Structure and Dynamics of Sialic Acid at the Surface of a Magnetically Oriented Membrane System†

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ABSTRACT: A structural analysis of a sialic acid containing glycolipid α-dodecyl-*N*-acetylneuraminic acid anchored to the surface of a phospholipid-based membrane-like fragment is presented. The analysis is based on measurement of dipolar interactions between ^{13}C–^{13}C and ^{13}C–^{1}H spin pairs in a magnetically oriented membrane phase and the interpretation of data using an order matrix formalism. Structural analysis in this medium allows an assessment of properties in an environment approximating the environment where similar molecules function as cell surface receptors. The results indicate an extended headgroup conformation with the carboxyl group of the sialic acid near the membrane interface. Anisotropic motion of the sialic acid headgroup occurs with the most restriction along an axis which is significantly out of the plane of the sialic acid ring suggesting an important role for the carboxylate group in membrane surface interaction. The effect of calcium on the headgroup orientation was also investigated, but the results showed no significant change in the preferred conformation, possibly due to a weak binding under the conditions of the experiment.

Sialic acid (*N*-acetylneuraminic acid) occurs frequently as a terminal residue of carbohydrate chains found on the glycolipids and glycoproteins of cell membranes (Wiegandt, 1985). In addition to the functionalities offered by neutral sugars, sialic acids have a carboxylate substituent at the anomeric carbon opening the possibility that they can contribute to the charge distribution at a membrane surface. With this enhanced functionality, sialic acid terminated carbohydrates are known to play major roles in phenomena such as regulation of cell growth, binding of specific proteins to membrane receptors, and adhesion of cells (Gennis, 1989; Philips et al., 1990; Waltz et al., 1990). A knowledge of the orientation, dynamics, and interactions of sialic acid moieties in these systems would obviously be useful in gaining a molecular level understanding of receptor function.

Nuclear magnetic resonance provides one approach to the study of the structure and dynamics of membrane-anchored systems. ^{2}H-NMR[1] and, more recently ^{14}N- and ^{15}N-NMR have provided a great deal of information on lipids and peptides in model membrane systems (Skarjune & Oldfield, 1982; Opella et al., 1987; Auger et al., 1990, Nicholson et al., 1991). Frequently, however, analysis relies on simplifying assumptions about the symmetry of motion. Detailed descriptions of anisotropic motions such as those which might be exhibited by carbohydrate headgroups require a great deal of information, and they are seldom attempted without data from a variety of measurements.

Measurement of dipolar interactions between several pairs of spin 1/2 nuclei (^{13}C–^{13}C, ^{1}H–^{13}C) within a single semirigid pyranose ring can contribute significantly to the necessary variety. The enhanced resolution of ^{13}C resonances obtained in oriented arrays as opposed to random dispersions of membrane fragments also helps in the measurement and assignment of sufficient numbers of interactions to pursue a more complete analysis. An approach based largely on ^{13}C-NMR data and employing a complete order matrix analysis to describe motional properties has been developed and applied to a number of dodecyl β-pyranoside derivatives (Sanders & Prestegard, 1991, 1992). These studies used uniformly ^{13}C-enriched glucose, which was readily available and easily incorporated into glycolipid analogs. Such an approach is in principle applicable to sialic acid, but ^{13}C-enriched sialic acids are not so easily obtained. Application, therefore, presents both a synthetic and spectroscopic challenge.

Sialic acid has been a target for spectroscopic study for some time, and both ^{2}H-NMR-based and ^{13}C-NMR-based studies have been conducted. Some time ago, ^{13}C-NMR spin-lattice relaxation data measured in solution showed that calcium binding to gangliosides occurs through the carboxyl group and the glycerol side chain of sialic acid (Jacques et al., 1977, 1980; Czarniecki & Thornton, 1977). These studies done at natural abundance and in solution, however, cannot directly address the nature of changes occurring in a membrane environment. It has been proposed that in a membrane environment calcium modulates the reorientation of glycolipid headgroups in order to induce membrane fusion (Hoekstra, 1985, 1986). Thus, interactions at a membrane surface are important.

Recently, a study on the effects of calcium on the dynamic behavior of a sialylglycerolipid in multilamellar dispersions by deuterium NMR was reported. Spin-relaxation data combined with quadrupolar splittings showed a small calcium dependence to the ring orientation (Frenske et al., 1991). But the difficulty in labeling the pyranose ring of the sialic acid moiety with deuterium anywhere other than the C_3 position limits the degree to which the details of structure and dynamics can be determined.

The limitations experienced with deuterium incorporation and use of natural abundance ^{13}C-NMR suggest that a synthesis of ^{13}C enriched sialic acid and direct measurement of dipolar interactions in a membrane phase might be pursued. Here, the synthesis and the NMR study of α-dodecylsialic

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[1] Abbreviations: NMR, nuclear magnetic resonance; DMPC, L-α-dimyristoylphosphatidylcholine; CHAPSO, 3-[(cholamidopropyl)dimethylamino]-2,2-dihydroxy-1-propanesulfonate; ADSA, α-dodecylsialic acid.

