Articles

Membrane Thickness and Molecular Ordering in *Acholeplasma laidlawii* Strain A Studied by ^{2}H NMR Spectroscopy†

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Received July 1, 1994; Revised Manuscript Received August 25, 1994⊗

ABSTRACT: Since *Acholeplasma laidlawii* can be restricted to incorporating fatty acids from the growth medium into its membrane lipids, it is possible to study the effects of the length of the acyl chains on the properties of the membrane of the organism. *A. laidlawii* strain A-EF22 was grown with mixtures of one perdeuterated saturated fatty acid and one monounsaturated fatty acid. The average length ($\langle C_n \rangle$) of the acyl chains in the membrane lipids varied from 14.6 to 19.9, and the degree of unsaturation ranged from 21 to 79 mol %. ^{2}H nuclear magnetic resonace (NMR) spectra were recorded on whole cells, on intact membranes, and on lipids extracted from these membranes. It was found that the NMR spectra for all three cases were very similar, yielding deuterium quadrupolar splittings typical for the lamellar liquid-crystalline phase (L_α) found in model membrane systems. The use of a perdeuterated acyl chain as a reporter molecule allowed for the calculation of order parameters averaged over the entire system. These measurements yielded a wide range of average order parameters varying from 0.136 to 0.186 for the membranes and from 0.137 to 0.181 for the extracted lipids. From the order parameters the average acyl chain length can be calculated, which is related to the average membrane thickness. This value ranged from 23.2 to 30.6 Å. When either the order or the membrane thickness of the intact membranes was compared to that of the extracted lipids, only slight or even undetectable differences were found. This implies that the proteins associated with the membranes do not have any large effect on the overall packing of the membrane lipids, even though the membrane thickness varied by approximately 8 Å over the series studied. A decrease in the ordering of the acyl chains was observed when the length of the acyl chains incorporated from the growth medium was increased in either the membranes or the extracted lipids. This decrease correlated with the decrease in the fraction of monoglucosyldiacylglycerol (MGlcDAG) found in the membrane. Since both the average order and the membrane thickness varied, it is proposed that by changing the mole fraction of MGlcDAG the organism regulates either the membrane curvature energy or the permeability, both of which are related to lipid packing in the bilayer.

There has been much progress in understanding the physical properties of membranes and how different factors affect these properties. Much of this work has involved the use of model membrane systems. It therefore is of interest to study how these ideas apply to biological membranes. The mycoplasma *Acholeplasma laidlawii* is an attractive system for such studies since it readily incorporates exogenous fatty acids into the acyl chains of its membrane lipids. Furthermore, since the organism cannot synthesize essential unsaturated fatty acids, and since the synthesis of saturated fatty acids can be nearly inhibited, the makeup of the acyl chains is largely under experimental control. In this work, these advantages are used to biosynthetically incorporate perdeuterated fatty acids into the membrane in order to carry out deuterium nuclear magnetic resonance spectroscopy (^{2}H NMR).[1]

Deuterium NMR has emerged as a powerful tool in characterizing the physical properties of lipids and membranes (Bloom, 1988; Davis, 1983; Lindblom & Rilfors, 1992; Seelig & Seelig, 1980; Thurmond & Lindblom, 1994). It provides information on the molecular level about both orientational order and dynamics. These data yield insight

† This work was supported by the Swedish Natural Science Research Council and the Knut and Alice Wallenberg Foundation. R.L.T. received financial support from the Swedish Institute during this work.

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: ^{2}H NMR, deuterium nuclear magnetic resonance spectroscopy; S_{CD}, carbon−deuterium bond order parameter for an individual segment of the acyl chain; $\langle S \rangle$, carbon−deuterium bond order parameter averaged over the acyl chain; $\langle L \rangle$, average acyl chain length projected onto the bilayer normal; $\langle C_n \rangle$, average number of carbons in the acyl chain; L_α, lamellar liquid-crystalline phase; T_m, chain-melting phase transition temperature; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MGlcDAG, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; DGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; MAMGlcDAG, 1,2-diacyl-3-*O*-(6-*O*-acyl-α-D-glucopyranosyl)-*sn*-glycerol; PG, phosphatidylglycerol; DAG, diacylglycerol; fatty acids are indicated by *x*:*y*, where *x* is the number of carbons and *y* is the number of cis double bonds; the addition of the notation -d_z indicates that the fatty acid contains *z* deuterium atoms.

0006-2960/94/0433-13178$04.50/0

into the structure and packing of lipids in biomembranes. Furthermore, using simple statistical models (De Young & Dill, 1988; Ipsen et al., 1990; Salmon et al., 1987; Schindler & Seelig, 1975; Thurmond et al., 1991, 1993), estimates of physical properties such as the effective acyl chain length and average surface area per molecule can be made.

Many advances have been made in the understanding of which membrane properties are of importance by studying the function of membrane proteins reconstituted into controlled lipid environments (cf. Devaux & Seigneuet, 1985; Keller et al., 1993; Newton, 1993). These studies have implicated membrane physical properties such as thickness, surface charge, molecular dynamics, lipid packing, and curvature stress as being important for membrane protein function. It is crucial, however, to understand whether these properties have the same influence in living organisms. One way to do this is to force an organism to grow under different conditions and determine how it changes its membrane composition and structure. For many eukaryotic as well as prokaryotic organisms, this can mainly be accomplished by growing the cells at different temperatures or in media with various salt concentrations. However, some microorganisms offer the possibility to change the above-mentioned quantities at will by using mutants in the lipid synthesis or by taking advantage of their limited capacity of lipid synthesis. Examples of such microorganisms are *A. laidlawii* (McElhaney, 1992; Rilfors et al., 1993), *Escherichia coli* (Raetz, 1986; Rietveld et al., 1993), *Clostridium butyricum* (Goldfine et al., 1987), and *Saccharomyces cerevisiae* (Lands & Davis, 1984).

One important factor for protein function may be the thickness of the membrane [Baldwin & Hubbell, 1985; Carruthers & Melchior, 1988; George et al., 1989; references in Rilfors (1985)]. One can imagine that it is very important for the hydrophobic thickness of both the protein and the membrane to match. Any deviation in either direction would be expected to be unfavorable due to hydrophobic effects and due to displacing the lipid acyl chains from their equilibrium lengths. Additionally, it is known that thinner bilayers are more permeable (McElhaney, 1992), which could have a large effect on cell viability.

In this work, the length of the lipid acyl chains has been varied by growing *A. laidlawii* strain A-EF22 with mixtures of one saturated fatty acid and one monounsaturated fatty acid, where the number of carbons in both cases varied from 14 to 20. Both intact membranes and their extracted lipids were studied. The data imply that the membrane proteins have only a very small, if any, effect on the overall packing of the acyl chains.

MATERIALS AND METHODS

Organism and Growth Media. *A. laidlawii* A-EF22 was grown in a bovine serum albumin/tryptose medium, in which the tryptose had been prepared with an improved lipid-depletion procedure (Wieslander et al., 1994). The medium was supplemented with one perdeuterated, saturated fatty acid (SFA) and one unsaturated fatty acid (UFA), at a total concentration of 150 μM and usually in the proportions 120:30 (Table 1). The deuterated acids were tetradecanoic (14:0-d_{27}), hexadecanoic (16:0-d_{31}), octadecanoic (18:0-d_{35}), and eicosanoic (20:0-d_{39}), respectively. The unsaturated acids were *cis*-9-tetradecenoic (14:1c), *cis*-9-hexadecenoic (16:1c), *cis*-9-octadecenoic (18:1c), and *cis*-11-eicosenoic (20:1c), respectively (Larodan, Malmö, Sweden). The SFA and UFA in a fatty acid pair were of equal chain length, or the UFA was either two carbons longer or two carbons shorter (Table 1).

