Biochimica et Biophysica Acta, 679 (1982) 361–368
Elsevier Biomedical Press

BBA 41028

CHLOROPHYLL *a* IN BILAYER MEMBRANES

II. INTERACTION WITH DISTEAROYLPHOSPHATIDYLCHOLINE BY NMR *

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(Received July 10th, 1981)

Key words: Lipid bilayer; NMR; Chlorophyll-lipid interaction; Photosynthetic membrane model; Differential thermal analysis

Bilayer membranes containing distearoylphosphatidylcholine and chlorophyll *a* have been investigated via ^{1}H- and ^{31}P-NMR. Measurements as a function of temperature and composition support the basic interpretation of the phase diagram for this mixed bilayer system advanced in the preceding report. The ^{31}P-NMR spectrum of mixed vesicles above the solidus temperature of the phase diagram shows a single resonance, but below the solidus two peaks are observed. Observation of two distinct ^{31}P resonances from the phospholipid at 46°C conclusively demonstrates that phase separation occurs at temperatures below the solidus. Observation of a 5.8 ppm upfield shift for one of the ^{31}P resonances at low temperature provides strong evidence for an interaction between the phospholipid headgroup and chlorophyll *a* in one of the low-temperature phases. This conclusion is supported by the observation of increased ^{1}H linewidths for the lipid and a longer ^{1}H T_1 for the choline *N*-methyl resonance of the lipid below the solidus temperature, indicating that headgroups of lipid molecules in at least one of the phases are motionally restricted. The observation of an interaction between chlorophyll *a* and lipid molecules in a bilayer membrane lends considerable support to the idea that chlorophyll molecules in the chloroplast antenna are physically separated from each other by strongly coordinating lipid molecules.

Introduction

Although there is a great deal of data concerning the interactions of chlorophylls with other molecules in solution, relatively little is known about the intermolecular interactions of chlorophyll with other components in surface films and bilayer membranes. Yet such interactions determine (to a large degree) the properties of chlorophyll which is confined to a lamellar matrix, as it may be in model and biological membranes. In the previous paper [25] we considered the thermodynamic phase diagram of chlorophyll *a*/DSPC bilayer membranes. We now examine in more detail the organization of chlorophyll *a* in the bilayer for each of the various regions of the phase diagram, with the goal of ascertaining the molecular basis behind this interesting and unusual phase behavior. In particular, we wish to verify independently that thermodynamic phase separation occurs within the two-phase regions, and to determine the intermolecular interactions responsible for the observed compound formation between chlorophyll *a* and lipid.

Based on structural and chemical considerations alone, one might initially expect chlorophyll *a* to be surface-active and to associate with lipid membranes. Comparison of the structures and relative dimensions of chlorophyll *a* and distearoylphosphatidylcholine (DSPC), a typical phospholipid with two saturated

Abbreviations: DSPC, distearoylphosphatidylcholine.

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18-carbon fatty acyl chains similar in length to the phytol chains of chlorophyll *a*, shows that the interface between hydrophobic chains and hydrophilic headgroups imparts an amphiphilic character. As a result each is surface active and forms aggregates in aqueous solution by virtue of the so-called hydrophobic effect [1]. The tendency of pure phospholipids such as DSPC to self-associate into aggregated lipid bilayers is well known and has been extensively studied. Although chlorophyll *a* can form monolayers at an air/water interface [2,3], it does not by itself form bilayer membranes. However, it has previously been established that in conjunction with other lipids chlorophyll *a* can form black lipid films [4,5], multilayers and small unilamellar vesicles [6–10]. The chlorophyll phytol group can thus serve as a lipophilic anchor binding chlorophyll to the host membrane.

In addition to the phytol chain the central magnesium atom of chlorophyll *a* is potentially important in determining its interactions with other lipid molecules. The central magnesium atom is coordinatively unsaturated in the porphyrin tetrapyrrole system of chlorophyll [11–14] and has always been observed to be complexed to one and sometimes two nucleophilic axial ligands. This coordination requirement must also be satisfied in the bilayer.

