ca^{2+} (NH_4^+ salt forms) suggested that the N−H of the HFA species is involved in a bent intramolecular hydrogen bond, probably with the fatty acid hydroxyl group and the glycosidic oxygen, while that of the NFA species is involved in a linear intermolecular hydrogen bond with the C═O and/or other oxygens. Ca^{2+} caused a rearrangement of the hydrogen-bonding network in the interfacial region of the HFA species involving the amide group. The results suggested increased hydrogen bonding of the C═O and a shift in hydrogen bonding of the N−H of the Ca^{2+} salt form of the HFA species from a bent intramolecular hydrogen bond to a linear intermolecular hydrogen bond, probably with the C═O of neighboring molecules, similar to the NFA species. The involvement of the fatty acid α-hydroxyl group in the rearranged network was indicated by a reduction in mobility of the α-CH group of the HFA species, in contrast to that of the NFA species. Participation of the α-OH group in hydrogen-bonding networks in the interfacial region of both the NH_4^+ and Ca^{2+} salt forms caused a significant increase in the interchain packing, as evident from correlation field splitting of the HFA−CBS methylene scissoring mode, while this did not occur for the NFA species. The absence of intramolecular hydrogen bonding of the N−H with the glycosidic oxygen for both salt forms of the NFA species and for the Ca^{2+} salt form of the HFA species may destabilize the "bent shovel", bilayer planar conformation of the sugar and cause it to be in the extended, bilayer perpendicular conformation. Calculations of the three-dimensional interaction energy of Ca^{2+} with CBS showed strong binding around the sulfate and the surface of galactose facing the bilayer in the bent shovel conformation. Ca^{2+} binding at this surface would disrupt intra- and intermolecular hydrogen-bonding interactions of the head group, thus accounting for its effect in inducing a transition to the extended conformation.

Calcium-mediated interactions between cell surface carbohydrates including those on glycolipids have recently been implicated as a basis of cell recognition and adhesion and have therefore been the subject of increasing interest (Eggens et al., 1989; Hakomori, 1991; Misevic & Burger, 1993). A Ca^{2+}-mediated interaction between cerebroside sulfate (CBS)[1] and galactosylceramide (GalCer), the two major glycolipids of myelin, has recently been found to occur (Hakomori, 1991; Stewart & Boggs, 1993; Koshy & Boggs, 1996). This interaction may play a role in adhesion of the extracellular surfaces of myelin. However, the structure of the complex and its relationship to its function in myelin are still unknown.

These glycolipids contain numerous hydroxyl groups plus the sulfate group on CBS which could chelate with Ca^{2+} and hydrogen bond with each other. In myelin, they contain both α-hydroxylated fatty acids (HFA) and non-hydroxy fatty acids (NFA) (Norton, 1977). Using X-ray crystallography, Pascher and Sundell (1977) showed that the head group of crystalline HFA−GalCer is bent almost perpendicular to the acyl chain (the "bent shovel" conformation) and capable of participating, along with the amide moiety and the fatty acid α-hydroxyl group, in an intermolecular hydrogen-bonding network. No crystal structures of CBS have been reported,

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* To whom correspondence should be addressed at the Research Institute, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8. Telephone: (416) 813-5919. Fax: (416) 813-5022. E-mail: jmboggs@sickkids.on.ca.

[‡] The Research Institute.

[§] Department of Molecular and Medical Genetics.

[||] Department of Clinical Biochemistry.

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[1] Abbreviations: CBS, cerebroside sulfate; NFA, non-hydroxy fatty acid species; HFA, α-hydroxy fatty acid species; FT-IR, Fourier transform infrared spectroscopy.

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but recent studies of semisynthetic NFA and HFA species of CBS by differential scanning calorimetry indicate that the hydroxy fatty acid increases the phase transition temperature, suggesting that it contributes to the stability of the gel phase by participating in the hydrogen-bonding network (Boggs et al., 1988). The HFA species of both CBS and GalCer were found to result in greater Ca^{2+}-mediated interaction between liposomes containing these lipids than the NFA species (Stewart & Boggs, 1993).

Fourier transform infrared (FT-IR) spectroscopy is an excellent technique for investigation of the structure of cation−lipid complexes and elucidation of groups which participate in interaction with cations or hydrogen bonding (Casal et al., 1987). In order to understand the role of the fatty acid hydroxyl group in the conformation of the lipid and its interactions with Ca^{2+}, and as a first step toward elucidation of the structure of the heterotypic complex of Ca^{2+} with CBS and GalCer, in the present study we compare the FT-IR spectra of the HFA C18:0h and NFA C18:0 semisynthetic species of CBS and analyze the conformational changes that occur during their interactions with Ca^{2+}. We were able to determine the effect of the α-hydroxyl group on the intramolecular and intermolecular hydrogen-bonding interactions of CBS, localize some of the molecular sites which interact with Ca^{2+}, and determine the effect of Ca^{2+} on the hydrogen-bonding interactions of the interfacial region and on the conformation of the carbohydrate head group.

MATERIALS AND METHODS

Chemicals. Semisynthetic stearoyl species of C18:0h−CBS (HFA−CBS) and C18:0−CBS (NFA−CBS) were prepared and purified according to the procedure reported earlier (Koshy & Boggs, 1983). In the absence of added cations they are in the ammonium salt form (Koshy & Boggs, 1996). D_2O (99.9%) was obtained from Merck, Sharp and Dohme (Montreal, Canada). $CaCl_2{\cdot}2H_2O$ was purchased from Fisher Chemical Co. (Fairlawn, NJ) and purified as described (Menikh & Fragata, 1993).

Sample Preparation. About 1.5 mg of dry lipid was dispersed by vortexing in 1 mL of D_2O. In order to ensure complete hydration, the multilamellar lipid vesicles were heated to about 90 °C, vortexed several times, and then cooled to room temperature. An aliquot of a 1.0 M $CaCl_2$ solution was added to the suspension in order to obtain a final salt concentration of 2 mM $CaCl_2$ and an approximately equimolar ratio of Ca^{2+} to CBS.

FT-IR Measurements. Both dry and hydrated samples were studied by infrared spectroscopy. Most of the results shown in the figures and discussed, unless otherwise noted, were conducted with dry films in order to avoid overlapping of the sulfate region with D_2O. Dry films of the lipid suspension were prepared as follows: a drop of the lipid suspension was layered on a CaF_2 window and allowed to dehydrate, under vacuum in a desiccator, over a period of about 24 h. Some bound water is probably still present although it was not detectable in the spectrum. Infrared spectra of these dried samples were measured in a Bruker FT-IR spectrometer, model IFS 48, equipped with a germanium-coated KBr beam splitter and DTGS detector. Typically, 100 interferograms were collected, coadded, and Fourier transformed to yield a resolution of 2 cm^{-1} after triangular apodization. Reference spectra were recorded under the same conditions with media containing no lipid. Hydrated samples were studied by sedimenting the lipid in a suspension prepared as described above, by centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was removed, and the wet pellet was mounted between two CaF_2 windows. The temperature was controlled by means of a cell jacket of circulating water connected to a thermocouple. During data processing, the spectrometer was continuously purged with high-purity argon to eliminate the spectral contribution of atmospheric vapor. For data analysis, all spectral files were imported to Spectra Calc (Galactic Industries Corp., Salem, NH) which makes use of the Savitzky−Golay convolution method (Savitzky & Golay, 1964). Resolution enhancement techniques were used in order to separate the unresolved bands. Spectra were subjected to both deconvolution and second derivative routines. Only features observed by both techniques were assigned and analyzed.