^{14}C-labeled fatty acids (Amersham International or New England Nuclear) were added to 1.0 μCi/L, and the extent of endogenous synthesis of saturated fatty acids was monitored by the addition of 20 μCi/L [^{3}H]acetate.

Growth and Harvest of Cells. Cultures were adapted to the different supplements by five consecutive transfers after growth at 37 °C for approximately 24 h. Cells that incorporated the shortest fatty acids into their membrane lipids could only grow for a limited number of generations at 37 °C; they swelled and eventually lysed as observed previously (Wieslander et al., 1994). For these fatty acid supplements, the adaptation procedure was performed at 30 ° C; however, the cells used for the NMR experiments were grown at 37 °C.

For the NMR experiments of the intact membranes or the extracted lipids, cell batches of 1.5−3.0 and 2.5−5.0 L were grown for 20 h, respectively. Cells were harvested by centrifugation at 5 °C, washed once in β-buffer, and frozen at −80 °C (for lipids) or lysed by stirring in deionized water at 37 °C for 60 min (for membranes). Membranes were collected by centrifugation at 5 °C and washed twice in diluted β-buffer (1:20), and the final wash was performed with buffer in deuterium-depleted water. Membrane pellets were transferred to NMR glass tubes by a spatula followed by low-speed centrifugation, and the tubes were stored on ice both before and between the NMR experiments. For certain batches washed, fresh cell pellets were transferred directly to NMR tubes for the studies on whole cells.

Extraction and Analysis of Lipids. Membrane lipids used in the NMR investigations were extracted from frozen cells by 2:1 (v/v) chloroform/methanol followed by methanol and purified from nonlipid contaminants by Sephadex chromatography (Eriksson et al., 1991). Lipids used for the analyses of the acyl chain and polar head group compositions were extracted from small cell batches according to Bligh and Dyer (1959). After separation by thin-layer chromatography, the polar lipids were quantified by liquid scintillation counting (Wieslander et al., 1994) or in an isotope imager. The acyl chain composition of the membrane lipids was determined by gas−liquid chromatography (GLC) after the acyl chains were converted to their methyl esters (Rilfors et al., 1978). The analysis was performed with a Carlo Erba Instruments Model HRGC 5300-HT apparatus connected to an integrator and equipped with a 15 m capillary, fused silica column coated with Supelcowax 10 (Supelco Inc., Bellefonte, PA). With the response factors and retention times obtained with standardized reference mixtures (Supelco), the molar fractions of individual SFA and UFA chains and the average acyl chain length, $\langle C_n \rangle$, in the lipid mixtures were calculated.

Preparation of NMR Samples. The extracted lipids were first dried to a film with a stream of N_2 in an 8 mm (outer diameter) glass tube and then dried further to constant weight in a vacuum ($\sim 10^{-3}$ Torr). To each sample was added 50% (w/w) deuterium-depleted water. The samples were first centrifuged at low speed and then flame-sealed. The samples were centrifuged again at low speed several times to mix them.

Measurement of ^2H NMR Spectra. The ^2H NMR spectra were acquired using a Bruker AMX2 500 spectrometer with a ^2H frequency of 76.77 MHz. The probe used was a selective high-power probe tuned to deuterium with an 8 mm horizontal solenoid coil (Model No. 500/8/X, Cryomagnetic Systems Inc., Indianapolis, IN). The temperature was controlled with a Eurotherm B-VT 2000 unit and was calibrated by the use of a standard curve based on standard parameter settings. A phase-cycled quadrupolar echo pulse sequence (Bloom et al., 1980; Davis et al., 1976) with a 4.9−8.0 μs $\pi/2$ pulse, a 50−60 μs pulse separation, a recycle time of 0.5−1.0 s, and a 3 μs dwell time was used to collect the data. Between 17 500 and 25 000 transients were recorded for the whole cell and intact membrane samples, whereas spectra for the extracted lipids consisted of between 2500 and 20 000 transients. The data were transferred via a local Ethernet in binary form with the use of FTP to a personal IRIS 4D/25 workstation. The data were then transformed to Felix format by the use of a home-written Fortran program Brutof. In this format, the free induction decays (FIDs) were fractionally left-shifted with the use of a home-written Fortran program FRLS. The FIDs were processed in FELIX (Hare Research Inc., Woodinwill, WA). Typically, the imported file sizes were reduced from 2048 points to 256 or 512 points and the FIDs were then left-shifted by the appropriate number of points. The FIDs were next zero-filled to 4096 points, and a line-broadening corresponding to 50 Hz in the frequency domain was applied. A 7 (or 11) points binomial smoothing was applied to the spectra. The spectra could then be de-Paked using the algorithm of Bloom et al. (Bloom et al., 1981; Sternin et al., 1983) to obtain the subspectra corresponding to the $\theta = 0°$ orientation of the bilayer normal with respect to the magnetic field.

A crude estimation of the amount of the gel phase was performed as follows. A simulated gel phase spectrum was obtained from a recorded spectrum consisting of the L_α and gel phases. The gel phase spectrum showed similarities to previously recorded gel phase spectra of, for example, dipalmitoylphosphatidylcholine (Barry et al., 1992). By subtraction of the corresponding gel phase spectrum of the appropriate intensity, an NMR spectrum of an L_α phase was obtained. From the areas of the different spectra, the amount of the gel phase was estimated.

Analysis of ^2H NMR Spectra. In the L_α phase, the ^2H NMR spectra represent axially symmetric motions, since the motions are averaged around the bilayer normal. In this case, the observed quadrupolar splittings, $\Delta\nu_Q$, are directly related to the C−^{2}H bond order parameter, S_{CD}, by the equation

$$\Delta\nu_Q = \frac{3}{2}\left(\frac{e^2qQ}{h}\right) S_{CD}\left(\frac{3\cos^2\theta - 1}{2}\right) \qquad (1)$$

where $e^2qQ/h = 170$ kHz and θ is the angle between the bilayer normal and the main magnetic field. In this equation, S_{CD} can be defined as

$$S_{CD} \equiv \langle P_2(\cos\beta)\rangle = \frac{1}{2}\langle 3\cos^2\beta - 1\rangle \qquad (2)$$

in which $\langle\cos^2\beta\rangle$ is a time-average, where β is the angle between the C−^{2}H bond direction and the bilayer normal, and P_2 is the second Legendre polynomial. On the basis of geometric considerations, the values of S_{CD} are assumed to be negative; however, for practical reasons we will always refer to the absolute value. Evaluation of S_{CD} can be made directly from the de-Paked subspectra where $\theta = 0°$. The assignments of individual carbon segments to a particular peak can be made by assuming that the splittings decrease from the α-carbon to the terminal methyl and that the area under each peak is proportional to the number of deuterons it represents. By doing this, the largest splitting is assigned to the α-carbon. This method has been shown to hold for phospholipids. However, the application to mixtures of lipids, and in particular glucolipids, clearly represents an assumption.