The studies described here rely on NMR techniques, which have already provided a host of information on both membrane structure and dynamics. NMR methods are particularly useful for investigating intermolecular interactions of chlorophylls because the ring current of the chlorophyll macrocycle causes a shift in the resonance position of nearby nuclei. The magnitude and direction of the shift depends on the distance and azimuthal angle of the shifted nucleus with respect to the porphyrin plane. Thus the measured ring current shifts may be used to deduce the orientation of interacting molecules with respect to the chlorophyll macrocycle.

The ring current shift effect has been used to good advantage in studies of the interaction between chlorophyll *a* and other molecules in solution [11,12,14]. In the following experiments we have exploited this effect to investigate the interactions of chlorophyll with molecules in a bilayer phase. In addition, linewidth and relaxation measurements have been used to study changes in the dynamics of the host membrane through the various phase transitions.

Materials and Methods

Chlorophyll *a* was isolated from spinach by techniques cited in the previous paper. Molar extinction coefficients at 660 nm and 428 nm were measured in diethyl ether and indicate that the purified chlorophyll *a* is substantially free of impurities. Synthetic β-γ-distearoyl-L-α-phosphatidylcholine (DSPC) was purchased from Calbiochem, checked for impurities by thin-layer chromatography, and used without further purification.

Small unilamellar vesicles were prepared by sonication. Weighed amounts of chlorophyll *a* and DSPC were dissolved together in fresh chloroform in order to assure their complete mixing. The chloroform solutions of chlorophyll *a* and DSPC were evaporated by a stream of nitrogen gas and residual solvent was completely removed under vacuum. The dried chlorophyll *a*/DSPC film was then suspended in an appropriate volume of 99.8%-*d* ^2H_2O by a vortex mixer. The resulting multilayer suspension was transferred to a centrifuge cone and sonicated with a Heat Systems-Ultrasonics, Inc. model W-225R cell disruptor using a stepped microtip and the highest possible power setting in order to minimize the sonication time. Best results were obtained by continuous sonication for 3 min in a cool glycerol bath followed by 10 min sonication on a 50% duty cycle with the glycerol bath removed so as to allow the temperature to rise slightly above the bilayer phase transition of about 55°C. The solution was then centrifuged to remove titanium particles eroded from the sonicator tip and also small, usually negligible, amounts of large bilayer structures.

Vesicles produced by the foregoing procedure are large enough to elute entirely in the void volume when chromatographed on Sepharose 4B; additional sonication does not change the elution pattern. This sets a minimum limit on their size of approx. 500 Å radius. However, the vesicles show the well-resolved ^{1}H-NMR spectrum characteristic of small sonicated liposomes. Thus they can be no larger than about 1000 Å radius.

NMR experiments were performed on vesicle solutions which had been hydrated and sonicated just prior to their use. The solution concentrations were 30 mg lipid (chlorophyll *a* + DSPC) per ml for ^{31}P experiments, and 10 mg/ml for the ^{1}H experiments.

We are confident that the vesicle suspensions were stable during the course of our experiments for the following reasons: (1) the solutions remain optically clear at the end of experiments; (2) vesicle spectra are easily distinguished from multilayer spectra which give noticeably broadened bulk-methylene resonances; and (3) all spectral changes with temperature are fully reversible.

^{31}P-NMR spectra at 40.5 MHz and ^{1}H-NMR spectra at 100 MHz were obtained on Varian XL-100 spectrometers using standard Fourier transform techniques. Sample temperatures were regulated by Varian temperature controllers which had been previously calibrated with a thermocouple. ^{31}P spectra were proton-decoupled by continuous broadband irradiation over the range of proton frequencies. 360 MHz ^{1}H-NMR spectra were obtained at the Stanford Magnetic Resonance Laboratory on a Bruker HXS-360 spectrometer. The Bruker temperature controller was calibrated prior to each set of experiments by measuring the frequency difference between the resonances of ethylene glycol at several temperatures. Quoted temperatures are accurate to within 1°C.