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acid (neuraminic acid) ^{13}C-enriched (99%) at C_1, C_2, and C_3 in a magnetically oriented membrane system is presented. Enrichment at these sites provides three possible ^{13}C–^{13}C interactions and two large one bond ^1H–^{13}C interactions at the C_3 carbon. These five interactions are in principle sufficient to allow a complete order matrix analysis. Moreover, the large chemical shift anisotropy of the C_1 carboxyl carbon can produce orientation-dependent shifts which provide confirmatory data on conformational and motional properties (Sanders & Prestegard, 1992).

The membrane system used consists of a mixture of L-α-dimyristoylphosphatidylcholine (DMPC) and 3-[(cholamidopropyl)dimethylamino]-2,2-dihydroxy-1-propanesulfonate (CHAPSO) at a molar ratio of 3:1. Under these conditions, the lipids are believed to form bilayer-like discs that retain many of the properties of the liquid crystalline (L_α) state of pure DMPC. ^{31}P-NMR studies have shown that the discs orient in a strong magnetic field with their normal perpendicular to the applied field. The homogeneity of this oriented phase allows dipolar couplings and chemical shift anisotropy to be measured and the average structure and motions of the sialic acid headgroup to be calculated.

MATERIALS AND METHODS

DMPC, CHAPSO, *N*-acetyl-β-D-mannosamine and *N*-acetylneuraminic acid aldolase (EC 4.1.3.3) were obtained from Sigma (St. Louis, MO). Pyruvic acid (sodium salt) ^{13}C-labeled at C_1, C_2, and C_3 (99%) was purchased from MSD-Isotopes (Montréal, Canada), and all other reagents were from Aldrich (Milwaukee, WI).

[1,2,3-^{13}C]Sialic Acid. The procedure described by Simon et al. (1988) was used to prepare sialic acid from *N*-acetylmannosamine and ^{13}C-labeled pyruvate. Because pyruvate becomes the more expensive reagent in the synthesis of labeled material, the procedure was modified as follows, to ensure a complete reaction of the pyruvate. A solution of 1.3 g (5.88 mmol) of *N*-acetylmannosamine, 70 mg (0.6 mmol) of labeled sodium pyruvate, 1 mg of bovine serum albumin, 2.5 mg of sodium azide, and 0.5 unit of *N*-acetylneuraminic acid aldolase in 5 mL of water was stirred gently at room temperature for 4 days (^1H-NMR at 500 MHz showed >95% conversion of pyruvate to product at this time). The reaction mixture was loaded directly on an anion-exchange AG1X-2 formate form column (5.5 mL of resin), and unreacted *N*-acetylmannosamine eluted with 80 mL of water (≈90% was recovered). A 0–2 N formic acid gradient (100 mL:100 mL) was then run, and the fractions containing the labeled sialic acid as detected by TLC (silica gel, 1-propanol/water 7:3) were pooled and lyophilized to give 158–180 mg (85%–93% yield on the basis of pyruvate). The purity was >98% as assessed by ^1H-NMR.

α-Dodecyl-N-acetyl-D-neuraminic Acid (^{13}C-Labeled). The sialic acid was peracetylated according to the procedure described by Myers (Myers et al., 1980), and a benzyl ester protecting group was added to the carboxylic acid. The latter group also favors the formation of the α-glycosidic linkages with a very high α/β ratio (Ratcliffe et al., 1990) and, hence, set the stage for subsequent reactions of the chloro derivative with alcoholic aglycones. The chlorination reaction was performed using the conditions described by Myers (Myers et al., 1980) to produce benzyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-chloro-2-deoxy-D-neuraminate (^{13}C-labeled). Reaction of the chloride (15 mg, 79 μmol) with dodecyl alcohol (17 mg, 92 μmol) was effected following the procedure of Osawa (Miyazawa et al., 1990). After separation of α and B anomers of the dodecylneuraminic acid on silica gel (acetone/benzene 2.5:8.5) and saponification to remove acetate groups, the α anomer was chromatographed on a Sephadex G-25 column and the fractions containing the sialylated glycolipid were lyophilized to yield 10 mg (a total yield of 20% from pyruvate). The expected product, α-dodecylsialic acid (ADSA), was identified through the chemical shift of the H3-eq proton at 2.62 ppm using well-documented empirical rules (Paulsen & Deesen 1984; Ito & Ogawa, 1987; Marase et al., 1989).

Preparation of NMR Samples. In order to observe dipolar couplings among protons and carbons near ^{13}C-labeled sites, NMR spectra were acquired in a field-ordered liquid crystal medium. A complete description of the oriented system (DMPC/CHAPSO) has been published elsehwere (Sanders & Prestegard, 1990). Briefly, a mixture of 6.5 mg of α-dodecylsialic acid (ADSA) (sodium salt), 34 mg of CHAPSO, and 112 mg of DMPC was mixed in 300 μL of phosphate buffer (50 mM/D_2O at pH 7.3) by a combination of centrifugation and sonication until a clear and homogeneous sample was obtained. This sample contained 35% (w/w) of lipid in buffer and a ratio of ADSA/DMPC/CHAPSO of 0.25:3:1.