The value of S_{CD} yields information on the orientational order of the acyl chain. However, conformational information can be extracted through the use of geometric models (Schindler & Seelig, 1975). A diamond lattice model (Jansson et al., 1992; Salmon et al., 1987; Schindler & Seelig, 1975) can be used to relate the order parameter to the average acyl chain length projected onto the bilayer normal using the equation

$$\langle L\rangle = l_0\left[\left(\frac{n-m+1}{2}\right) + \sum_{i=m}^{n-1}\left|S_{CD}^{(i)}\right| + 3\left|S_{CD}^{(n)}\right|\right] \qquad (3)$$

Here, $l_0 = 1.25$ Å is the length of one carbon−carbon bond in the all-trans state, n is the number of carbons in the acyl chain, m is the number of the carbon segment where the hydrocarbon region is considered to begin ($m = 2$ for the *sn*-1 chain corresponding to the α-methylene group of the acyl chain), $S_{CD}^{(i)}$ is the C−^{2}H bond order parameter of position i in the acyl chain, and $S_{CD}^{(n)}$ the order parameter of the methyl segment.

RESULTS

Membrane Lipid Composition. The fatty acids supplemented to the growth medium were, in most cases, efficiently incorporated into the membrane lipids of *A. laidlawii.* With the exception of a few cell cultures grown with the shortest and the longest fatty acids, the supplemented acids constituted between 91 and 97 mol % of the lipid acyl chains. When the cells were grown with the fatty acid pair 14:0/14:1c, these acids constituted only 55 mol % (intact membranes) or 78 mol % (extracted lipids) of the acyl chains. In this case the cells elongated above all the UFA, and 16:1c, 18:1c, and 20:1c made up 28 mol % (intact membranes) or 19 mol % (extracted lipids) of the chains. Likewise, the acyl chain pair 14:0/16:1c in one experiment constituted 88 mol % (intact membranes) or 78 mol % (extracted lipids) of the acyl chains; 16:1c was elongated and 18:1c and 20:1c constituted 8−9 mol % of the chains. When *A. laidlawii* was grown with 30 μM 20:0 and 120 μM 20:1c, these acids made up 67 mol % (intact membranes) or 74 mol % (extracted lipids) of the acyl chains. In this case, the organism responded by synthesizing shorter SFA, and acyl chains between 13:0 and 18:0 made up the rest of the chains. However, when the organism was grown with 50 μM 20:0 and 100 μM 20:1c, these acids made up 95−97 mol % of the acyl chains. The observations of both the elongation of incorporated fatty acids and the *de novo* synthesis of short fatty acids are completely in line with recent results from an extensive investigation of the membrane lipid composition of *A. laidlawii* cells grown with fatty acids of various lengths (Wieslander et al., 1994).

Table 1: Lipid Composition for ^{2}H NMR Samples of Membranes Derived from *A. laidlawii* Grown at 37 °C with Different Fatty Acid Compositions

supplementation			lipid composition			
fatty acids[a]	μM/μM	⟨C_n⟩	% unsaturated chains	MGlcDAG (mol %)	DGlcDAG (mol %)	nonbilayer-forming lipids (mol %)
14:0-d_{27}/14:1c	75/75	14.7	47.4	48.7	5.3	53.3
14:0-d_{27}/16:1c	120/30	14.9	38.2	50.2	3.2	56.7
14:0-d_{27}/16:1c	120/30	15.7	73.5	57.0	5.7	61.6
16:0-d_{31}/16:1c	120/30	16.0	21.4	31.7	15.2	58.0
16:0-d_{31}/18:1c	120/30	17.1	54.4	32.9	21.4	57.4
18:0-d_{35}/16:1c	120/30	17.3	25.0	21.9	13.7	64.2
18:0-d_{35}/18:1c	120/30	17.9	27.0	21.5	18.1	58.5
20:0-d_{39}/20:1c	30/120	18.1	55.5	12.6	39.4	15.3
20:0-d_{39}/18:1c	120/30	18.7	46.5	12.0	46.3	15.4
20:0-d_{39}/20:1c	50/100	19.9	78.6	11.7	46.9	14.9

[a] The fatty acids are designated *x*:*y*, where *x* is the number of carbons and *y* is the number of cis double bonds present; the addition of the notation -d_z indicates *z* deuterium atoms.

Table 2: Lipid Composition for ^{2}H NMR Samples of Extracted Lipids Derived from *A. laidlawii* Grown at 37 °C with Different Fatty Acid Compositions

supplementation			lipid composition			
fatty acids[a]	μM/μM	⟨C_n⟩	% unsaturated chains	MGlcDAG (mol %)	DGlcDAG (mol %)	nonbilayer-forming lipids (mol %)
14:0-d_{27}/14:1c	75/75	15.0	52.6	46.5	1.3	50.8
14:0-d_{27}/16:1c	120/30	14.6	22.1	50.9	1.9	53.6
14:0-d_{27}/16:1c	120/30	16.0	66.2	56.2	2.6	60.8
16:0-d_{31}/16:1c	120/30	16.0	22.3	24.5	14.3	54.3
16:0-d_{31}/18:1c	120/30	16.8	40.6	25.3	20.3	56.8
18:0-d_{35}/16:1c	120/30	17.5	25.2	21.7	14.6	51.9
18:0-d_{35}/18:1c	120/30	17.9	29.6	19.2	18.3	60.4
20:0-d_{39}/20:1c	30/120	18.5	65.3	9.0	48.6	10.7
20:0-d_{39}/18:1c	120/30	18.8	48.3	10.6	41.6	17.1
20:0-d_{39}/20:1c	50/100	19.9	79.2	10.7	49.4	13.0

[a] The fatty acids are designated *x*:*y*, where *x* is the number of carbons and *y* is the number of cis double bonds present; the addition of the notation -d_z indicates *z* deuterium atoms.

The polar head group composition of the membrane lipids was adjusted in relation to the length and the degree of unsaturation of the acyl chains incorporated into the lipids (Tables 1 and 2). The ⟨C_n⟩ value of the acyl chains varied from 14.6 to 19.9, and the degree of unsaturation varied from 21 to 79 mol %. The fraction of 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDAG) decreased from about 50 to 55 mol % for the shortest chains and to about 10 mol % for the longest chains. There was a drastic drop in this lipid fraction when 16:0, instead of 14:0, was incorporated from the growth medium. The fraction of 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDAG) varied in the opposite direction compared to MGlcDAG; DGlcDAG constituted about 1−5 mol % for the shortest chains and it increased to about 48 mol % for the longest chains. This lipid fraction exhibited a pronounced increase when 20:0, instead of 18:0, was incorporated from the growth medium. Phosphatidylglycerol (PG), which is the major phospholipid in the membranes of *A. laidlawii* strain A when the ⟨C_n⟩ value of the acyl chains is ≥17, increased from 13 to about 30−34 mol % with increasing length of the acyl chains. It has been shown that *A. laidlawii* strain A is able to synthesize and accumulate at least three different lipids having the potential ability to form nonbilayer aggregates under biological conditions (Lindblom et al., 1993; Rilfors et al., 1993; Wieslander et al., 1994). These lipids are MGlcDAG, 1,2-diacyl-3-*O*-(6-*O*-acyl-α-D-glucopyranosyl)-*sn*-glycerol (MAMGlcDAG), and diacylglycerol (DAG). The total fraction of the nonbilayer-forming lipids varied between 53 and 64 mol % (intact membranes) and between 51 and 61 mol % (extracted lipids), with ⟨C_n⟩ less than 18. This fraction then fell drastically to about 11−17 mol % when ⟨C_n⟩ increased above 18 (Tables 1 and 2).