Molecular Modeling. Atomic coordinates for HFA−CBS were generated on the basis of the crystal structure of β-D-galactosyl-*N*-(2-D-hydroxyoctadecanoyl)-D-dihydrosphingosine (HFA−GalCer) (Pascher & Sundell, 1977). A sulfate group was added to the 3-position of galactose, and the torsion angles of the galactose 3-*O*-sulfate moiety were adjusted to conform to the conformation in the crystal structure of methyl α-D-galactopyranoside-3-(sodium sulfate) monohydrate (Polvorinos et al., 1994). With regard to the galactose−ceramide linkage, two different conformations were considered, the bent shovel conformation which exists in the crystal structure of HFA−GalCer (Pascher & Sundell, 1977) and a conformation ($\psi = 180°$, $\theta_1 = 180°$) in which the galactose is parallel to the lipid chains and extended away from the ceramide. These two conformations have been calculated using molecular mechanics (MM3) to be the two lowest energy conformations for both NFA and HFA−glucosylceramide at values of the solvent dielectric constant of 4−80 [see conformers 2 and 5 in Nyholm and Pascher (1993a,b)]. Coordinates for the NFA species of CBS in its bent shovel and extended conformations were generated by deletion of the α-OH group of the corresponding HFA−CBS conformations.

In order to locate favorable binding sites for Ca^{2+} and NH_4^+ on the CBS head group, calculations were carried out with the GRID program (Goodford, 1985). A solvent dielectric constant of 10 was used to approximate the dielectric properties of the microenvironment at the membrane surface. The interaction energy between the ionic probe and the target molecule was evaluated every 0.5 Å in the *x*, *y*, and *z* directions. The results were visualized using three-dimensional contours superimposed on the molecular structure. All molecular manipulation and graphics were carried out using the ChemX program (Chemical Design, Ltd., Oxford).

RESULTS

Interfacial Region—Amide Group. The amide group linking the fatty acid chain to sphingosine provides an important potential site for investigation of the intramolecular and/or intermolecular hydrogen-bonding interactions of the interfacial region of CBS by FT-IR spectroscopy. The amide I band arises principally from the C═O stretching vibration with a small contribution of C−N stretching and N−H bending (Bandekar, 1992), while the amide II band between 1580 and 1500 cm^{-1} is essentially an N−H bending vibration strongly coupled to C−N stretching (Lee et al., 1986). An X-ray crystallography study on a synthetic HFA species of GalCer (Pascher & Sundell, 1977) indicated that the amide C=O is involved in intermolecular hydrogen bonding with both the hydroxyl of the sphingosine chain and the 3-OH group of the sugar head group of neighboring molecules while the amide N−H forms a bifurcated intramolecular hydrogen bond with the fatty acid hydroxyl group and the oxygen of the glycosidic linkage. In addition to intramolecular hydrogen bonding with the N−H group, the fatty acid hydroxyl group is hydrogen bonded intermolecularly to the 4-OH group of the sugar. FT-IR spectra of the HFA species of GalCer in water were consistent with the involvement of the N−H in an intramolecular hydrogen bond but indicated that in the NFA species the N−H was involved in a linear intermolecular hydrogen bond, possibly with the C=O, since the C=O of the NFA species was involved in stronger hydrogen-bonding interactions than that of the HFA species (Lee et al., 1986).

The effect of the hydroxyl group of the fatty acid of CBS on the strength of the hydrogen-bonding interactions of the C=O can be determined by comparison of the spectra of the amide I region of C18:0h−CBS and C18:0−CBS. The amide I band of a dry film of the ammonium salt form of C18:0−CBS was shifted to a lower frequency relative to C18:0h−CBS (Figure 1A), indicative of a more strongly hydrogen-bonded C=O group for the NH_4^+−NFA species.

Deconvolution of the amide I spectral region of the NFA species revealed two intense bands located at 1614 and 1641 cm^{-1} (Figure 1B, curve 3) while that of the HFA species revealed three different bands at 1622, 1632, and 1652 cm^{-1} (Figure 1C, curve 5). The frequencies of these bands are summarized in Table 1. Site symmetry splitting of IR bands can occur either when the motional freedom of the molecule is reduced, as a consequence of crystallization, or if the symmetry of the molecule is lowered (Nokamoto, 1978). The former was found to occur for short-chain species of phosphatidylcholine or glycosyldiacylglycerol in a stable subgel phase in which the acyl chains were in a rigid crystalline structure (Lewis & McElhaney, 1990; Lewis et al., 1990). However, hydrated samples of the ammonium salt form of CBS had similar spectral behavior as dry films, although the bands were shifted to higher frequencies (Table 1), suggesting that restriction of the mobility of the C=O was not the cause of the appearance of two or more spectral components.

In an FT-IR study of hydrated natural bovine brain CBS containing a mixture of the NFA and HFA species by Tupper et al. (1992), the amide I spectral region also displayed two components, at 1619 and 1647 cm^{-1}. On the basis of their spectra of anhydrous NFA and HFA species of GalCer, the authors concluded that the low-frequency band originated from the HFA species of CBS and the high-frequency component originated from the NFA species of CBS. In contrast, our results show clearly that this spectral region of each species of CBS has two or more components. We believe that this effect is due to the existence of two or more populations of amide C=O groups for each of the NFA and HFA species. The low-frequency component corresponds to a strongly hydrogen-bonded C=O, and the higher frequency components correspond to more weakly hydrogen-bonded C=O groups (Wong & Mantsch, 1988). Similar results have been observed for the ester C=O groups of both the *sn*-1 and *sn*-2 chains of phosphatidylcholine, indicating that this lipid also occurs as two instantaneous subpopulations of lipid whose C=O groups are involved in different degrees of hydrogen bonding (Blume et al., 1988; Lewis & McElhaney, 1992). Similar results were also reported for hydrated samples of GalCer (Lee et al., 1986) and glucosylcerebroside (GluCer) (Mueller & Blume, 1993) and for the C26:0 species of CBS (Nabet et al., 1996).