NMR Spectroscopy. ^{13}C-NMR spectra were recorded on a Bruker AM-500 spectrometer at 125.67 MHz at 40 °C. WALTZ-16 was used to achieve heteronuclear decoupling of ^1H from ^{13}C during acquisition of ^{13}C–^{13}C coupled spectra, and MREV-8 (Webb & Zilm, 1989) was used for homonuclear decoupling of protons during acquisition of ^{13}C–^1H coupled spectra. A 1D-INADEQUATE experiment was also employed to filter out the strong background arising from the natural abundance of ^{13}C in the DMPC/CHAPSO system. All spectra were acquired without sample spinning and without a field frequency lock. Details of the experiments are given in the legends of the figures.

THEORY AND CALCULATIONS

The splittings observed in spectra of the above samples are the result of scalar couplings (when spins are directly bonded) and residual dipolar couplings that are not completely averaged when molecules tumble anisotropically. The separation of the scalar part and the analysis of the residual dipolar part have been described in detail elsewhere (Sanders & Prestegard, 1992), but basic features will be outlined here.

The dipolar interaction between two spin 1/2 nuclei, i and j, is related to the distance r separating them and to the angle θ between the **ij** vector and the magnetic field by the expression.

$$D_{ij} = \frac{-\gamma_i\gamma_j}{2\pi^2 r^3} S_{\text{bilayer}} \left\langle \frac{3\cos^2(\theta) - 1}{2} \right\rangle \qquad (1)$$

Here the brackets represent the average over all orientations sampled on a time scale short compared to the inverse dipolar splitting. Three distinct types of motions contribute to the partial averaging of the dipolar coulings: (1) the wobbling of the DMPC/CHAPSO discs, (2) the fast rotation along the glycolipid chain axis, and (3) the anisotropic motion of the headgroup about bonds near the membrane interface. The first two are lumped into the S_{bilayer} term, and are usually evaluated on the basis of ^2H and ^{31}P data taken from measurements on typical phospholipid groups at or near the bilayer interface (Sanders & Prestegrad, 1991). The latter is the focus of our study and is explicitly treated in the averaging represented by the brackets.

The angular averaging of dipolar couplings arising from internal motions can be expressed in terms of an order matrix (Saupe, 1968; Diehl & Khetrepal, 1969; Emsley, 1985). A

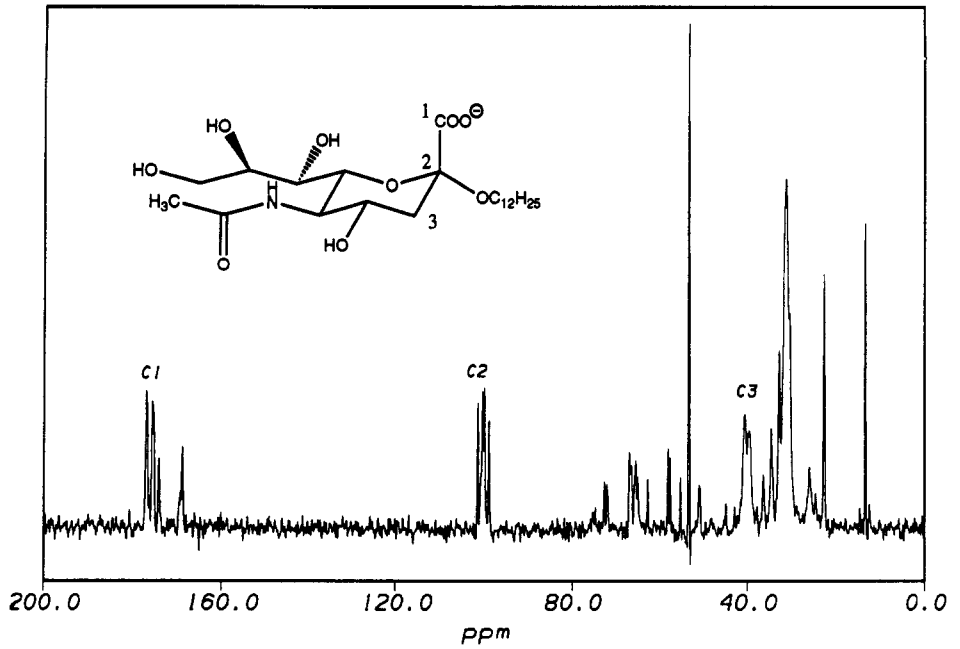

FIGURE 1: ^{1}H-decoupled inverse gated ^{13}C-NMR spectrum (125.67MHz) of α-dodecyl-*N*-acetylneuraminic acid ^{13}C-labeled at C_1, C_2, and C_3 at 99% dissolved in a DMPC/CHAPSO 3:1 at 35% (w/w) lipid content in phospate buffer at pH 7.5 recorded at 40 °C. A Gaussian multiplication with a line broadening factor of −1.0 Hz and a Gaussian broadening factor of 0.4 Hz were applied prior to Fourier transform. The spectrum was referenced on the methylene resonances at 32 ppm.