Results

The thermal phase diagram which was reported in the previous paper suggests that the interactions of chlorophyll *a* with phospholipid in a bilayer are manifested differently at high and low temperatures. At high temperatures the phase diagram indicates that chlorophyll *a* and DSPC form a single-phase solution within the bilayer. However, below a thermal phase transition temperature of about 50°C, the phase diagram predicts that two distinct phases are present, each containing both chlorophyll *a* and DSPC. One of these phases is a solid solution while the other is an inter-lipid compound phase thought to be formed by some specific chemical interaction between chlorophyll *a* and DSPC. These conclusions from the phase diagram are supported by the following NMR experiments. The NMR results also provide some insight into the intermolecular interaction responsible for the formation of the compound.

^{31}*P-NMR*

Fig. 1 shows 40.5 MHz ^{31}P-NMR spectra of a 20% chlorophyll *a*/DSPC vesicle suspension at two different temperatures corresponding to two different regions of the phase diagram. The spectrum at 57°C, in the one-phase homogeneous solution region of the phase diagram, does indeed show a single phospholipid resonance. At 46°C, corresponding to the two-phase compound region below the solidus, there are two phospholipid resonances observed. One of these two peaks has a chemical shift nearly the same as that of the single peak obtained in the higher temperature spectrum, whereas the second, additional peak is shifted 5.8 ppm upfield. Within experimental error the two resonances have the same area; the phase diagram predicts that for a composition which is 20 mol% chlorophyll *a* the pure DSPC and compound phases should be approximately equal in magnitude. The total absolute intensity of the phosphorus resonance is approximately equal at high and low temperatures, indicating that all of the lipid is portioned into only two environments at low temperature. The ob-

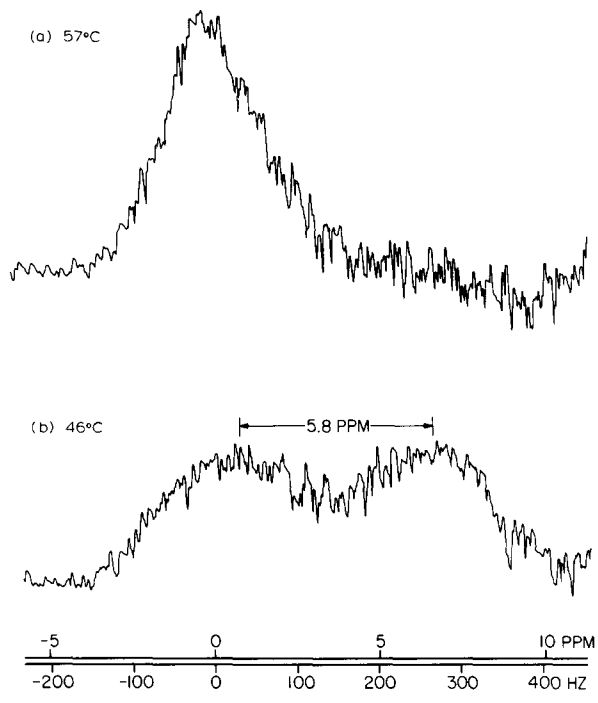

Fig. 1. Proton decoupled ^{31}P-NMR spectra at 40.5 MHz of sonicated 20% chlorophyll *a*/DSPC single-walled bilayer vesicles at 57°C (a) and 46°C (b). At 57°C the membranes are in the solution region of the phase diagram. At 46°C the phase diagram predicts phase separation into solid DSPC and compound phases.

servation of two distinct phospholipid resonances in the 46°C spectrum conclusively demonstrates that phase separation occurs at temperatures below the solidus of the phase diagram.

The additional resonance in the 46°C spectrum must presumably correspond to phosphate in phospholipid of the compound phase. There are two possible mechanism to explain the upfield shift of the resonance due to this portion of the lipid. Each suggests that the lipid phosphate moiety is in close proximity to the central magnesium atom of chlorophyll in the compound phase.