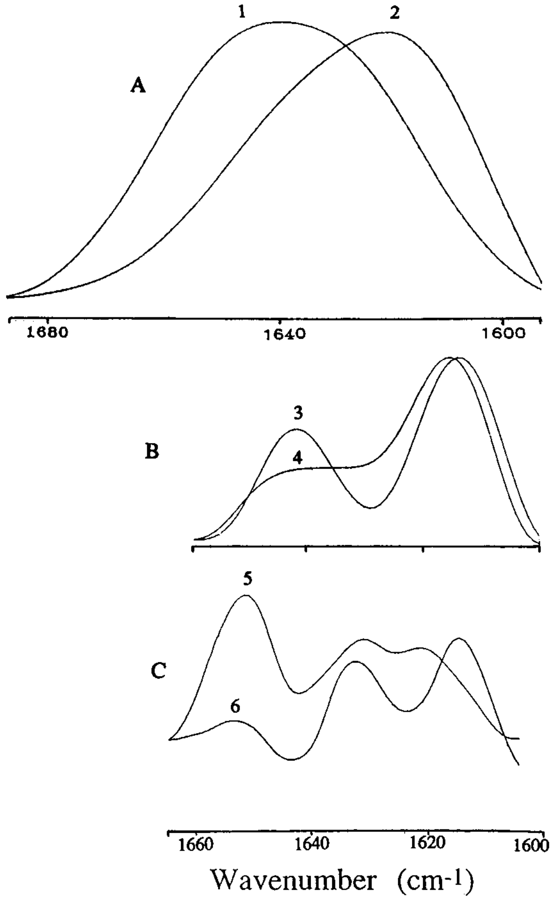

FIGURE 1: Amide I C=O stretching region of the infrared spectra of a dry film of HFA− and NFA−CBS. (A) Original spectra of NH_4^+ salt of HFA−CBS (1) and NFA−CBS (2). (B and C) Deconvolved spectra of (3) NH_4^+ salt of NFA−CBS, (4) Ca^{2+} salt of NFA−CBS, (5) NH_4^+ salt of HFA−CBS, and (6) Ca^{2+} salt of HFA−CBS. The spectra were deconvoluted using a break point of 0.3 in the Fourier domain. Curves in (A) are plotted at the same scale as those in (B) and (C), but those in (A) are shown over a wider range.

The higher frequency of all components of the amide I band of the HFA species relative to the major component of the NFA species at 1614 cm^{-1} indicates that all populations of the ammonium salt form of HFA−CBS have more weakly hydrogen-bonded C=O groups than the predominant NFA−CBS population. The amide II band of a dry film of NFA−CBS occurred at 1549 cm^{-1} (Figure 2C), considerably higher than that for HFA−CBS at 1541 cm^{-1} (Figure 2A). The higher frequency of the amide II band of NFA−CBS indicates participation of the N−H in a linear intermolecular hydrogen bond, i.e., in a plane perpendicular to the N−H bending vibrations. This is most likely with the C=O of neighboring molecules, consistent with the lower frequency of the amide I region of the NFA species, as concluded for NFA−GalCer (Lee et al., 1986). The formation of a linear

Table 1: Frequencies of Amide I Band, Sulfate Antisymmetric Stretching Vibrational Band, and C−O and O−H Stretching Vibrational Band Regions of Dry Films and Hydrated Samples of NFA−CBS and HFA−CBS in the Presence and Absence of Ca^{2+}

	ν (CO) amide I[a] (cm^{-1})	ν ($O{=}S{-}O^-$) (cm^{-1})	ν (C−O−S) (cm^{-1})	ν (C−OH and/or C−OC) (cm^{-1})	ν (O−H) (cm^{-1})
dry films					
NH_4^+−NFA−CBS	1641, $\underline{1614}$	1214	992	1144	3417
Ca^{2+}−NFA−CBS	1646, $\underline{1615}$	1219	990	1147	3427
NH_4^+−HFA−CBS	$\underline{1652}$, $\underline{1632}$, 1622	1216	992	1145	3352
Ca^{2+}−HFA−CBS	1654, 1633, $\underline{1614}$	1218	990	1147	3474
hydrated samples					
NH_4^+−NFA−CBS	1652, $\underline{1627}$	1214			
Ca^{2+}−NFA−CBS	$\underline{1659}$, 1643, $\underline{1625}$	1220			
NH_4^+−HFA−CBS	1661, ~1645, $\underline{1627}$	1216			
Ca^{2+}−HFA−CBS	$\underline{1668}$, $\underline{1626}$, 1610	1219			

[a] Spectra of this region were deconvolved; frequencies of major bands are underlined.

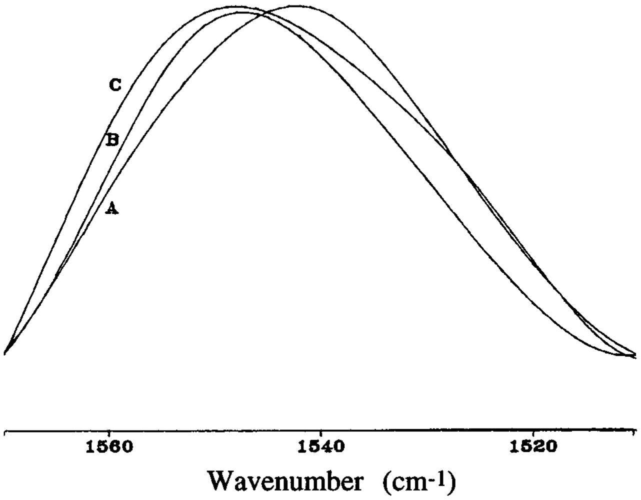

FIGURE 2: Infrared absorbance spectra in the amide II region of (A) NH_4^+ salt of HFA−CBS, (B) Ca^{2+} salt of HFA−CBS, and (C) NH_4^+ salt of NFA−CBS. Films were dried from a D_2O suspension.

hydrogen bond is expected to increase the amide II frequency because the hydrogen bond constrains the bending deformation. As a result the force constant for this mode increases and the frequency shifts to higher values. The lower frequency for HFA−CBS is consistent with participation of the NH of HFA−CBS in a bent hydrogen bond, i.e., in a plane parallel to the N−H bending vibrations. This is most likely an intramolecular hydrogen bond with the adjacent α-OH group of the fatty acid and the oxygen of the glycosidic linkage as in HFA−GalCer (Pascher & Sundell, 1977; Lee et al., 1986). This involvement of the amide N−H of the HFA species in intramolecular hydrogen bonding would attract electrons from the C═O, resulting in less contribution from the polar form (Norman et al., 1990), and thus would account for the increase in frequency of the amide I band and weaker hydrogen bonding of the C═O of the HFA species.

Effect of Ca^{2+} on the Amide Region. Addition of Ca^{2+} had only a small effect on the amide I spectral region of NFA−CBS (Figure 1B, curve 4). However, it significantly affected both the frequency and relative intensities of the bands in the deconvoluted spectra of HFA−CBS (Figure 1C, curve 6). The frequency of the most strongly hydrogen-bonded C═O group of HFA−CBS shifted from 1622 to 1614 cm^{-1} (Figure 1C, curve 6), similar to that of NFA−CBS, and the intensity of this band increased relative to that of the component due to the most weakly hydrogen-bonded C═O group at 1654 cm^{-1}. This indicates that Ca^{2+} caused most of the amide groups of HFA−CBS to be located in an environment of high dielectric constant, reflecting their involvement in hydrogen bonding, while a small population of amide groups was still located in a lower polarity interfacial environment. The bands due to the C═O groups also became more sharply resolved, indicating a reduction in their mobility. The amide II band at 1549 cm^{-1} for NFA−CBS completely disappeared upon addition of Ca^{2+} due to exchange of the amide proton for deuterium. However, the N−H of HFA−CBS was more resistant to exchange, and its amide II band clearly shifted to higher frequency, from 1541 to 1548 cm^{-1} (Figure 2B). This shift of the amide II band to higher frequency, together with the shift of the amide I band to lower frequency, suggests a rearrangement of the hydrogen-bonding network of the HFA species, possibly formation of a linear intermolecular C═O···HN bond in the presence of Ca^{2+}, similar to that which occurs for both the NH_4^+ and Ca^{2+} salt forms of the NFA species.