description of the average motional restriction of a molecular fragment in an arbitrary cartesian frame relative to a principal order frame uses nine order matrix elements. Because the order matrix is traceless ($S_{zz} = S_{xx} - S_{yy}$) and symmetric ($S_{ij} = S_{ji}$), only five of these elements are independent (Emsley, 1985). Each element (order parameter) is defined as

$$S_{ij} = \left\langle \frac{3\cos\rho_i\cos\rho_j - \delta_{ij}}{2} \right\rangle \quad (2)$$

where ρ_i defines the angle between the i molecular frame axis and the principal order frame axis (i.e., the bilayer normal). As usual, $\delta_{ij} = 1$ for $i = j$ and 0 otherwise. If the arbitrary frame of each fragment is fixed in a semirigid molecular structure such as a pyranose ring, the averaging of all vectors in this structure can be expressed in terms of a single set of order parameters and angles describing the orientation of an ij vector relative to a single set of fragment axes. Thus, the angular term of expression (eq 1) becomes

$$\left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle = \cos^2(\phi_x)(-S_{yy} - S_{zz}) + \cos^2(\phi_y)(S_{yy}) + \cos^2(\phi_z)(S_{zz}) + 2\cos(\phi_x)\cos(\phi_y)(S_{xy}) + 2\cos(\phi_x)\cos(\phi_z)(S_{xz}) + 2\cos(\phi_y)\cos(\phi_z)(S_{yz}) \quad (3)$$

where the ϕ_i are the angles between the i molecular frame axes and the internuclear vector.

The order matrix can be diagonalized to yield three eigenvalues as principal order parameters: namely, S_{XX}, S_{YY}, and S_{ZZ}, and corresponding eigenvectors which define the orientation of the order frame (X, Y, Z) in the molecular frame (x, y, z) axis system. The direction of the greatest order is chosen along the Z axis (i.e., along the bilayer normal).

Chemical shift anisotropy can be analyzed in a similar manner. The observed chemical shift is defined as the sum of isotropic and anisotropic terms:

$$\sigma_{obs} = \frac{1}{3}(\sigma_{11} + \sigma_{22} + \sigma_{33}) + \frac{2}{3}\sigma_{an} \quad (4)$$

The anisotropic part, σ_{an}, which contains geometrical information, can be expressed in terms of the same parameters we have presented above (Sanders & Prestegard 1992).

The extraction of initial order parameters from a set of dipolar couplings is accomplished using a FORTRAN program (ORDERTEN) (Sanders & Prestegard, 1991, 1992) that searches through all the possible values of S_{ij} for those that reproduce experimental dipolar splittings using eq 1. Chemical shift offsets calculated from the choice of S_{ij} and an input chemical shift tensor are reported for comparison with experimental data. Diagonalization routines and routines that convert eigenvectors to Euler angles relating the molecular frame to the order frame are also contained in this program.

RESULTS AND DISCUSSION

Measurement of Dipolar Couplings. The inverse gated ^{1}H-decoupled ^{13}C-NMR spectrum of α-dodecylneuraminic acid (ADSA) dissolved in DMPC/CHAPSO at 40 °C is shown in Figure 1. The three labeled carbons, C_1, C_2, and C_3 resonate at 175, 98, and 40 ppm, respectively. Three ^{13}C–^{13}C coupling constants can be measured directly from this spectrum. Both C_1 and C_2 resonances are doublets of doublets showing splittings, of 181Hz and 35Hz for C_1 and 181Hz and 100Hz for C_2. The C_3 resonance is poorly resolved due to incomplete removal of ^{1}H coupling but this contains only redundant ^{13}C–^{13}C coupling information.

Figure 2 shows a 1D-INADEQUATE ^{13}C-NMR spectrum with ^{1}H-heterodecoupling during evolution using WALTZ-16 and ^{1}H-homodecoupling during acquisition using MREV-8. The latter sequence removes the strong ^{1}H–^{1}H dipolar couplings and reduces both the dipolar and scalar coupling of ^{13}C–^{1}H pairs by a theoretical factor of 0.54 (Webb & Zilm, 1989). Large ^{13}C–^{1}H dipolar splittings could arise from both directly bonded axial and equatorial protons on C_3. Only a single splitting (2200 Hz after correction for the effects of MREV-8) is observed, which suggests that the second coupling is smaller or equal to the 150Hz line width. The results of all coupling measurements are summarized in Table I.