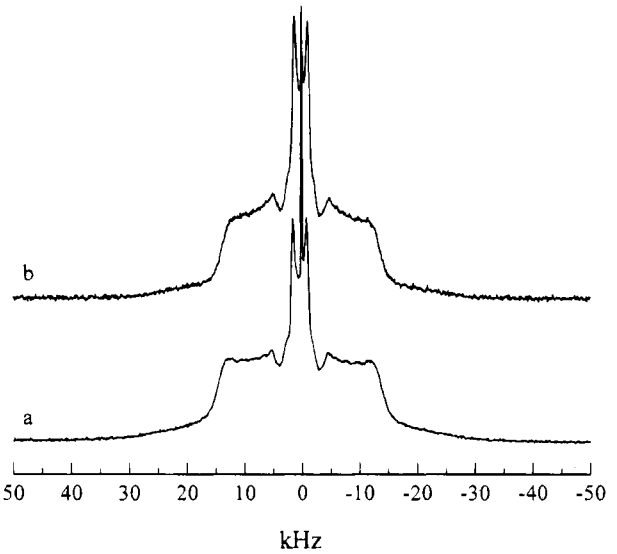

FIGURE 1: Deuterium NMR powder-type spectra at 37 °C recorded on whole cells (a) and intact membranes (b) prepared from *A. laidlawii* grown with a mixture of 120 μM 16:0-d_{31} and 30 μM 18:1c fatty acids.

^{2}H NMR Measurements. Figure 1 shows the ^{2}H NMR spectra at 37 °C for whole cells and intact membranes of *A. laidlawii* grown with 120 μM 16:0-d_{31} and 30 μM 18:1c. It

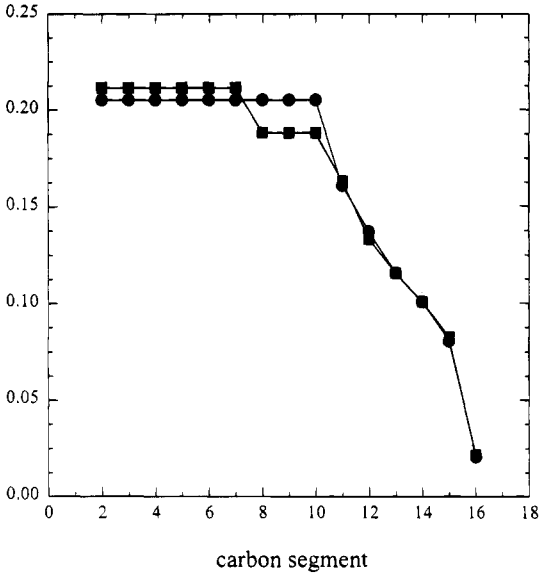

FIGURE 2: Carbon–deuterium bond order parameters, S_{CD}, extracted from de-Paked spectra derived from the powder spectra for whole cells (●) and intact membranes (■) in Figure 1.

appears that the spectra for either the whole cells or the intact membranes are identical except for the large narrow peak in the spectra of the whole cells. Since these differences were minimal, only the intact membranes will be considered. It is obvious from the shape of the spectra that the structure of the membranes is similar to that seen in the L_α phase in model membranes (Thurmond & Lindblom, 1994; A. E. Niemi, L. Rilfors, and G. Lindblom, manuscript in preparation). The maximum splittings indicate that even in the presence of membrane proteins the lipid bilayer is in a fluidlike state with intermediate order. Furthermore, the shape of the spectra indicates that there is a distribution of order along the acyl chain. All of these points are evident when the order parameters are extracted from the de-Paked spectra (Figure 2). Here, it is obvious that the order parameters, S_{CD}, of the whole cells and those of the intact membranes are virtually the same and that the degree of ordering is intermediate between the completely ordered all-trans state ($|S_{CD}| = 0.5$) and a completely disordered state ($|S_{CD}| = 0$). It should be noted that the ^2H label is only on the saturated acyl chain and will basically act as a reporter molecule of the order parameters averaged over the entire system. Similar approaches have been used before in studying intact membranes (Davis et al., 1980; Jarrell et al., 1982; Monck et al., 1992; Rance et al., 1980, 1982; Smith & Mantsch, 1979; Stockton et al., 1977) and have been found to be valid in model membrane systems (Lafleur et al., 1989; Thurmond & Lindblom, 1994).

In order to understand how acyl chains of different lengths affect the structure and properties of biomembranes, *A. laidlawii* was grown with a series of different fatty acid mixtures of various lengths (Table 1). Figure 3 shows a series of representative ^2H NMR spectra for intact membranes from *A. laidlawii*. The spectrum for the membranes grown with 14:0-d_{27}/16:1c is typical of that for pure L_α phase (Figure 3a). However, spectra from membranes grown on several of the other fatty acid combinations also exhibit a very broad component (Figure 3b,c). This component has a large splitting of ≈125 kHz, which is the theoretical splitting of the 0° orientation ($|S_{CD}| = 0.5$). Such spectra have been observed for many phospholipids when they are

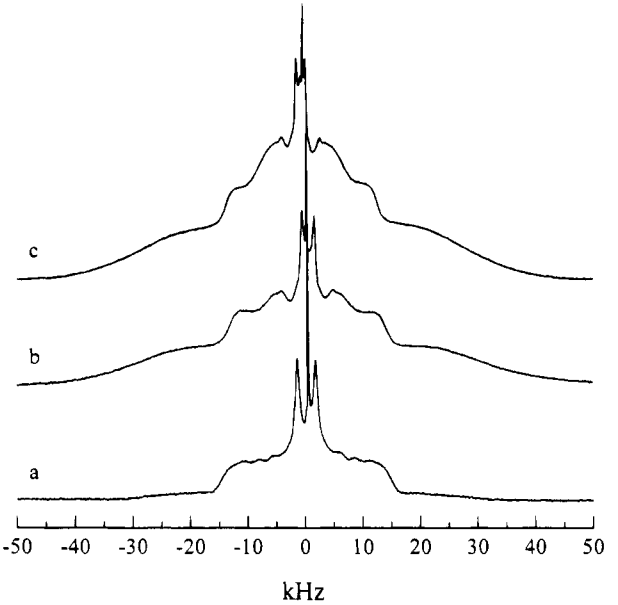

FIGURE 3: Representative ^2H NMR spectra of intact membranes from *A. laidlawii* grown with 14:0-d_{27}/16:1c (a), 16:0-d_{31}/16:1c (b), and 18:0-d_{35}/16:1c (c) at 37 °C.