For hydrated samples where the amide I bands of both species were shifted to higher frequencies, reflecting weakening of intermolecular hydrogen bonding of the C═O, possibly due to hydrogen bonding with water, addition of Ca^{2+} decreased the frequency of the lower frequency band for both the NFA and HFA species (Table 1). Thus greater charge shielding by Ca^{2+} increased the hydrogen bonding of the C═O of both species. However, the frequency of the most strongly hydrogen-bonded population of the HFA species decreased to 1610 cm^{-1}, significantly lower than that of the NFA species when dry or hydrated (Table 1). This indicated stronger intermolecular hydrogen bonding of the C═O of the Ca^{2+} salt form of the HFA species, at least for a fraction of the lipid, than that of the NFA species in excess water.

The more prolonged exposure of the samples to D_2O required for measurement of hydrated samples of the Ca^{2+} salt forms caused the amide II band to disappear completely for the HFA species and partially for the NFA species, indicating accessibility of the N−H to D_2O. The rate of exchange was not compared quantitatively for the NH_4^+ and Ca^{2+} salt forms of the two species. However, no exchange was noted for the NH_4^+ salt forms in D_2O over the time period used for preparation and measurement of hydrated samples. The frequency of the amide II band for the hydrated NFA species was 1552 cm^{-1} for the NH_4^+ salt and 1555 cm^{-1} for the Ca^{2+} salt, reflecting linear intermolecular hydrogen bonding or hydrogen bonding of the N−H to water. However, for the NH_4^+ salt of the HFA species, the frequency of the amide II band was 1542 cm^{-1}, still lower than that of the NFA species, reflecting the persistence of a bent intramolecular hydrogen bond for the NH_4^+−HFA species in the presence of water.

Head Group—Sulfate, C−O, and Hydroxyl Groups. The antisymmetric double bond O=S−O$^-$ stretching vibration ranging between 1200−1265 and 1040−1080 cm^{-1}, respectively, together with the stretching mode of the C−O linking the sulfate group, ranging between 920 and 1040 cm^{-1} (Bellamy, 1975; Kates, 1986), provides important information about the environment of the sulfate region. The O=S−O$^-$ symmetric stretching vibration at 1040−1080 cm^{-1} was precluded from analysis since it overlapped with many C−O−H stretching bands from other groups in the lipid. The sulfate antisymmetric stretching band for a dry film of the ammonium salt form of the NFA species appeared at 1214 cm^{-1}, a somewhat lower (but reproducible) frequency than that of the HFA species at 1216 cm^{-1} (Table 1), suggesting that the sulfate of the NH_4^+−NFA species interacts more with some group, either water, the sugar OH groups of neighboring lipid molecules, or the N−H groups of ammonium ion, than that of the HFA species. Upon addition of Ca^{2+}, the O=S−O$^-$ antisymmetric stretching band shifted to higher frequency for both the HFA and NFA species, in the dry film and also in the hydrated samples (Table 1). The increase in frequency indicates displacement of some group from the sulfate on binding Ca^{2+}. An increase in frequency of the phosphate symmetric and antisymmetric stretching bands of phospholipids is associated with dehydration of the lipid head group, either upon binding Ca^{2+} or on evaporation of water (Casal & Mantsch, 1984; Arrondo et al., 1984; Dluhy et al., 1983; Casal et al., 1987). The frequencies of the Ca^{2+} salt forms of the NFA and HFA species were more similar to each other than those of the NH_4^+ salt forms, suggesting a more similar environment of the sulfate of the two species when bound to Ca^{2+}.

The O=S−O$^-$ spectral region could be deconvoluted into at least two bands (not shown) as reported by Tupper et al. (1992) for both the HFA and NFA species in the presence of both ammonium and Ca^{2+}. NMR spectroscopy showed that all of the lipid was in the NH_4^+ salt form in the absence of added cation. Since Ca^{2+} has a higher affinity for the sulfate than NH_4^+, the addition of an equimolar amount of Ca^{2+} should result in all of the lipid being in the Ca^{2+} salt form. Therefore, the presence of two or more bands may be due to splitting caused by lowering of the site symmetry on coordination of sulfate to NH_4^+ or Ca^{2+} (Nokamoto, 1978), rather than to different populations of the sulfate group. Site symmetry splitting of the phosphate symmetric stretching band of phosphatidylserine on binding Ca^{2+} has also been observed (Dluhy et al., 1983).

Addition of Ca^{2+} shifted the stretching vibration of the C−O linking the sulfate to galactose to lower frequency for both the NFA and HFA species, indicating that Ca^{2+} interacted indirectly with the C−O (Table 1). However, the stretching mode C−O of C−O−H or C−O−C groups at 1144 cm^{-1} for the NFA species and at 1145 cm^{-1} for the HFA species shifted to 1147 cm^{-1} for both species on addition of Ca^{2+} (Table 1). This suggested that Ca^{2+} weakened hydrogen-bonding interactions with these oxygens. Moreover, the hydroxyl group (OH) stretching vibrations increased for both species, but especially for the HFA species, upon complexation with Ca^{2+} (Table 1), indicating that Ca^{2+} weakened the hydrogen-bonding network in which the hydroxyl groups were involved. The frequency of the NH_4^+−HFA species was lower than that of the NH_4^+−NFA species, suggesting further that the hydroxyl groups of the NH_4^+−HFA species were more involved in hydrogen bonding than those of the NH_4^+−NFA species. In contrast, in the presence of Ca^{2+} the frequency was higher for the HFA species than the NFA species, indicating that the hydroxyl groups of the Ca^{2+}−HFA species were less involved in hydrogen bonding than those of the Ca^{2+}−NFA species.

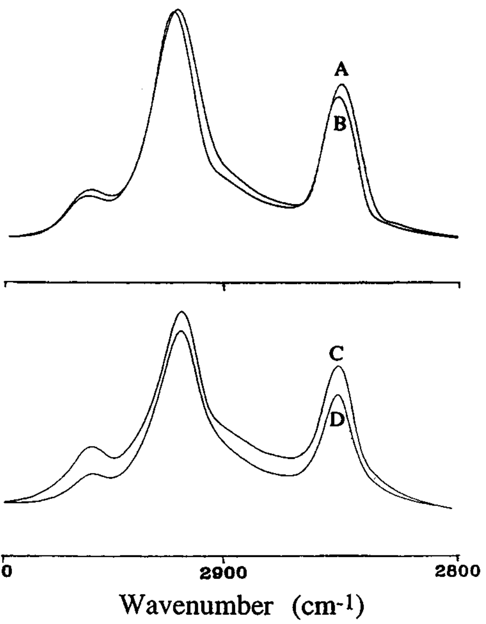

FIGURE 3: Infrared spectra of the C−H stretching region for (A) NH_4^+ salt of HFA−CBS, (B) Ca^{2+} salt of HFA−CBS, (C) NH_4^+ salt of NFA−CBS, and (D) Ca^{2+} salt of NFA−CBS. A linear base line extending from 3000 to 2800 cm^{-1} has been subtracted.