First, the upfield shift of the phosphate could be due to the ring current effect of an adjacent chlorophyll molecule. If we assume that the only source of shift is the ring current magnetic anisotropy of chlorophyll *a* the magnitude and direction of the observed shift may be used to deduce the relative orientation of the phosphate with respect to the porphyrin macrocycle. Fig. 2 illustrates the chlorophyll shift map calculated according to the approximate empirical expression of Shulman et al. [15], which is nearly accurate for distances greater than 3 Å. If we presuppose that the phosphorus of DSPC is perpendicular to the central magnesium atom of chlorophyll, the observed 5.8 ppm upfield shift would correspond to a phosphorus to magnesium distance of 3.4 Å. A shift of the same magnitude could also be produced by a smaller angle and a correspondingly shorter distance, but for a deviation of more than 30° from the perpendicular direction the distance becomes unreasonably small, less than the sum of the P, O and Mg covalent radii.

Second, the upfield shift could result from electrostatic interactions between the phosphate and the chlorophyll magnesium. Grasdalen et al. [16] measured the ^{31}P chemical shift of the phosphate moiety in sonicated vesicles of egg yolk lecithin as a function of Ca^{2+} concentration. The authors were able to fit their data assuming that Ca^{2+} binding to the lecithin phosphate produced an upfield shift of 4.25 ppm. Since the electrostatic effect on chemical shift falls off with distance at about the same rate as the ring current shift mentioned earlier, in order to have a significant electrostatic effect on the shift, the phosphate and chlorophyll magnesium must once again be in close proximity. Thus while it is not possible with the data in hand to partition the

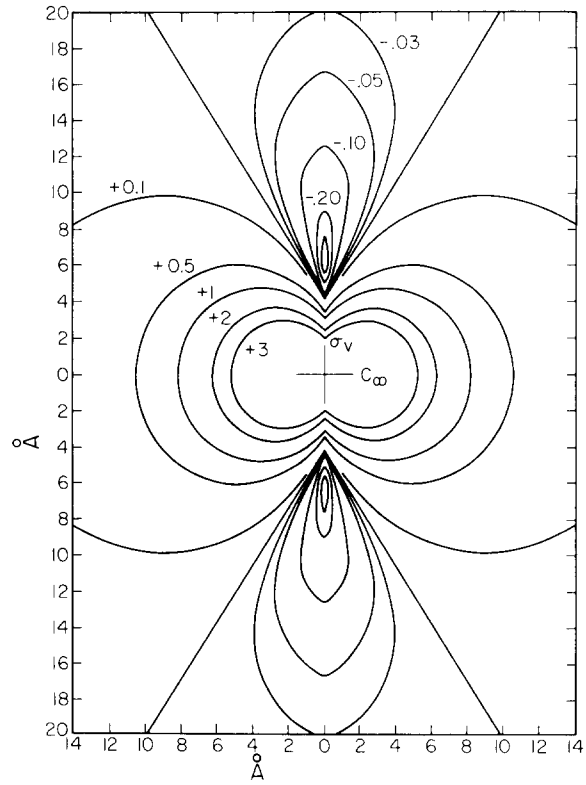

Fig. 2. Ring current shift map of chlorophyll *a* calculated according to the empirical expression of Shulman et al. [15] (iso-shielding lines labeled in part per million). The porphyrin plane lies on a vertical plane perpendicular to the page (σ_v) and is assumed to have rotational symmetry about C_∞ and reflection symmetry through σ_v.

5.8 ppm shift between ring current and electrostatic effects, the observation of a large upfield shift clearly requires that the lipid phosphate and chlorophyll magnesium lie in close proximity in the compound phase, probably within 4 Å.

The observation of two resonances in the 46°C spectrum also suggests that the rate of exchange of DSPC molecules between the compound-phase and solution-phase environments is slow. If the chemical exchange were faster than the timescale set by the static chemical shift differences between the two environments, then a single averaged resonance would be observed. The condition for coalescence of resonances from two equal populations in chemical exchange is given [17] by $k_{\text{exchange}} = 2\pi\Delta\nu/\sqrt{2}$, from which we may calculate an upper bound for the

exchange rate of DSPC between compound and solution phases of less than 1000 s^{-1}.