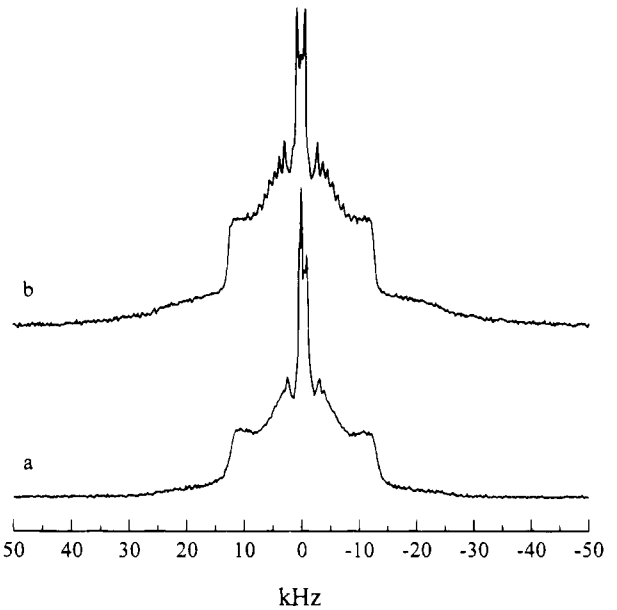

FIGURE 4: Comparison of the ^2H NMR spectra recorded at 37 °C from intact membranes (a) and an aqueous dispersion of the extracted lipids (b) from *A. laidlawii* grown with 20:0-d_{39}/20:1c (50 μM/100 μM).

below the temperature of the gel to liquid-crystalline phase transition (T_m) (Barry et al., 1991; D'Orazio et al., 1989; Davis, 1979) and have also been seen previously in *A. laidlawii* membranes (Smith et al., 1979). It was observed that the membranes from cells grown with 14:0-d_{27}/14:1c, 14:0-d_{27}/16:1c, and 20:0-d_{39}/20:1c contained no gel phase. Membranes prepared from cells grown with the other fatty acid combinations contained between 31% and 48% gel phase. However, even with a significant portion of gel phase present the cells still grow, as has been observed previously for *A. laidlawii* strain B (McElhaney, 1974).

Interestingly, the spectra for the extracted lipids are very similar to those of the intact membranes (Figure 4), which would imply that the membrane proteins do not play a major role in the organization and molecular ordering of the

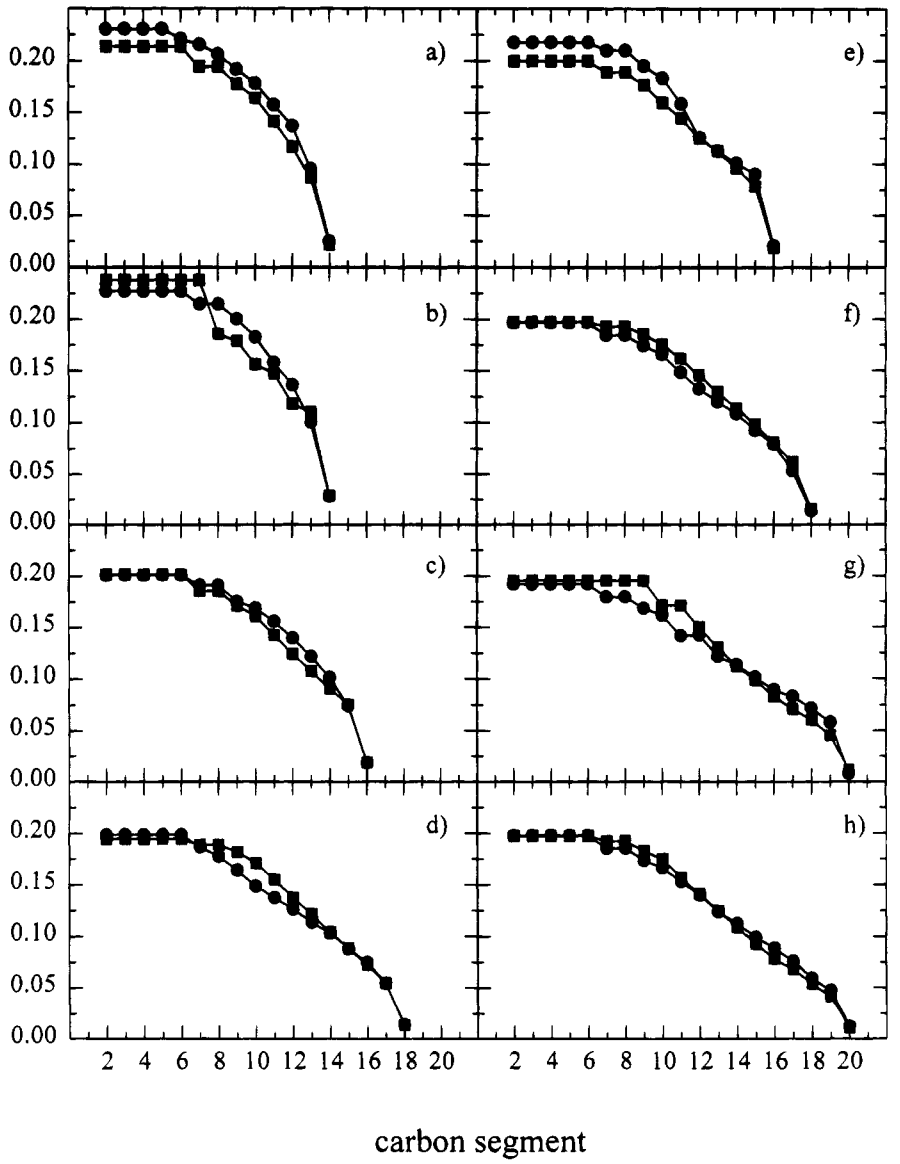

FIGURE 5: Effects of acyl chain length on the order parameter profiles of intact membranes (●) and extracted lipids (■) from *A. laidlawii* grown with different mixtures of fatty acids. The order parameters, S_{CD}, were extracted from the de-Paked ^{2}H NMR spectra recorded at 37 °C. The fatty acid mixtures shown are (a) 14:0-d_{27}/14:1c, (b) 14:0-d_{27}/16:1c, (c) 16:0-d_{31}/16:1c, (d) 18:0-d_{35}/16:1c, (e) 16:0-d_{31}/18:1c, (f) 18:0-d_{35}/18:1c, (g) 20:0-d_{39}/18:1c, and (h) 20:0-d_{39}/20:1c (30 μM/120 μM).

membrane lipids. The spectra recorded from the extracted lipids from cells grown with the fatty acid pairs 14:0-d_{27}/14:1c and 20:0-d_{39}/20:1c show only an L_α phase. Lipid extracts prepared from cells grown with the other fatty acid pairs contained between 17% and 53% gel phase. However, it was not always true that the amount of gel phase was equal in the membranes and the extracted lipid samples. It appears that the presence of membrane proteins had no influence on the lipid phase equilibria, but rather the identity of the lipid species themselves determined the phase properties.

Since it can be assumed that the L_α phase portion of the membrane is the most biologically active and that there is a large L_α component in all of the systems, the spectra can be de-Paked and order parameters extracted. The order profiles are shown in Figure 5 for all of the pairs of fatty acids studied. In all cases, the order parameters of the membranes are similar to those of the extracted lipids, suggesting that the membrane proteins do not play a large role in lipid organization and packing. The absolute values of the parameters are in the general range found for many other lipids in the L_α phase (Eriksson et al., 1991; Lafleur et al., 1990; Thurmond et al., 1991). To better quantify and compare the molecular ordering of the different systems, the order parameter averaged over all segments in the acyl chain, $\langle S \rangle$, was calculated and tabulated in Tables 3 and 4. Furthermore, since the organism does have some control over which fatty acids are incorporated into the membranes and for very short chains, in particular, it has the ability to elongate the supplied fatty acids, the average number of carbons in an acyl chain, $\langle C_n \rangle$, was determined. In general, as $\langle C_n \rangle$ is increased, both the average order parameters and the order parameter of the C_α methylene group decrease (Figure 6). This is contrary to what might have been expected from studies of pure phospholipid systems, where increases in the chain length of the lipids increase the order parameter, mainly due to an increase in the T_m value (Barry et al., 1991; Dodd & Brown, 1989; Morrow et al., 1992). These observations are also found for the lipids extracted from the membranes. This trend is not strictly held however, especially when directly comparing membranes and extracted