Hydrocarbon Chains. Infrared spectroscopy is a sensitive method for monitoring changes in the hydrocarbon chain packing. The parameters generally used to characterize the acyl chain regions of lipids are the frequencies and bandwidths of methylene stretching bands (Cameron et al., 1980; Auger et al., 1988). An increase of conformational disorder and motional freedom of the acyl chain can be monitored from an increase in stretching vibrational frequencies and the bandwidth of the methylene groups. The most useful information regarding the chain packing and interchain interactions in the gel phase of lipids can be obtained from the CH_2 scissoring mode (Lewis & McElhaney, 1993).

The symmetric and antisymmetric stretching vibrations of dry films of CBS gave rise to bands at 2850 and 2919 cm^{-1}, respectively (Figure 3). The phase transition temperatures of hydrated samples of CBS were determined from the increase in frequency of the symmetric stretching vibration with temperature (not shown). Those of the ammonium salt forms of the NFA and HFA species were 47 and 58 °C, respectively. This increase in transition temperature caused by the α-OH group also occurred in the presence of other cations as determined by differential scanning calorimetry (Boggs et al., 1988, 1984). Ca^{2+} increased the transition temperature of the NFA species to 55 °C, in agreement with previously published results on C18:0−CBS in the presence of Ca^{2+} (Boggs et al., 1984). The effect of Ca^{2+} on the phase transition temperature of the HFA species was not determined, but previous DSC studies showed that the transition

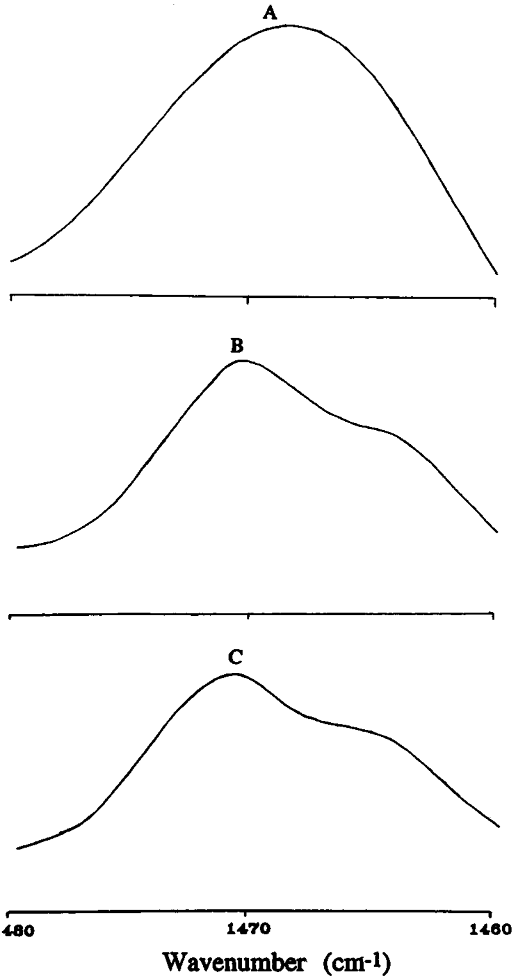

FIGURE 4: Infrared spectra in the methylene scissoring region between 1460 and 1480 cm^{-1}. Original spectrum for (A) NH_4^+ salt of NFA−CBS. The spectrum of the Ca^{2+} salt form of NFA−CBS was similar. Deconvolution of the spectrum in (A) did not reveal more than one band. Deconvoluted spectra for (B) NH_4^+ salt of HFA−CBS and (C) Ca^{2+} salt of HFA−CBS. The spectra were deconvolved using a break point of 0.3 in the Fourier domain.

temperature of the C16:0h−CBS HFA species was a few degrees higher than that of the C16:0−CBS NFA species in the presence of Ca^{2+} (Boggs et al., 1984).

The frequencies of the symmetric stretching vibration of the dry films of the HFA and NFA species were within 1 cm^{-1} of each other for both the NH_4^+ and Ca^{2+} salt forms, indicating similar conformational order of the hydrocarbon chains of both species. However, close examination of the CH_2 scissoring region from 1460 to 1480 cm^{-1} of dry films of the lipids showed significant differences in the behavior of the NFA and HFA species (Figure 4). After resolution enhancement, only a single major band located at 1468 cm^{-1} (Figure 4A) was observed for both the NH_4^+ and Ca^{2+} salt forms of the NFA species. This is typical of systems with the acyl chains packed in a hexagonal lattice (Casal & Mantsch, 1984) but has also been observed for short acyl chain species of phosphatidylcholine and of glycosyldiacylglycerol in a stable subgel phase in which the acyl chains are in a rigid crystalline structure but are orientationally disordered (Lewis & McElhaney, 1990; Lewis et al., 1990). However, for the NH_4^+ and Ca^{2+} salt forms of the HFA species, the peak observed in this region could be resolved into two bands located around 1471 and 1465 cm^{-1} (Figure 4B,C); these may arise from correlation field splitting (Lewis & McElhaney, 1993).

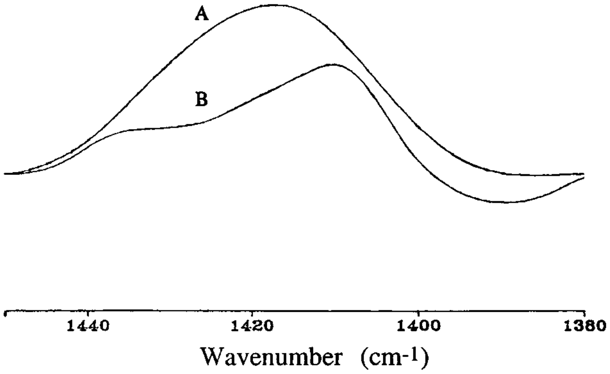

FIGURE 5: Original spectra of HFA−CBS in the methylene bending mode of (A) Ca^{2+} salt and (B) NH_4^+ salt. Spectra were smoothed with 11 data points.

Methylene scissoring band splitting has also been observed with other lipids and hydrocarbons in solid or crystalline phases (Bellamy, 1975), in subgel phases (Lewis & McElhaney, 1990; Lewis et al., 1990), or in the gel phase at high pressure (Auger et al., 1990; Wong et al., 1988) and has been attributed to lipid interchain interactions, with nonequivalent orientation. The two chains of the lipid may be arranged with nonparallel zigzag planes or the chains of one molecule may be perpendicular to the chains of neighboring molecules. Similar splitting has been detected using surface enhanced Raman spectroscopy to study monolayers of hydrocarbon chain compounds self-assembled onto Ag or Au substrates with highly ordered orthorhombic subcell acyl chain packing (Chumanov et al., 1995; Menikh & Bouraoui, 1996). The splitting observed here for HFA−CBS indicates that it has a higher degree of orientational order than the NFA species. Similar results were observed for hydrated samples of HFA−CBS (not shown) although the splitting of the scissoring band was not as strong as for dry films.

