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LIPID-ASSOCIATED CHLOROPHYLL

EVIDENCE FROM ^{13}C-NMR OF THE PHOTOSYNTHETIC SPINACH THYLAKOID MEMBRANE *

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Summary

The ^{13}C-NMR spectrum at 90.5 MHz has been obtained for the photosynthetic thylakoid membrane of spinach. Specific lipid and chlorophyll resonances can be assigned in the high resolution spectrum, although protein resonances are not observed. It can be estimated from resonance intensities that at least 30% of the plant chlorophyll contributes to the high resolution ^{13}C spectrum with the remainder broadened by incomplete motional averaging. The resonance linewidths of the observed chlorophyll phytol chains are approximately the same as those of the lipid hydrocarbon chains, indicating a similar motional state and suggesting that this particular pool of chlorophyll is lipid-bound or at most only loosely associated with proteins.

Understanding the molecular architecture of the photosynthetic membrane is an essential step toward unraveling the details of its function. Although the spectroscopic heterogeneity [1] of chlorophyll *a* suggests that nature has found it expedient to diversify the process of light absorption and transduction by structural means, the organization and distribution of chlorophyll in the membrane is still an issue which is not totally settled. Much of our knowledge regarding the location of chlorophyll in the photosynthetic membrane has been

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based on studies [2—4] wherein one attempts to dissect the membrane with various detergents. Such studies indicate that much of the chlorophyll is associated with membrane proteins although a significant fraction is usually obtained as a protein-free pigment band by gel chromatography. While these studies are valuable, they suffer several disadvantages. Detergent solubilization must necessarily cause gross disruption of the membrane structure resulting in a loss of information concerning membrane organization. In addition, it is possible that membrane components become artifactually associated as a result of the detergent solubilization. Clearly, some of these difficulties can be alleviated by studying the organization of the photosynthetic membrane by in situ techniques. In particular, NMR allows one to investigate the structure and dynamics of a biological system such as the photosynthetic membrane without structural perturbations. In this paper we report ^{13}C-NMR studies of intact thylakoid photosynthetic membranes. Our results show that well-resolved spectra can be obtained from whole thylakoid membranes, with resonances which can be assigned to specific membrane components. The linewidths and apparent intensities of these resonances have allowed us to draw some limited conclusions concerning the organization of chlorophyll in the thylakoid membrane.

For these studies, thylakoid membranes from spinach were obtained by procedures [5] which do not alter their native structure. Whole chloroplasts were harvested from leaves of fresh spinach by homogenization in isotonic sucrose buffer and centrifugation to remove cell debris. Thylakoid membranes were isolated from the intact chloroplasts by osmotic lysis of the double outer envelope membrane in a hypotonic buffer medium. The thylakoids were pelleted by centrifugation and resuspended several times to effect separation of the thylakoid and envelope fractions. All buffer media contained cations (5 mM $MgCl_2$/75 mM NaCl/10 mM $Na_4P_2O_7$, pH 7.2) which prevent the dissociation and swelling of the thylakoid grana [6]. The optical spectrum of thylakoid membranes isolated in this manner was found to be essentially indistinguishable from that of whole chloroplasts.

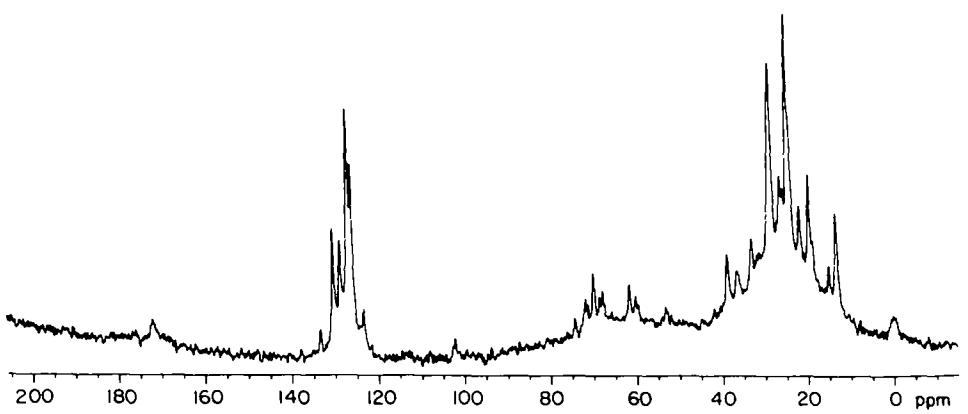

Fig. 1. Proton-decoupled natural abundance 90.5 MHz ^{13}C Fourier transform NMR spectrum of isolated spinach thylakoid membranes at 25°C. The sample is a loosely packed pellet of thylakoid membranes in ^2H_2O in a 10 mm tube. A total of 26 875 transients were accumulated and Fourier transformed with a 15 Hz exponential window to yield the above spectrum. The chemical shift scale is in ppm relative to tetramethylsilane.