Table 3: Average Order Parameter, $\langle S \rangle$, Acyl Chain Length, $\langle L \rangle$, and Number of Carbons in the Acyl Chains, $\langle C_n \rangle$, for Membranes Derived from *A. laidlawii* Grown with Different Fatty Acid Compositions (Spectra Were Recorded at 37 °C)

FA composition[a]	$\langle C_n \rangle$	$\langle S \rangle$	$\langle L \rangle$ (Å)
14:0-d_{27}/14:1c	14.7	0.181	11.56
14:0-d_{27}/16:1c	14.9[b]	0.182[b]	11.72[b]
	15.7[b]	0.186[b]	12.28[b]
16:0-d_{31}/16:1c	16.0	0.156	12.35
16:0-d_{31}/18:1c	17.1	0.166	13.23
18:0-d_{35}/16:1c	17.3	0.140	13.20
18:0-d_{35}/18:1c	17.9	0.144	13.65
20:0-d_{39}/20:1c[c]	18.1	0.138	13.98
20:0-d_{39}/18:1c	18.7	0.136	14.31
20:0-d_{39}/20:1c[d]	19.9	0.138	15.32

[a] The fatty acids are designated *x*:*y*, where *x* is the number of carbons and *y* is the number of cis double bonds present; the addition of the notation -d_z indicates that the fatty acid contains *z* deuterium atoms. [b] The results from two different cultures are shown. [c] Concentrations were 30 μM 20:0-d_{39} and 120 μM 20:1c. [d] Concentrations were 50 μM 20:0-d_{39} and 100 μM 20:1c.

Table 4: Average Order Parameter, $\langle S \rangle$, Acyl Chain Length, $\langle L \rangle$, and Number of Carbons in the Acyl Chains, $\langle C_n \rangle$, for Extracted Lipids Derived from *A. laidlawii* Grown with Different Fatty Acid Compositions (Spectra Were Recorded at 37 °C)

FA composition[a]	$\langle C_n \rangle$	$\langle S \rangle$	$\langle L \rangle$ (Å)
14:0-d_{27}/14:1c	15.0	0.167	11.51
14:0-d_{27}/16:1c	14.6[b]	0.181[b]	11.51[b]
	16.0[b]	0.172[b]	12.23[b]
16:0-d_{31}/16:1c	16.0	0.151	12.26
16:0-d_{31}/18:1c	16.8	0.153	12.78
18:0-d_{35}/16:1c	17.5	0.144	13.41
18:0-d_{35}/18:1c	17.9	0.149	13.77
20:0-d_{39}/20:1c[c]	18.5	0.150	14.22
20:0-d_{39}/18:1c	18.8	0.137	14.49
20:0-d_{39}/20:1c[d]	19.9	0.142	15.23

[a] The fatty acids are designated *x*:*y*, where *x* is the number of carbons and *y* is the number of cis double bonds present; the addition of the notation -d_z indicates that the fatty acid contains *z* deuterium atoms. [b] The results from two different cultures are shown. [c] Concentrations were 30 μM 20:0-d_{39} and 120 μM 20:1c. [d] Concentrations were 50 μM 20:0-d_{39} and 100 μM 20:1c.

lipids for any pair of acyl chains, which most likely reflects the fact that for a living system every parameter cannot be controlled.

As described in the Materials and Methods section, the average acyl chain length, $\langle L \rangle$, can be estimated from the order parameter profiles. In order to do this, we have to make a few additional assumptions beyond those made when deriving the equation (Jansson et al., 1992; Thurmond & Lindblom, 1994). In all cases we are measuring the order parameter of only the perdeuterated chain, although other acyl chains are present. In some cases this becomes even more complicated when the organism elongates the shortest deuterated fatty acids, although this is not much of a problem because at the most this represents only a small fraction of the total deuterated acyl chains. Nevertheless, the order parameters represent an average over the whole system. Therefore, if we replace *n* in eq 3 with $\langle C_n \rangle$, an estimate of the average acyl chain length can be made. This approximation has been shown to be valid for mixtures of phosphatidylcholines (Thurmond & Lindblom, 1994). One additional assumption is that the double bonds can be ignored and approximated by single bonds. It can be estimated that a double bond will shorten the chain length by less than 1 Å

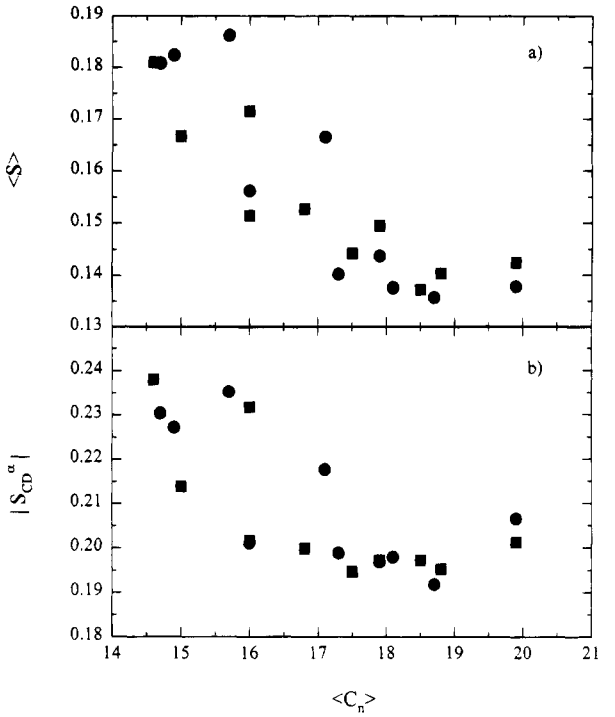

FIGURE 6: Dependence of (a) the average order parameter, $\langle S \rangle$, and (b) the order parameter at the C_α methylene, S_{CD}^{α}, on the average number of carbons in the acyl chain, $\langle C_n \rangle$, for both intact membranes (●) and extracted lipids (■). The points represent every fatty acid composition used to grow *A. laidlawii* (Tables 1 and 2).

compared to the fully saturated chains (Israelachvili et al., 1977). With these assumptions in mind, the values of $\langle L \rangle$ have been tabulated in Tables 3 and 4. As expected, an increase in the number of carbons in an acyl chain corresponds to an increase in the calculated average acyl chain length. $\langle L \rangle$ is approximately one-half of the bilayer thickness. Therefore, for this series the thickness of the membranes spans a range of 23.2−30.6 Å.

DISCUSSION

A vast pool of knowledge exists concerning membrane physical properties in model systems (Thurmond & Lindblom, 1994). However, there have been relatively few applications of these ideas to living systems. Using the unique features of *A. laidlawii*, we have explored the influence of the acyl chain length on the physical properties of the intact membranes and compared them to the extracted lipids. The extracted lipids are equivalent to the model systems mentioned earlier, whereas the intact membranes represent a more real biological system.

The ^{2}H NMR spectra for the membranes all contained components that were similar to those found for model systems in the L_α phase. Therefore, it appears that the structure of intact membranes can be approximated by model membrane systems of lipids. In some of the cases a significant gel phase component was found. It may be surprising that such a phase separation is found in a living system; however, it can be assumed that the biologically active part of the membrane is the fluid lipid bilayer, and for the rest of the analysis only the lipids in the L_α phase will be considered.