The band due to the bending mode of the α-CH adjacent to the carbonyl HFA−CBS was significantly affected by addition of Ca^{2+}. In the absence of Ca^{2+}, a weak band in the spectrum of the dry film at around 1408 cm^{-1} was observed (Figure 5b). On addition of Ca^{2+}, a single sharp band at 1418 cm^{-1} appeared (Figure 5A). Lewis and McElhaney (1990) observed a similar effect when the Lc subgel phase of diacylphosphatidylcholine formed. They attributed the appearance of a single sharp α-methylene band in the Lc phase to a conformational change which resulted in a reduction of the mobility of the α-methylene group. For the NH_4^+ and Ca^{2+} salt forms of the NFA species, there was no distinct band corresponding to the α-methylene, indicating greater mobility of this group in the NFA species (not shown). This difference in behavior of the α-CH, adjacent to the amide carbonyl of the HFA species, from that of the NFA species must be due to the presence of the α-hydroxyl group located on the same carbon of the fatty acid. We suggest that the sharpening and increase in frequency of the α-CH band in HFA−CBS are due to the formation of an extensive intermolecular hydrogen-bonding network involving the α-OH group as well as the amide N−H and C═O groups at the polar/apolar interfacial region in the presence of Ca^{2+}.

Hydrated samples of the NFA and HFA species had similar hydrocarbon chain conformational order as in dry films, but

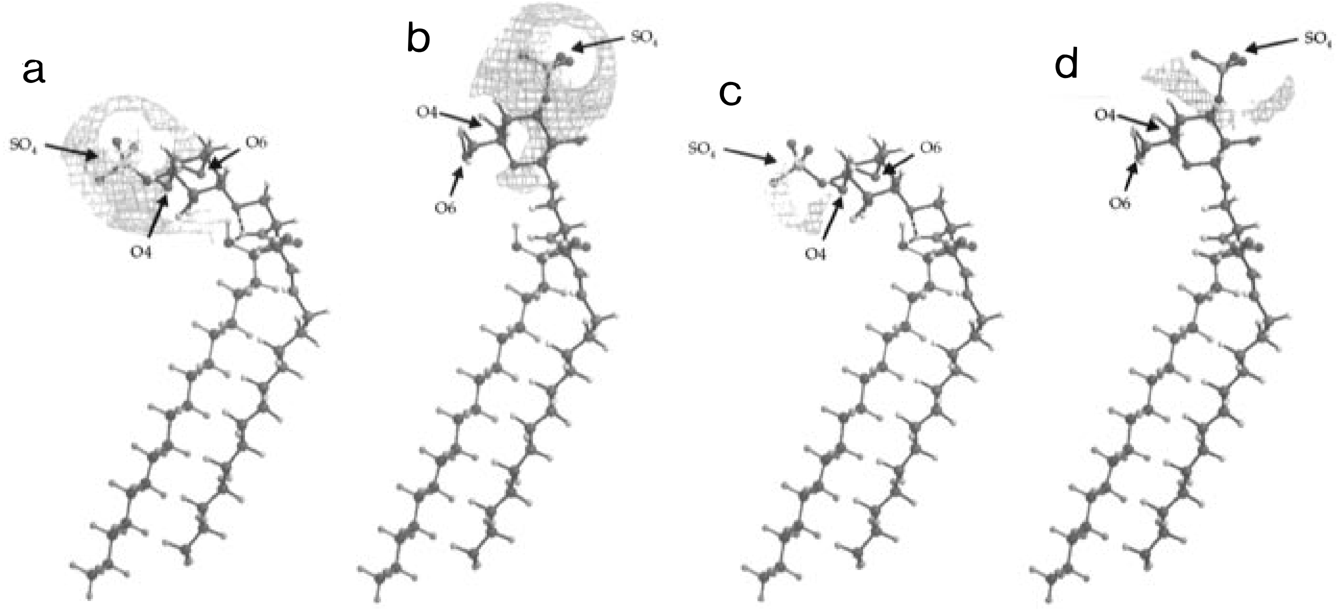

FIGURE 6: Ball and stick models of CBS superimposed with three-dimensional contours of interaction energies with Ca^{2+} and NH_4^+ probes according to calculations by the GRID program. Interaction of a Ca^{2+} probe with HFA−CBS in (a) the "bent shovel" conformation and (b) the extended conformation. The contour of the interaction energy at a level of −58 kcal/mol is shown in light blue. Contours for interaction of Ca^{2+} with NFA−CBS in these two conformations are similar. Interaction of an NH_4^+ probe with (c) HFA−CBS in the bent shovel conformation and (d) NFA−CBS in the extended conformation. The contour of the interaction energy of NH_4^+ is shown at a level of −19 kcal/mol. At a level of −16 kcal/mol the contour of NH_4^+ more closely resembled that of Ca^{2+} at −58 kcal/mol in (a) and (b). The bifurcated hydrogen bond of the amide hydrogen with the fatty acid hydroxyl group and the glycosidic oxygen, which occurs in the bent shovel conformation of HFA−CBS, has been indicated by dotted lines in (a) and (c). Carbons are shown in dark gray, hydrogens are in light gray, oxygens are in red, the nitrogen is in blue, and the sulfur is in yellow. C−C and C−H bonds of the acyl chain are shown in green and other C−C and C−H bonds are shown in dark blue. The CBS molecule is oriented according to the crystal structure of HFA−GalCer (Pascher & Sundell, 1977).

the bands due to the symmetric and antisymmetric stretching vibrations become broader, indicating an increase in motional freedom of the chains. Weak methylene scissoring band splitting still occurred for the Ca^{2+} salt form of the HFA species. However, the shift in the frequency of the α-CH bending mode of HFA−CBS on addition of Ca^{2+} observed for dry films did not occur in the presence of excess water (H_2O), suggesting that intermolecular hydrogen bonding of the fatty acid OH is weaker in the presence of water.