Determination of the Sign of the Dipolar Couplings. According to eq 1, dipolar couplings can be either positive or

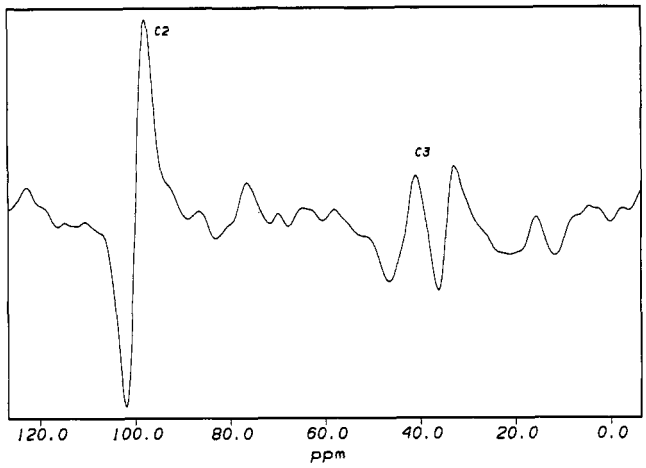

FIGURE 2: ^{13}C 1D-INADEQUATE (125.67 MHz) on the sample described in the legend of Figure 1 with ^{1}H-broadband decoupling using WALTZ-16 during evolution and ^{1}H-homodecoupling using MREV-8. The delay required for the generation of the double-quantum coherence was optimized for a coupling of 125 Hz.

Table I: Dipolar splittings and CSA for the Carboxyl Group for ADSA Enriched in ^{13}C at C1, C2, and C3 in DMPC/CHAPSO 35% (w/w) at 40 °C at pH 7.5

	observed (Hz)	predicted (Hz)
C_1–C_2	−221 ± 20	−224 ± 20
C_2–C_3	−140 ± 20	−140 ± 20
C_1–C_3	−35 ± 20	−36 ± 20
C_3–H_{3a}	−2200 ● 300	−2593 ± 300
C_3–H_{3e}	150 ± 150	194 ± 150
δ_{COO}–CSA	5 ppm	5.5 to −1.0 ppm[a]

[a]Value depends on the rotameric state of the carboxyl group.

negative depending on the range of angles sampled during motional averaging. Knowing the sign of the coupling is of significant value in working back from measured couplings to order parameters in either molecular or principal order frames. Signs are not apparent from a single spectrum such as that show in Figure 1. However, recognizing that dipolar contributions scale with overall order, while scalar couplings can often be assumed to be fixed and of known sign, one can use any systematic variation in order to separate the dipolar part and determine the sign.

It has been shown (Sanders & Prestegard, 1992; unpublished results) that the DMPC/CHAPSO 3:1 system at 20% total lipid content undergoes a gradual conversion to isotropic behavior ($S_{bilayer} = 0$) as temperatures are lowered to 20 °C. This conversion is easily monitored by observing the lines for the two carbonyl groups of DMPC. Two well-resolved resonances are present at 40 °C in a 20% by weight lipid sample, because one ester carbonyl of the phospholipid is oriented on average at a significantly different angle than the other ester carbonyl (Hauser et al., 1981). At 20 °C a single line at the isotropic ^{13}C shift of an ester carbonyl is observed as the initially oriented discs adopt more isotropic properties. Thus, diluting the sample with enough water to lower the total lipid content to 20% and recording the ^{13}C-NMR spectrum at several temperatures provides a way to reduce $S_{bilayer}$ gradually. If couplings observed from the sialic acid carbons collapse to zero and then reemerge as pure scalar coupling, the dipolar and scalar contributions must have been of opposite sign (dipolar negative). If the couplings simply converge to the scalar value the dipolar and scalar couplings must have been of the same sign (dipolar positive).

Figure 3 shows ^{13}C-NMR spectra of ADSA in a DMPC/CHAPSO 3:1 as a function of temperature. The C_2 resonance