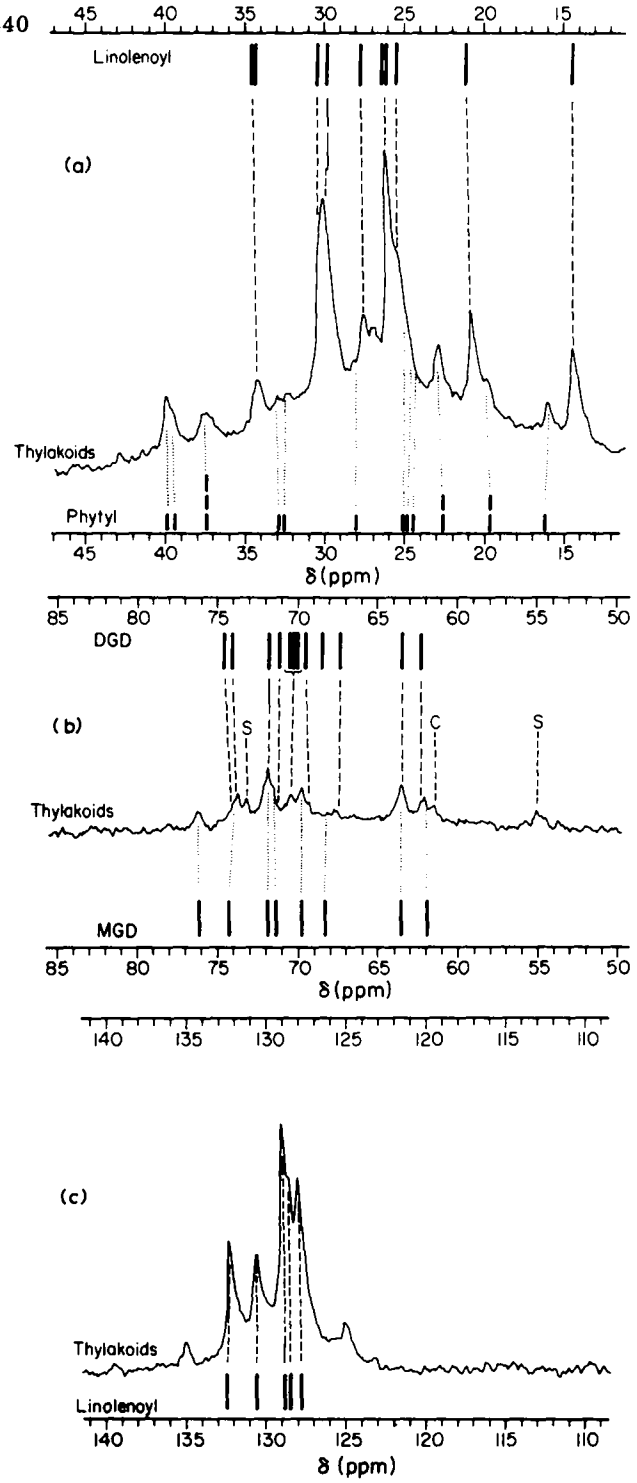

Fig. 2. Assignments of the ^{13}C-NMR spectrum from spinach thylakoids. The selected regions of Fig. 1 are expanded for ease of comparison. (a) Saturated carbon region (10—45 ppm). Positions of phytol and linolenic fatty acid resonances taken from Refs. 9 and 12 are indicated by vertical bars. (b) Galactolipid headgroup and glycerol backbone region (50—85 ppm). Positions of MGD and DGD resonances from Ref. 12 are indicated by the bars. The letters S and C indicate peaks assignable to sulfolipid (S) and chlorophyll (C). (c) Unsaturated carbon region (110—140 ppm). Positions of linolenic acid resonances from Ref. 12 are again indicated by bars. Resonances assigned but not shown: galactosyl C-1 of MGD (104.8 ppm); fatty acid carbonyls (172 ppm). Abbreviations: MGD, monogalactosyl diacylglycerol; DGD, digalactosyldiacylglycerol; linolenoyl, 9Z, 12Z, 15Z-octadecatrienoate.