Molecular Modeling. Models of the HFA and NFA−CBS species based on the crystal structures of HFA−GalCer (Pascher & Sundell, 1977) and methyl α-D-galactopyranoside 3-sulfate (Polvorinos et al., 1994), with the galactose−ceramide linkage in the two minimum energy conformations calculated for GluCer (Nyholm & Pascher, 1993a,b), i.e., with the sugar either extended away from the bilayer and nearly parallel to the acyl chains or with the sugar bent over nearly perpendicular to the acyl chains, are shown in Figure 6. In the bent shovel conformation (Figure 6a,c) a bifurcated intramolecular hydrogen bond between the amide N−H and the fatty acid hydroxyl group and the glycosidic oxygen can occur as in the crystal structure of HFA−GalCer. Grid calculations using Ca^{2+} as a probe demonstrated strong binding of Ca^{2+} around the sulfate group and at the surface of the galactose, particularly in proximity to the axially oriented O4 hydroxyl group, as shown at an interaction energy contour of −58 kcal/mol in Figure 6a,b. The most favorable interaction points for Ca^{2+} with HFA−CBS had interaction energies in the range of −70 to −75 kcal/mol. In the bent shovel conformation, favorable interaction points are located between the galactose and the amide group of the ceramide. In a lipid bilayer, this region would be between the bilayer surface and the sulfated galactose. Similar results were obtained in calculations on the corresponding two conformations of the NFA species (not shown). Comparative calculations with an NH_4^+ probe revealed much weaker interactions (see Figure 6c,d) with minima in the range of −20 to −25 kcal/mol, although the spatial distribution of these regions was similar to that found for Ca^{2+}.

DISCUSSION

The α-hydroxyl group on the acyl chain of CBS increased the phase transition temperature of both the ammonium and Ca^{2+} salt forms as shown previously for other cations (Boggs et al., 1984, 1988). This suggests that the α-hydroxyl group stabilizes the gel phase or destabilizes the liquid crystalline phase. Analysis of the FT-IR spectra of CBS in a low temperature phase provided information on how the α-hydroxyl group affects interactions between the lipid molecules which stabilize this phase. Although some of the spectra were obtained on lipid films from which the excess water (D_2O) had been evaporated, a small amount of bound water was probably still present. Furthermore, in the presence of excess water, relatively similar differences in the behavior of the NFA and HFA species were obtained for all regions of the spectra, although the carbonyl groups were less hydrogen bonded than in dry films and there was increased accessibility of the N−H of the Ca^{2+} salt forms to water as indicated by H/D exchange.

The spectra of the amide I region of NFA and HFA species of CBS indicated the coexistence of at least two different instantaneous populations of both the HFA and NFA species in which the carbonyl was involved in hydrogen bonds of different strengths, as found earlier for both the *sn* 1 and *sn*

2 chains of phospholipids (Blume et al., 1988; Lewis & McElhaney, 1992) and for the acyl chain of glycosphingolipids (Lee et al., 1986; Mueller & Blume, 1993). This disagrees with the conclusion of Tupper et al. (1992) that two different populations with different degrees of hydrogen bonding found in a natural bovine brain mixture of CBS molecules arose from the HFA and NFA species. In phospholipids the carbonyl must be involved in hydrogen bonding with water because no other groups are present in the interfacial region to hydrogen bond with them. However, for sphingolipids, other hydrogen bond donating groups are present in the interfacial region, i.e., the N−H group and the sphingosine and fatty acid hydroxyl groups (the latter for the HFA species only), and hydroxyl groups from the sugar, if it is bent over in a shovel conformation as in the crystal structure of HFA−GalCer. Different degrees of hydrogen bonding of the carbonyl of CBS with some of these groups in neighboring lipid molecules, and/or with water, may account for the presence of several lipid populations.

Ammonium Salt Forms of CBS. For the ammonium salt form, the spectra of the amide I region indicated that the carbonyl of the NFA species is more involved in hydrogen bonding than that of the HFA species. The spectra of the amide II region indicated that the N−H of the NFA species is also involved in a linear hydrogen bond whereas that of the HFA species is involved in a bent hydrogen bond. These results suggest that the N−H of NFA−CBS is involved in an intermolecular hydrogen bond, probably with the carbonyl, as concluded from FT−IR spectra of a stable gel state of NFA−GalCer (Lee et al., 1986), while that of NH_4^+−HFA−CBS is involved in a bifurcated intramolecular hydrogen bond with the fatty acid hydroxyl group and the glycosidic oxygen as in the crystal structure of HFA−GalCer (Pascher & Sundell, 1977) and in a gel phase of HFA−GalCer (Lee et al., 1986). This may stabilize the bent shovel conformation of the sugar for HFA−CBS, shown in Figure 6a. Intramolecular hydrogen bonding of the N−H of HFA−CBS persisted in the presence of excess water.

In spite of the lack of involvement of the amide N−H group in intermolecular interactions and the weaker intermolecular interactions of the C═O group of the ammonium salt form of HFA−CBS, compared to the NFA species, the methylene scissoring band splitting for the HFA species indicates that the hydrocarbon chains of HFA−CBS have a higher degree of orientational order than those of the NFA species. This splitting is caused by interchain coupling of vibrational modes when acyl chain motion is damped. The lower packing order of the hydrocarbon chains of NFA−CBS reflects perturbation of the van der Waals interactions between the hydrocarbon chains and increase in the distance between them caused by the charged head group in the absence of the fatty acid hydroxyl group.

Vibrational stretching bands due to the OH groups of the fatty acid and sphingosine cannot be distinguished from those of the sugar; however, given the large number of sugar hydroxyls, the hydroxyl bands are probably dominated by those. The frequencies of bands arising from O−H stretching vibrations indicate that those of the NH_4^+−HFA species are more involved in hydrogen bonding than those of the NFA species. This could be due to greater hydration of the HFA species or to greater intermolecular hydrogen bonding with groups on neighboring lipid molecules. The higher transition temperature of the HFA species suggests that greater intermolecular hydrogen-bonding interactions between lipid head groups occur, either directly involving sugar OH groups of neighboring molecules or indirectly via hydrogen-bonded water bridges.

Thus even though the fatty acid hydroxyl is apparently involved in intramolecular hydrogen bonding and it weakens involvement of the amide region in intermolecular hydrogen bonding, it causes ordering of the hydrocarbon chains, suggesting that it causes closer or more highly organized packing of the lipid molecules. It also increases hydrogen bonding of hydroxyl groups of the glycolipid and decreases interaction of the sulfate with some group suggesting decreased accessibility of the sulfate to solvent. In addition to causing intramolecular hydrogen bonding of the N−H to the glycosidic oxygen, it may have these effects by hydrogen bonding with the 4-OH of a sugar group in a neighboring molecule as in the crystal of HFA−GalCer, thus further stabilizing the sugar in the bent shovel conformation in which other sugar oxygens can then be involved in numerous intermolecular interactions with other groups on neighboring lipid molecules. Thus FT-IR spectroscopy indicates that the ammonium salt form of the HFA species of CBS has a structure similar to that of the crystalline form of HFA−GalCer. For NFA−CBS on the other hand, the weaker hydrogen bonding of the hydroxyl groups, including the sugar hydroxyls, greater accessibility of the sulfate to solvent, and lack of intramolecular hydrogen bonding of the glycosidic oxygen with the N−H indicated by the present FT−IR results suggest the sugar may be in the extended conformation as shown in Figure 6d.

Effect of Ca^{2+}. Ca^{2+} increases the phase transition temperature of CBS relative to the ammonium salt form, but not to the extent observed for acidic phospholipids, and it does not increase the transition temperature of CBS as much as the monovalent cations Na^+ and K^+ (Boggs et al., 1984, 1988), suggesting that, despite its divalent charge, it does not cross-link two CBS molecules as it does acidic phospholipids. Rather, it probably increases the transition temperature by the same mechanism as monovalent cations, by shielding the negative charge of the sulfate. It has a higher affinity for sulfate than ammonium has, as indicated by the GRID calculations, and shields the charge better because of its divalency.