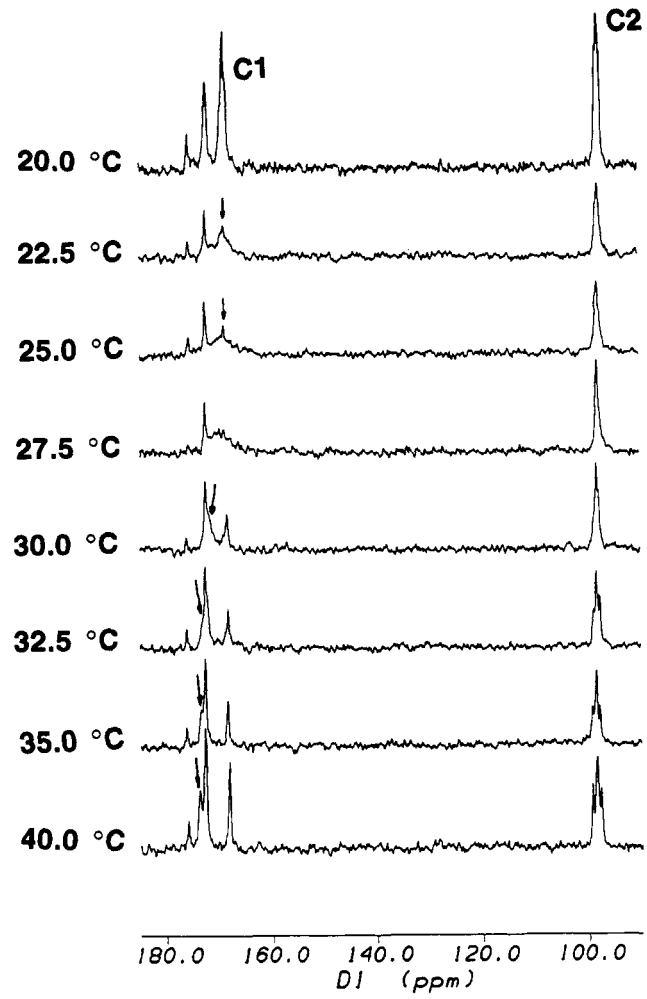

FIGURE 3: ^{1}H-decoupled inverse gated ^{13}C-NMR (125.67 MHz) spectra of the sample described in the legend of Figure 1 except that it was diluted with D_2O to bring the lipid content to 20% (w/w) and recorded at variable temperature. Note that the C2 resonance at 100 ppm, a doublet of a doublet, collapses to a singlet at 27.5 °C and changes to a doublet of a doublet with a splitting of about 40 Hz.

at 98 ppm is most easily observed. It shows a single peak at 27.5 °C resulting from the cancellation of the scalar couplings ($^{1}J_{C1,C2}$ and $^{1}J_{C2,C3}$) by their counterpart dipolar couplings ($D_{C1,C2}$ and $D_{C2,C3}$). Hence, $D_{C1,C2}$ and $D_{C2,C3}$ are negative. It is also clear from Figure 3 that the carbonyl resonances of the C_1 sialic acid carbon shifts up field by approximately 5 ppm in this process. As with the more pronounced shift of the DMPC sn2 carbonyl, it most likely reflects orientational preferences.

Analysis of Dipolar Coupling. Analysis of the above couplings in terms of an order tensor requires definition of a molecular frame in terms of atom positions in a rigid sialic acid ring structure. The crystallographic structure of *N*-acetylneuraminic acid methyl ester reported by O'Connell (O'Connell, 1973) was used for this purpose. It is assumed that only the low energy conformer with the carboxylate in the axial position is significantly populated. The *z* axis was defined as passing through the C_2 (anomeric) and C_5 carbons. The *x* axis was chosen perpendicular to *z* and bisecting the C_3–C_4 bond. The *y* axis is perpendicular to both the *x* and *z* axes. Vectors between coupled pairs of atoms in this coordinate system were used as input to the program ORDERTEN, and a search for a set of order parameters that fit the data was conducted. The resulting order matrices were then diagonalized to yield the three principal axis order parameters S_{XX}, S_{YY}, and S_{ZZ} (−0.12, −0.24, 0.37). These

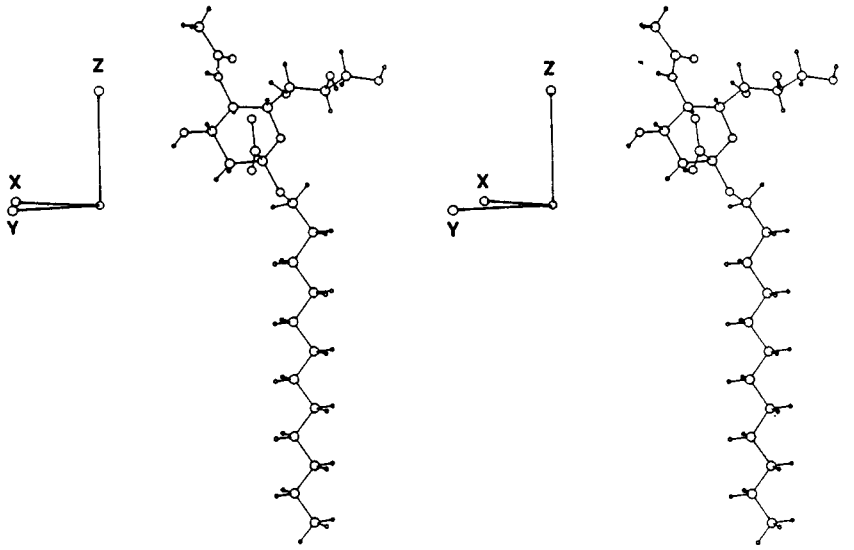

FIGURE 4: Stereoview of the structure of α-dodecyl-*N*-acetylneuraminic acid calculated with the order matrix approach. The *Z* axis that defines the bilayer normal is along the lipid chain axis. This structure illustrated here represents the average of 22 allowed solutions which were consistent with the NMR data. The orientation of the side chains is as described by the crystal structure (see text).