The observed ^{31}P linewidths of about 150–200 Hz are substantially larger than the 50 Hz linewidth normally obtained in spectra of pure DSPC vesicles. The most likely explanation is that the inclusion of chlorophyll *a* into the bilayer alters the normal intermolecular headgroup interactions of pure DSPC bilayers which are thought to govern the ^{31}P linewidth.

^{1}H-NMR

Proton spectra of chlorophyll *a*/DSPC vesicles at several compositions were obtained as a function of temperature at 100 MHz and 360 MHz. The spectra shown in Fig. 3 are typical. In their gross features these spectra resemble those of pure phospholipid vesicles, but upon closer scrutiny they reveal new and useful information. Because of the rigidity of the chlorophyll *a* porphyrin macrocycle and the additional motional restriction imposed by binding chlorophyll *a* to the membrane, no porphyrin resonances contribute to the high resolution spectra. Phytomethyl and methylene resonances do contribute to the spectrum, however. This can be seen by comparing the methyl and methylene intensities in a series of spectra of different chlorophyll *a* compositions as shown in Fig. 4. Since chlorophyll *a* has comparatively more methyl groups than does DSPC, increasing its content relative to DSPC increases the methyl to methylene ratio. Other than this difference and some subtle changes in linewidths, the spectra are virtually indistinguishable from pure phospholipid vesicle spectra.

In contrast to the ^{31}P-NMR spectra, which show clear evidence of two classes of phospholipid below the solidus, the ^{1}H-NMR spectra show only a single choline *N*-methyl resonance over a temperature range encompassing all regions of the phase diagram. It cannot be determined whether resonances from other protons are split at lower temperatures since they become obscured by broadened choline *N*-methyl and chain methylene resonances. The observation of a single choline resonance allows us to set an upper bound for the chemical shift difference between *N*-methyl resonances in the solution and compound phases. The nature of the observed resonance depends on both the chemical shift difference and the rate of chemical exchange between the two environments. If

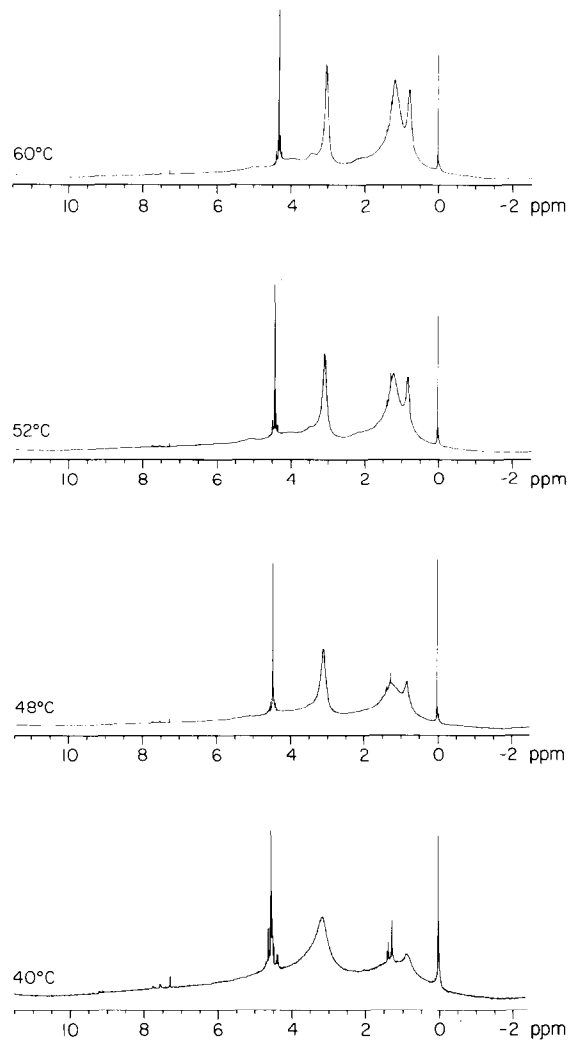

Fig. 3. 360 MHz proton NMR spectra of 15% chlorophyll *a*/DSPC vesicles at selected temperatures. The sharp resonance at 0 ppm (TMS) and other low intensity sharp resonances are from the reference capillary.