When the average order parameters are extracted from the spectra of the membranes, they are found to vary between

0.136 and 0.186. This range is within that expected from studies of model membrane systems (Davis, 1979; Dodd & Brown, 1989; Eriksson et al., 1991; Lafleur et al., 1990; Thurmond et al., 1991; Thurmond & Lindblom, 1994), and it appears that the ordering of the membrane is not drastically perturbed by any nonlipid components. It should be noted that *A. laidlawii* does not contain cholesterol, which would be expected to have a dramatic effect on the ordering of the membrane (Davis et al., 1980; Rance et al., 1982). As a general trend, the order parameters seem to decrease with an increasing number of carbons in the acyl chains (Figure 6). This is the opposite of what would be expected from studies of phosphatidylcholines, where increases in the number of carbons in the acyl chain lead to an increase in the order parameters at the same absolute temperature (Barry et al., 1992; Morrow et al., 1992; Salmon et al., 1987). Therefore, some other factor must be influencing the packing of the lipids in the membranes.

From the ^{2}H NMR order profiles, an estimate of the bilayer thickness can be obtained (Salmon et al., 1987; Seelig, 1977; Thurmond et al., 1991; Thurmond & Lindblom, 1994). This quantity varies over a significant range (23.2−30.6 Å), which is approximately what would be expected from the change in the number of carbons in the chains [this can be estimated using eq 3, setting the order parameter at 0.5 and then adjusting this value by 0.7 to account for the melted state of the acyl chains (Tanford, 1980)]. Here, it appears that the organism can only accomplish minor regulation of the thickness of the membranes (see Results) and must rely mainly on the fatty acids in the growth medium.

Given that there is a large variation in the thickness of the membranes, one might expect that the incorporation of proteins into the membrane could be a problem. It is commonly assumed that the membrane thickness must match the hydrophobic thickness of the membrane proteins (Mouritsen & Bloom, 1984). It may also be expected that the proteins would contribute to the ordering of the membranes. If the membrane thickness is smaller than the hydrophobic thickness of the protein, then the chains would have to be more extended, resulting in an increase in the molecular ordering, and for membranes that are too thick the acyl chains would have to be made less ordered. In either case, the acyl chains would be displaced from the equilibrium lengths adopted in the absence of the protein, and it might be expected that when the proteins are removed the acyl chains will relax back to equilibrium. This would be observed as changes in the average order parameters. When the average molecular ordering in the membranes is compared to that of the extracted lipids, there do appear to be slight differences. Short chains yield higher order parameters in the membranes than in the extracted lipid samples, whereas for long chains the trend is reversed. These small differences are evident only when comparing the average order parameters and are not seen when comparing the order parameters at the C_α methylene position (Figure 6). This is not surprising, since the ordering at the C_α position is expected to be more sensitive to the properties at the lipid/water interface than to the thickness of the membrane (Thurmond & Lindblom, 1994).

Monck et al. (1992) carried out a similar study using *A. laidlawii* strain B, although they studied only a narrow range of bilayer thicknesses (25.2−26.7 Å). They found that $\langle S \rangle$ varied over the range 0.140−0.176, which is very similar to the range for *A. laidlawii* strain A investigated by us. In their case, they found virtually no difference between membranes and extracted lipids for a variety of mixtures with 16:0-d_{31} and 18:1c chains, as we find here. However, when studying samples with a 1:1 mixture of 16:0-d_{31} and 14:0 chains, they found an increase in the ordering when going from membranes to extracted lipids, which is contradictory to our data and the preceding ideas.

The differences between the membranes and the extracted lipids are quite small, and it is difficult to judge whether or not they are significant. However, the numbers are consistent with recent molecular modeling of lipid/protein interactions (Fattal & Ben-Shaul, 1993; G. Orädd, G. Lindblom, K. Gunnarsson, and G. Arvidson, submitted for publication). In this formulation, the disruption of lipid packing by the protein occurs only over a distance of approximately 8 Å, which is roughly equivalent to one lipid molecule [assuming the average surface area occupied by a lipid is 70 Å^2 (Eriksson et al., 1991; Thurmond et al., 1991)]. Fattal and Ben-Shaul give two cases: one where the protein is 7 Å longer than the bilayer thickness and the other one where it is 7 Å shorter. In the case where the protein is longer, the order should be increased by approximately 35% for one lipid layer around the protein (8 Å). If we assume that a large membrane protein has a diameter of 50 Å [based on the proteins of the photosynthetic reaction center with a molecular mass of 150 kDa (Deisenhofer & Michel, 1989)], then one lipid layer would correspond to 20 lipid molecules. In *A. laidlawii* membranes, the lipid to protein ratio is around 60:1 as calculated from Wieslander and Rilfors (1977), and therefore, the 35% increase would only occur in one-third of the total lipids, yielding an increase of 12% when measured over the entire system. For our system, this would result in an increase of 0.02 in the average order parameter, $\langle S \rangle$, which is close to what is observed (Tables 3 and 4). The values chosen for the average membrane protein diameter and the lipid to protein ratio are considered to be extremes, and in actuality the effect could be much smaller due to smaller proteins (the average molecular mass in *A. laidlawii* is 40−45 kDa). Using the same argument for a protein with a shorter hydrophobic thickness than the membrane, the change is a reduction in the average order parameter of only 6% or 0.009. It should be noted that in our case the protein/membrane hydrophobic mismatch would not be expected to be as large as 7 Å, and so the changes would be even smaller. Thus, it appears from the current models of lipid/protein interactions that the perturbations caused by even a large protein/membrane mismatch would be quite small or even undetectable, as we observe.

Because only slight differences are seen between the membranes and the extracted lipids, we can make no judgement on the validity of the model of Fattal and Ben-Shaul or any other lipid/protein model [see Mouritsen and Bloom (1993)]. In these models the proteins are considered as rigid solutes, but it is possible that the protein itself could adjust to the different membrane thicknesses seen here by perhaps adjusting aromatic amino acids at the lipid/water interface (Schulz, 1993). It can be said conclusively that *membrane proteins do not have any significant effect on the average packing of the membrane lipids in A. laidlawii.*

It was found that the membrane lipid composition was influenced by the acyl chain length (Tables 1 and 2; Wieslander et al., 1994). MGlcDAG was produced in great

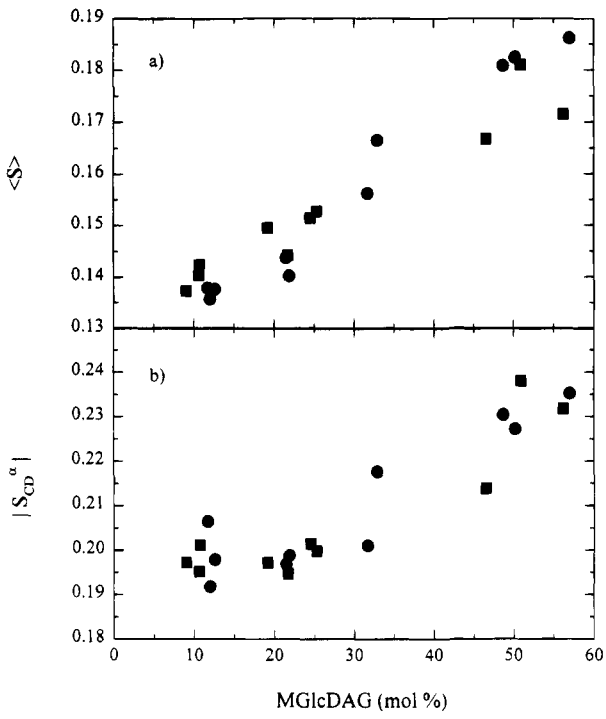

FIGURE 7: Dependence of (a) the average order parameter, $\langle S \rangle$, and (b) the order parameter at the C_α methylene, S_{CD}^{α}, on the mole percentage of MGlcDAG for both intact membranes (●) and extracted lipids (■). The points represent every fatty acid composition used to grow *A. laidlawii* (Tables 1 and 2).