Table II: Order Parameters for ADON in DMPC/CHAPSO 35% (w/w) at 40 °C Calculated from ORDERTEN

		direction cosines		
S_{ij}		x	y	z
S_{XX}	−0.12 ± 0.03	0.635	0.477	−0.608
S_{YY}	−0.24 ± 0.06	−0.634	0.772	−0.057
S_{ZZ}	0.37 ± 0.06	0.442	0.421	0.792

describe the degree of order for each axis of the order frame. The orientations of the order frame axes in the molecular frame are given for the best solution through the direction cosines of Table II.

The parameters in Table II cannot be determined with absolute precision, especially with the limited amount of data available from just three labeled sites. Variations in order parameters among 22 solutions fitting all measured couplings within 1/2 a line width are shown as error limits in the table. Variations in these parameters are correlated to some extent. We attempt to assess the degree of this correlation in Figure 5. Here, the Euler angles α and γ relating the molecular frame to the order frame are plotted versus β. The spread of β and α angles is more pronounced than for the γ angle (50° vs 25°). However, all three angles are reasonably well defined. The asymmetry of motion is described by the parameter η, defined as $(S_{XX} - S_{YY})/S_{ZZ}$. η is plotted vs S_{ZZ} in Figure 6. The plot shows a reasonably good grouping of the allowed solutions.

The principal order parameter of 0.37 is comparable to that observed for dodecyl β-glucoside suggesting that motions of both rings are restricted to a similar degree. An η of 0.4 shows a reasonable degree of asymmetry with motion about the *Y* axis (approximately perpendicular to the ring) being more restricted.

Chemical shift anisotropy predictions based on the above order tensor elements and orientational factors add confidence to our analysis. Predictions require values for the shift tensor elements and some assumptions about the orientation of the carboxyl group on the sialic acid ring. We used $\sigma_{11} = -110.5$ ppm, $\sigma_{22} = -61.7$ ppm, and $\sigma_{33} = 21.6$ ppm (Pines et al., 1974) for the shift tensor elements. Chemical shifts of 5.5 to −1.0 are then predicted depending on the rotameric state of the carboxyl group. Hence, reasonable agreement with observation can be achieved.

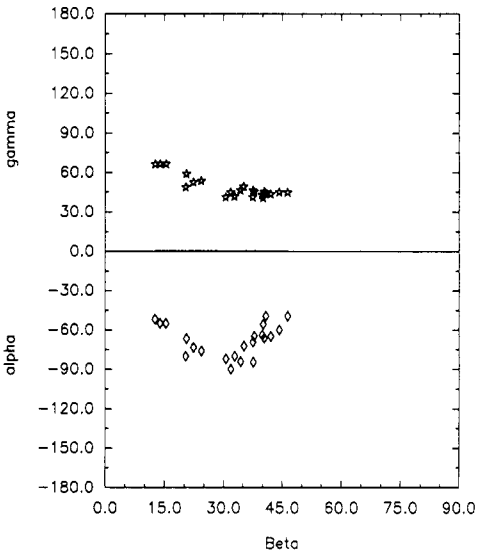

FIGURE 5: Euler angles for the 22 solutions describing the order frame in terms of the molecular axis frame coordinates. θ represents the angle between the *z* molecular axis and the *Z* order frame axis; φ describes a rotation.

Even though we have obtained no direct information about the conformation of the alkyl chain and the glycosidic torsion angles joining it to the sialic acid, knowledge of the orientation of the sialic acid ring frame relative to the bilayer normal, and an assumption that the alkyl chain lies along the normal can be used to generate a model for the average structure of α-dodecyl-*N*-acetylneuraminic acid. Thus, the structure of ADSA in a membrane-oriented system is shown in Figure 4.

There is also no direct information about the conformation of the glycerol side chain or the acetamido group. The ring orientation, however, suggests that these groups might be extended toward the aqueous media where they can interact with the solvent and not with the membrane interface. In the figure these groups are oriented as in the crystal structure but do extend naturally away from the apparent membrane interface.

The relatively large assymetry of motion and greater restriction about the *Y* axis suggest that interaction between the carboxyl group, which lies near this axis, and the membrane

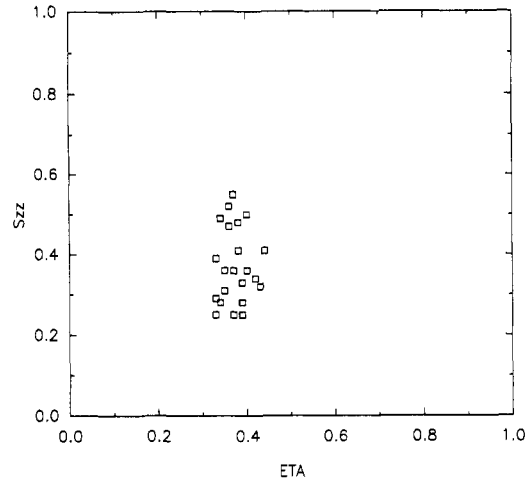

FIGURE 6: Plot of the asymmetry parameter (η) vs the principal order parameter (S_{ZZ}) for the 22 allowed solutions.