exchange is slow on the NMR timescale then the single observed resonance must be a superposition of two lines separated by less than the resonance linewidth, about 100 Hz or less than 0.3 ppm. On the other hand, if exchange is fast, i.e., $k_{\text{exchange}} > 2\pi\Delta\nu/\sqrt{2}$, then the observed resonance represents a dynamic exchange-averaged resonance intermediate between two static resonances separated by no more than $\sqrt{2}/2\pi$-times the exchange rate. From the upper

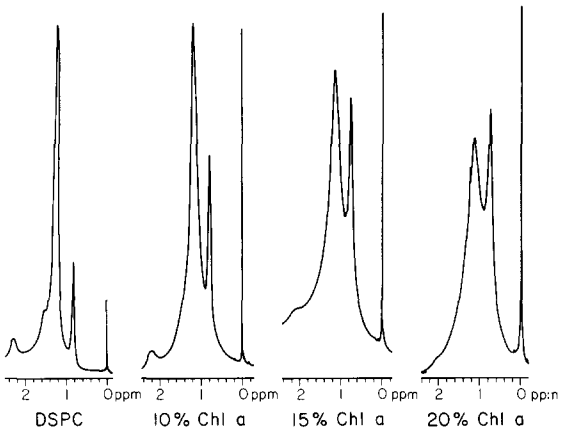

Fig. 4. Proton NMR spectra of chlorophyll *a*/DSPC sonicated vesicles at 0, 10, 15, and 20 mol% chlorophyll *a* in the methyl and methylene region.

bound for the exchange rate of 1000 s^{-1} obtained from the ^{31}P-NMR spectrum, we can estimate that the chemical shift difference could be no greater than 230 Hz, or about 0.6 ppm. Thus, the observation of a single choline peak implies that the difference in the chemical shifts of the choline *N*-methyl resonances of lipids in the compound and solution phases is small.

The chemical shift of the choline *N*-methyl resonance is temperature dependent and can be correlated with the predicted phase changes. Fig. 5 shows these resonance positions referenced to external TMS in C^2HCl_3 at 100 and 360 MHz. Inasmuch as the shifts in Fig. 6 are referenced to an external standard, corrections must be made for changes in bulk susceptibility with temperature. This is easily accomplished by making use of the fact that the change in bulk susceptibility is approximately linear over small temperature ranges [18], and is opposite in sign and twice the magnitude for solenoid versus electromagnet field/sample geometries [19]. Making the necessary corrections, we obtain the corrected data of Fig. 6. These data show a small shift in the peak position of the choline *N*-methyl resonance as the temperature is raised and a complete bilayer solution is formed between chlorophyll *a* and the lipid. This upfield shift is analogous to an aromatic solvent shift [20] resulting from the dissolution of a molecule into an aromatic solvent capable of producing a ring cur-