amounts when short lipid chains were present, and DGlcDAG and PG increased when the chain length increased. As noted earlier, the order parameter decreases with an increasing number of carbons in the acyl chain, as opposed to what may have been expected from studies of phosphatidylcholines. This decrease in the average order parameter seems to correlate with the decrease in the fraction of MGlcDAG present in the membranes (Figure 7a). This correlation is not nearly as strong with any other lipid component. The same conclusion can also be reached for the C_α methylene group (Figure 7b). The correlation between the fraction of MGlcDAG and $\langle S \rangle$ can be understood in terms of what is known about the forces that govern lipid acyl chain packing. The smaller head group of MGlcDAG (Eriksson et al., 1991; Lindblom et al., 1993) allows less accessible area for the acyl chains to occupy than the other major lipid head groups in the system (DGlcDAG and PG). This causes the chains to adopt a more extended conformation and, hence, an increase in the order parameter. This indeed has been seen when comparing ^2H NMR spectra of DGlcDAG and MGlcDAG (Eriksson et al., 1991). This idea of accessible areas has been useful in explaining the differences in order between phosphatidylcholines and phosphatidylethanolamines (Thurmond et al., 1991), as well as differences between the packing of different phases (Thurmond & Lindblom, 1994; Thurmond et al., 1993). Furthermore, the correlation with the order at the C_α methylene group also reflects the smaller head group size. It has been postulated that the order parameter of this carbon most accurately represents the area per lipid at the lipid/water interface (Thurmond et al., 1991, 1993). Therefore, an increase in the amount of MGlcDAG present would effectively decrease the area at the interface and increase the order parameter at the C_α methylene group.

It should be noted that S_{CD} is measured on only the L_α phase component of the spectra, and in some cases a significant portion of the gel phase component exists (up to 53%). The results for $\langle C_n \rangle$ and the lipid composition, on the other hand, are determined for the whole system. It may be expected that the distribution of the acyl chains and the head groups would be different in the two phases. One might imagine that the gel phase is enriched in saturated acyl chains and smaller head groups, such as MGlcDAG. However, taking into account an uneven distribution of the acyl chains and/or the head goups does not alter any of the trends seen here.

It then appears that *A. laidlawii* responds to having to incorporate different chain lengths into the membranes by changing the amount of MGlcDAG present. What then is the organism optimizing? As explained earlier, the incorporation of MGlcDAG would effectively increase the acyl chain order and increase the bilayer thickness. However, the cells seem to grow over a range of both acyl chain order and bilayer thickness, so it is doubtful that these factors are important.

It has been proposed that *A. laidlawii* maintains a balance between lipids that tend to form an L_α phase and those that can adopt nonlamellar phases and that the organism adjusts these lipids so that the membranes are rather close to a lamellar to nonlamellar phase transition boundary (Lindblom et al., 1986, 1993; Rilfors et al., 1994; Wieslander et al., 1986; Österberg et al., 1994). Although these nonbilayer structures would not be expected to be present in the membranes, a curvature stress (Anderson et al., 1989; Gruner, 1989; Helfrich, 1973; Rand et al., 1990) associated with them would still exist. This curvature stress seems to be a necessary physical property of the membrane that is regulated by the organism (Österberg et al., 1994). Indeed, it has been proposed that this property is important for the function of many membrane proteins (Epand et al., 1992; Eriksson et al., 1991; Gibson & Brown, 1991, 1993; Jensen & Schutzbach, 1988; Keller et al., 1993; Lindblom & Rilfors, 1989; Navarro et al., 1984; Wiedmann et al., 1988). It is well-known that short acyl chains are not favorable for the formation of nonlamellar phases (Lewis et al., 1989; Marsh, 1991; Rilfors et al., 1993; Tate & Gruner, 1987). Therefore, when forced to grow with short chain fatty acids, *A. laidlawii* increases the amount of MGlcDAG present in the membrane and may synthesize DAG and MAMGlcDAG, which would also favor nonlamellar phases (Lindblom et al., 1993). The opposite would be true for the long chain fatty acids, where the amount of MGlcDAG is reduced so that the membranes remain in the L_α phase. The size of the MGlcDAG head group relative to the acyl chains causes both the tendency to form nonlamellar phases and the increase in order of the acyl chains. However, it appears that it is the relationship to nonlamellar phases that is important for the organism, and the increase in acyl chain order, upon a decreasing number of carbons in the acyl chains, is only a consequence of this.

It is expected that there should be a relationship between the average molecular ordering in the L_α phase and the curvature stress. This curvature stress is generated when the equilibrium area of the head goup is smaller than the equilibrium area of the acyl chains. The balance between these two forces would result in a net repulsive pressure in the acyl chain region (and likewise a net attractive pressure in the head goup region). Such chain pressures have been

correlated in statistical mechanical models (Meraldi & Schlitter, 1981a,b) with the segmental order parameter, S_{CD}. Increases in lateral chain pressure result in increases in acyl chain order if all other factors are equal. Therefore, since increases in curvature stress would be predicted to increase the lateral chain pressure, this should correlate with increases in the segmental order parameter. Likewise, if *A. laidlawii* is regulating the curvature stress in the membranes (Österberg et al., 1994), it may be expected that the segmental ordering would also remain constant. However, this may not be observed here since many other factors that would influence the ordering, such as acyl chain length and unsaturation, are changing.

Another factor that could play a role is the membrane permeability. As the bilayer thickness decreases, it is expected that the permeability would increase (McElhaney, 1992). This would be highly unfavorable and the organism may compensate for this. One way to do this would be to pack the head groups tighter together. Hence, adding more MGlcDAG to the membrane could have the effect of decreasing the membrane permeability since it is known that this lipid packs tighter than DGlcDAG or PG (Eriksson et al., 1991; Lindblom et al., 1993). This decrease in permeability may help to offset the effect of the decrease in bilayer thickness. Therefore, as the number of carbons in the acyl chain decreases, the amount of MGlcDAG would increase and an increase in the molecular ordering would be observed, as seen here.

CONCLUSIONS

Since *A. laidlawii* can be restricted to incorporating fatty acids from the growth medium into its membranes, we have been able to study the effects of the length of the lipid acyl chains on the properties of the membranes of the organism. It appears that the proteins associated with the membranes do not have any large effect on the overall packing of the membrane lipids, since the differences in the order between the intact membranes and the extracted lipids were very slight or even undetectable. This is true, even though the bilayer thickness calculated from the ^{2}H NMR order profiles varied by approximately 8 Å. When acyl chains of different lengths were incorporated into the membrane lipids, the order parameter of either the membranes or the extracted lipids decreased as the length of the fatty acid increased. This decrease can be explained by the decrease in the fraction of MGlcDAG found in the cell. Since both the packing of the membrane lipids (as judged by the order parameters) and the bilayer thickness varies over the entire series, we propose that it is the membrane curvature energy that is regulated by the organism by changing the fraction of lipids that have the potential to form nonlamellar phases.

ACKNOWLEDGMENT

R.L.T. thanks the Swedish Institute for financial support during this work. We also thank Viola Tegman and Annelie Niemi for technical assistance.

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