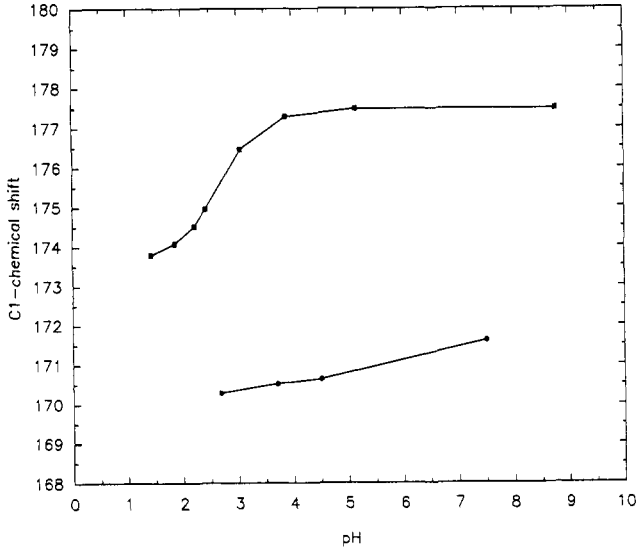

FIGURE 7: Influence of the pH on the chemical shift of the carboxyl group of free sialic acid (■) in D_2O and of ADSA (●) in a DMPC/CHAPSO 3:1 system 20% (w/w) lipid content at 22 °C.

interface may be particularly strong. A membrane interface constituted of DMPC and CHAPSO offers a wide array of hydrogen bond acceptor groups (phosphate groups, sulfonic groups, and ester carbonyls), but few hydrogen bond donor groups. In such an environment, preferred interactions may well involve a protonated carboxyl group as opposed to a fully ionized one. This hypothesis is supported by two pieces of experimental evidence: the effect of pH on the chemical shift of the carboxyl group and the very weak binding of Ca^{2+}.

The effect of pH on the chemical shift of the carboxyl group of free sialic acid is quite pronounced. There is an abrupt shift of about 3.5 ppm downfield as pH is lowered from 4.0 to 1.8. In contrast, the chemical shift of the carbonyl of ADSA dissolved in a DMPC/CHAPSO 3:1 system with 20% (w/w) lipid content (in order to achieve isotropic conditions) varies little (ca. 1.3 ppm) in the pH range 2.4–7.5. The relevant data are shown in Figure 7. Since changes in chemical shift on deprotonation should be reasonably independent of the local carboxyl group environment, and we know there is little change in ring orientation, it seems most reasonable that the small shift reflects resistance to a change in the level of protonation. Hence the apparent pK_a must be shifted from the solution value of 2.7. Such shifts for carboxyl group pK_as in membrane environments are well documented (Tsui et al., 1986). In all cases they are to higher values. The small shift supports a picture in which the group is totally protonated at pH 2.5 and deprotonated to less than 30% at pH 7. Either a low dielectric environment or stabilization of a protonated carboxyl through hydrogen bonds supports these effects.

Also, concentrations of Ca^{2+} up to 37 mM, which corresponds to a molar ratio of 1:1 sialic acid/Ca^{2+}, did not affect the orientation in a detectable way, nor did the carbonyl chemical shift change appreciably. This does not absolutely exclude the possibility of Ca^{2+} binding. But it seems unlikely that a divalent ion could substitute for a directly bonded proton without changing either the electron distribution (hence chemical shift) or the orientation, (hence dipolar coupling and CSA effects). ^{2}H-NMR as well as relaxation studies have shown that Ca^{2+} does affect the reorientation of 1,2-di-*O*-tetradecyl-3-*O*-α-D-sialyl-*sn*-glycerol in DMPC bilayer (Fenske et al., 1991). The differences in observation may be connected with the difference in the structure of our molecule and the diacylglycerol adduct. However, we also note that the changes observed by Frenske et al. are rather small (11% of observed ^{2}H splitting). Their observation may in fact be consistent with our conclusion that Ca^{2+}-induced changes in orientation are small.

CONCLUSION

The structure presented in this study resulted from consideration of five NMR dipolar measurements and a single CSAinduced shift. Five independent measurements is the minimum number required to define both average orientation and amplitude to potentially anisotropic motions using the order matrix approach. Nevertheless, a reasonably well-defined structure arises in which the sialic acid ring is extended into the aqueous phase with the carboxyl group remaining close to the membrane surface. The preferred orientation of ADSA, the pH studies, and the Ca^{2+} studies suggest that the headgroup may interact significantly with the membrane interface via a carboxyl group interaction with a hydrogen bond acceptor at the membrane surface.

ADDED IN PROOF

Phosphate buffer was present in the above studies. This limits the activity of free Ca^{2+} and may prohibit accurate assessment of binding to sialic acid.

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