Proton-decoupled Fourier transform ^{13}C spectra at 90.5 MHz of the resulting thylakoid preparation were obtained at 25°C on a Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory within 24 h of sample preparation. Transients were accumulated every 2.5 s using 16 K data points and a spectral width of 20 KHz.

The resulting ^{13}C-NMR spectrum of isolated spinach thylakoids, depicted in Fig. 1, shows a number of well-resolved resonances which may be identified by comparison with previous spectra [7,8] of chlorophyll *a* and other naturally occurring thylakoids lipids [9—11]. These assignments are indicated in Fig. 2a, b and c. Virtually all of the observed resonances may be assigned either to galactolipids, which are the predominant lipid constituent of the membrane [5], or to phytol chains of chlorophyll. The assignment of resonances is facilitated by the fact that polyunsaturated 16 : 3 and 18 : 3 fatty acids comprise about 90% of the esterified hydrocarbon chains of thylakoid lipids [12,13] and that resonances from the porphyrin headgroup of chlorophyll *a* are not observed because of the rigidity of the porphyrin macrocycle and the additional motional restriction imposed by association of the chlorophyll with the membrane. The relative intensities of the various galactolipid resonances are consistent with their known mole fractions in the membrane, although the intensities of the assigned chlorophyll resonances relative to those of the lipids are somewhat less than expected. Measurements of the absolute intensities of the chlorophyll phytol resonances indicate that not all of the chlorophyll is observable in the high resolution spectrum. By comparison of the spectral intensities of resonances in Fig. 1 with those of chlorophyll *a*/phospholipid model bilayer systems under equivalent instrumental conditions (after correction to equivalent concentrations and signal-to-noise ratios) it is estimated that a pool of 30 ± 10% of the total chlorophyll complement of the thylakoid is observed in the high resolution spectrum.

Notably absent are resonances from membrane proteins. This result is consistent with previous ^{13}C-NMR studies of biological membranes where the high resolution resonances arise from lipids rather than proteins [14]. Since lipid and protein are present in similar amounts this observation must be due to differences in the molecular motion of the two membrane fractions. ^{13}C relaxation mechanisms are predominantly intramolecular and therefore principally related to internal flexibility [15]. Thus, protein resonances are not observed (i.e. are broad) because of incomplete motional averaging resulting from restricted internal motion or long motional correlation times. On the other hand, the relatively narrow phytol resonances of chlorophyll must indicate more complete motional averaging. It may therefore be concluded that the motional state of the pool of chlorophyll observed in the ^{13}C spectrum is much different than the motional state of the protein, and that indeed it more closely approximates that of the lipid fraction of the membrane. Since the manner in which chlorophyll is bound to the membrane will influence both its lateral mobility and internal flexibility and, therefore, its overall motional state, the present observations may have some bearing on the question of how chlorophyll is distributed within the membrane.

Assume for the purpose of discussion that the observed chlorophyll is contained within membrane protein. This being the case, the chlorophyll must be

bound in such a way that the motional state of the phytol chain is not affected by the surrounding protein shell. If the chlorophyll were contained within a hydrophobic core which is sufficiently spacious to allow this type of motion, then aliphatic protein side-chains should have equally free motion. However, the lack of resonances from such groups in the ^{13}C spectrum suggests that this is not the case. Furthermore, in the case of the only chlorophyll containing protein for which the crystal structure has been determined, the authors concluded that both the porphyrin and phytol portions of chlorophyll are held firmly in position with little or no freedom of movement [16].

Since the motional state of the chlorophyll phytol chains observed here more closely resembles that of the lipid portion of the membrane, it is more reasonable to assume that this pool of chlorophyll is not embedded in protein, but rather is contained in the bilayer portion of the membrane or, perhaps, bound at the periphery of membrane protein with the phytol chains essentially free [17]. Such a conclusion is consistent with the observation of a free pigment band found in gel chromatography of detergent solubilized membrane and is further supported by evidence for the close association of galactolipids with chlorophyll in vivo [18—21]. The biological significance of this pool of chlorophyll is not certain, although studies of model systems of chlorophyll in lipid bilayers suggest that it can be involved as, or be a part of, a photosynthetic antenna.

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