The infrared data reported in this study indicate that Ca^{2+} interacts with the CBS head group of both the NFA and HFA species and more specifically with the charged sulfate group. This is made clear from the increase in frequency of the O═S−O^- antisymmetric stretching band caused by Ca^{2+}, indicating it displaces some group from the sulfate, most likely water. An interaction of Ca^{2+} with the oxygen of the C−O−S group could also be detected from the FT-IR spectra.

The most important effect of Ca^{2+} was observed on HFA−CBS. In that case, it increased the degree of hydrogen bonding of the C═O group and decreased its mobility. Furthermore, it caused a rearrangement of the hydrogen-bonding network involving the N−H group of the HFA species, most likely a shift from a bifurcated intramolecular hydrogen bond with the fatty acid OH and the glycosidic oxygen to a linear intermolecular hydrogen bond with the C═O. Since the N−H is no longer involved in an intramolecular hydrogen bond with the glycosidic oxygen, the bent shovel conformation may no longer be stable and the sugar may prefer the extended conformation instead (Figure 6b), as in NFA−CBS. This is supported by the reduced hydrogen-bonding interactions of the hydroxyl groups in the HFA species in the presence of Ca^{2+} and the similar environment of the sulfate as in the Ca^{2+} salt form of the NFA species.

However, the hydrocarbon chains of the Ca^{2+} salt form of the HFA species still had a greater degree of orientational order than the NFA species, and the mobility of the α-CH group was reduced on Ca^{2+} binding. The fatty acid hydroxyl group must be the cause of the differences in orientational order of the hydrocarbon chains, reduced mobility of the α-CH group, and higher transition temperature of the HFA species compared to the NFA, although the behavior of the α-OH group cannot be determined from these FT-IR spectra as it cannot be distinguished from the other OH groups in the molecule. Since intramolecular hydrogen bonding of the α-OH group with the N−H group is apparently reduced for the Ca^{2+} salt form, the fatty acid hydroxyl group may hydrogen bond intermolecularly with the C═O group or sphingosine hydroxyl of neighboring molecules. Thus it could stabilize the gel phase of the Ca^{2+} form by participating directly in the intermolecular hydrogen-bonding network rather than indirectly as for the NH_4^+ form where it stabilizes the shovel conformation in which intermolecular hydrogen bonding of sugar oxygens with other groups of neighboring molecules can occur.

Ca^{2+} may cause this change in the hydrogen-bonding network and conformation of HFA−CBS by decreasing charge repulsion between CBS molecules better than ammonium ion, or it may have a more specific effect by chelating some hydroxyl groups of the sugar in addition to sulfate. The GRID calculations indicate that, in the bent shovel conformation of HFA−CBS, favorable Ca^{2+} binding sites are located between the galactose and the amide group and on that surface of the galactose which would face the bilayer surface. Binding of Ca^{2+} at these sites would most likely disrupt the intra- and intermolecular hydrogen-bonding network characteristic of the HFA species in the bent shovel conformation, i.e., hydrogen bonds involving the N−H and the galactose hydroxyls. On the other hand, binding of Ca^{2+} to HFA−CBS in the extended conformation would not interfere with packing of the head groups and would still permit participation of the interfacial region of the lipid in intermolecular hydrogen bonding. Thus these calculations suggest an explanation for the conclusion from the FT−IR results that Ca^{2+} induces a transition of HFA−CBS from the bent shovel to the extended conformation. Further experimental as well as theoretical work will be required to further define the regions of the CBS head group involved in Ca^{2+} binding and to establish the effects of Ca^{2+} on the dynamics and intermolecular interactions of the CBS head group. Energy minimization must be performed in order to predict the geometry of the most favorable complexes of Ca^{2+} with HFA− and NFA−CBS.

Although the intermolecular interactions of the interfacial region in the extended conformation are weakened in the presence of excess water, as indicated by increased frequency of the amide I bands, increased H/D exchange of the N−H of both species in the presence of Ca^{2+}, and abolishment of the Ca^{2+}-induced shift in frequency of the α-CH bending mode of the HFA species, they are not eliminated. These interactions could account for the significant effect of hydroxylation of the fatty acid of hydrated CBS on its gel to liquid crystalline phase transition temperature and the sensitivity of its phase behavior to the type of cation present (Boggs et al., 1984).

CONCLUSIONS

The results presented in this study indicate that the conformations of the NH_4^+ salt forms of HFA and NFA−CBS differ from each other, with the sugar of NH_4^+−HFA−CBS being in the bent shovel bilayer planar conformation stabilized by a bifurcated intramolecular hydrogen bond between the N−H and the glycosidic oxygen and the fatty acid hydroxyl, while the sugar of NH_4^+−NFA−CBS is in the extended bilayer perpendicular conformation in which an intermolecular hydrogen bond between the N−H and C═O and possibly other groups occurs. Ca^{2+} has little detectable effect on the NFA species but induces a conformational change of the HFA species to an extended conformation like that of the NFA species. The fatty acid hydroxyl group promotes intermolecular hydrogen-bonding interactions for both the NH_4^+ and Ca^{2+} salt forms of the HFA species. It does this by stabilizing the bent shovel conformation of the NH_4^+ salt form in which sugar hydroxyls can hydrogen bond with other groups in neighboring lipid molecules and by participating directly in these intermolecular interactions in the extended conformation of the Ca^{2+} salt form. These intermolecular interactions increase the orientational and packing order and gel to liquid crystalline phase transition temperature of the HFA species. In the case of the Ca^{2+} salt form where the fatty acid hydroxyl is believed to be directly involved in these intermolecular interactions, they also decrease the mobility of the α-CH. Direct involvement of the sulfate and the galactose oxygens in these interactions cannot be conclusively ascertained. Although these groups are interacting with some other groups, it cannot be determined whether they interact with water molecules, hydroxyl groups of neighboring lipids, or cations. However, the results are consistent with the models described above in which the sulfate of NH_4^+−HFA−CBS is less hydrated than that of the NFA species and in which the hydroxyl groups of the HFA species are more involved in intermolecular hydrogen bonding. They are consistent with further dehydration of the sulfate of both species due to interaction with Ca^{2+} and decreased intermolecular hydrogen bonding or decreased hydration of the galactose hydroxyl groups of Ca^{2+}−HFA−CBS compared to the NH_4^+ salt form. GRID calculations of the sites of interaction of Ca^{2+} with CBS indicate that Ca^{2+} would destabilize the bent shovel conformation since it interacts with the surface of the sulfated galactose which participates in intermolecular and intramolecular hydrogen bonding. The conformational change of the sugar head group induced by interaction of Ca^{2+} with the HFA species suggests a mechanism by which Ca^{2+} binding increases the accessibility to and interaction of the sulfated galactose with other ligands at the cell surface, although it does not explain why Ca^{2+}−HFA−CBS interacts more strongly with GalCer in an apposing bilayer than the NFA species.

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