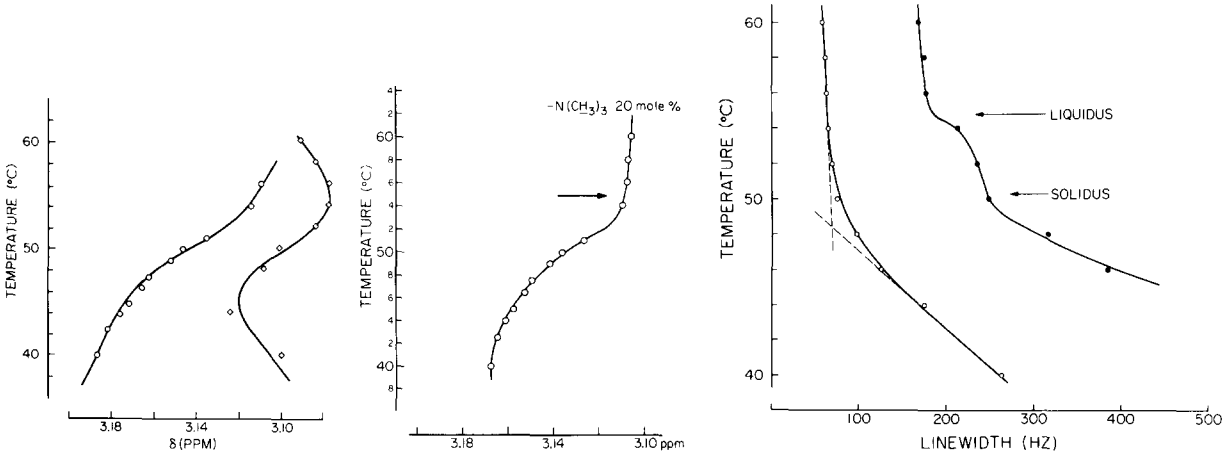

Fig. 5. (Left-hand figure.) Chemical shift of the DSPC choline *N*-methyl resonance (referenced to TMS) from 20% chlorophyll *a*/DSPC sonicated vesicles as a function of temperature. Circles: 360 MHz superconducting solenoid spectrometer. Diamonds: 100 MHz electromagnet spectrometer.

Fig. 6. (Center figure.) Data of Fig. 5 corrected for changes in the solvent bulk magnetic susceptibility with temperature. The arrow indicates the liquidus phase transition temperature.

Fig. 7. (Right-hand figure.) Linewidths at half height of the choline methyl (○) and bulk fatty acid methylene (●) lipid resonances from 20% chlorophyll *a*/DSPC vesicles as a function of temperature. The liquidus and solidus temperatures at this composition are indicated by arrows. These data were obtained from 360 MHz spectra.

rent shift which does not spatially average to zero.

The proton linewidths are also correlated with the predicted phase changes. Fig. 7 shows the measured linewidths of two lipid resonances from 20% chlorophyll *a*/DSPC vesicles. There is an abrupt change in the linewidth of the choline *N*-methyl resonance at about 49°C, corresponding approximately to the solidus of the phase diagram where compound is formed from the solution phase. The methylene resonance begins to broaden at a somewhat higher temperature, corresponding to the liquidus of the phase diagram, with a further break at the solidus. These changes all occur upon formation or elimination of a phase at a boundary of the phase diagram and correspond to changes in the motional state of the lipids. The linewidth of the methylene resonance changes at points where lipid is transformed from a gel phase to a liquid crystalline phase, with corresponding changes in the motional state of the hydrocarbon chains. The break in the choline *N*-methyl resonance linewidth at the solidus implies that there is a change in the motional state of the lipid headgroup in the transformation between compound and solution phases. This is consistent with the ^{31}P data which suggests a specific interaction between chlorophyll and the lipid headgroup in the compound phase.

Such a conclusion can also be reached by examining the spin-lattice relaxation times of pure DSPC vesicles and chlorophyll *a*/DSPC vesicles. Values of T_1 obtained at 360 MHz by the inversion recovery technique are given in Table I. These data indicate that T_1 of the choline *N*-methyl proton resonance is unaffected by increasing amounts of chlorophyll *a* when the temperature is 60°C. This is consistent with the phase diagram in that at 60°C only a single solution phase is present, regardless of composition. In contrast to the situation at 60°C, the T_1 data at 48°C show a substantial increase with increasing chlorophyll *a* content. This, too, is consistent with the phase diagram. Below 50°C the phase diagram predicts that the 15% chlorophyll *a* composition should contain 90% of the DSPC in the solution phase and only 10% in the compound phase. Thus the T_1 value for 15% chlorophyll *a*, which is only slightly higher than for pure DSPC, indicates that the relaxation rate in the solution phase is similar to that of DSPC. On the other hand, the 32% chlorophyll *a* composition should contain 65% of the DSPC in the compound phase. The larger T_1 value at 32% chlorophyll *a* indicates that T_1 for the compound phase is much longer than T_1 for the solution phase. Solving a simple set of simultaneous equations, we find that a pure compound phase should have $T_1 = 1.2$ s. This must indicate that the lipid headgroup is relatively restricted in the compound.

TABLE I

T_1 RELAXATION TIMES (IN SECONDS) OF THE CHOLINE *N*-METHYL PROTONS IN MIXED CHLOROPHYLL *a*/DSPC VESICLES AT TWO TEMPERATURES

Composition	48°C	60°C
DSPC	0.36 (± 0.13)	0.41 (± 0.02)
15% Chlorophyll *a*	0.49 (± 0.21)	0.51 (± 0.10)
32% Chlorophyll *a*	0.91 (± 0.07)	0.44 (± 0.15)

Discussion

The NMR studies presented here support the basic interpretation of the phase diagram for the chlorophyll *a*/DSPC bilayer which was advanced in the preceding report. They confirm that there is a homogeneous population of phospholipid in the solution region of the phase diagram, whereas below the solidus there is clear evidence of the predicted phase separation. In addition they provide strong evidence for an interaction between the DSPC headgroup and chlorophyll *a* in the compound phase, which in all likelihood involves a coordination interaction between the DSPC phosphate and the central magnesium atom of chlorophyll.

Evidence for a specific interaction between chlorophyll *a* and DSPC comes in two parts: first, the magnitude of the ^{31}P upfield shift, and second, linewidth and relaxation rate measurements of the choline *N*-methyl resonance. The linewidth change at the solidus temperature and the longer T_1 for the choline *N*-methyl resonance indicate that the headgroups of DSPC molecules involved in the compound phase are motionally restricted. This conclusion is also supported by the ^{31}P results which suggest that in the compound the phosphate of DSPC is situated sufficiently close to chlorophyll *a*, and remains there on a long enough timescale to produce a slow exchange

spectrum and yield a substantial upfield shift. The magnitude of this shift indicates that the phosphorus nucleus of the DSPC molecule is probably no more than 4 Å away from the central magnesium atom of chlorophyll *a*. The presence of this phosphate group would necessarily preclude any other axial ligand to chlorophyll *a*, and indeed it is likely that the nucleophilic phosphate is itself the axial ligand.

Interactions between lipid molecules and chlorophyll *a* in solution have been inferred from optical spectra [21,22] but to our knowledge the present studies are the first conclusive evidence that such interactions can occur within a lipid bilayer membrane. The observation of a compound formed between chlorophyll *a* and a lipid molecule lends considerable support to the proposal of Beddard and Porter [23] that chlorophyll molecules in the chloroplast antenna are physically separated from each other by strongly coordinating lipid molecules. This separation is necessary to prevent the formation of excitation traps from orbital overlap of two chlorophyll molecules. While phospholipids are only a minor constituent of chloroplast membranes, other lipid molecules could provide similar nucleophilic ligands. For example, the strong interaction of chlorophyll with alcohols suggests that galactolipids could coordinate with chlorophyll perhaps even more strongly than with phospholipids. In addition to serving as a means of separation, the interaction of chlorophyll *a* with lipid molecules could provide a means of ordering chlorophyll within the membrane. It is therefore interesting to note that a large portion of in vivo chlorophyll *a* is oriented with respect to the thylakoid membrane in a way which is consistent with its known orientation in model membranes. The ordering of chlorophyll within the membrane has important consequences with respect to its optical properties. This effect will be discussed in a future communication. There is as yet no conclusive proof as to whether or not chlorophyll *a*-lipid interactions such as we have observed actually occur in vivo, although we have recently found [24] that there is a population of chlorophyll in thylakoids with a motional state quite similar to that of the lipids.

Acknowledgments

This work was supported by the United States Public Health Service Grant GM-22432 and NIH National Research Service Awards GM-07616 and GM-01262 from the National Institute of General Medical Sciences. High-field ^{1}H-NMR spectra at 360 MHz were obtained at Stanford Magnetic Resonance Laboratory, which is supported by NSF Grant GP-23633 and NIH Grant RR-00711. ^{31}P-NMR spectra were recorded on a Varian XL-100 spectrometer in the laboratory of Professor J.H. Richards, whose kind generosity we gratefully acknowledge.

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