Lipid-Protein Interactions in Thylakoid Membranes of Chilling-resistant and -sensitive Plants Studied by Spin Label Electron Spin Resonance Spectroscopy*

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Lipid-protein interactions in thylakoid membranes from lettuce, pea, tomato, and cucumber have been studied using spin-labeled analogues of the thylakoid membrane lipid components, monogalactosyl diglyceride and phosphatidylglycerol. The electron spin resonance spectra of the spin-labeled lipids all consist of two components, one corresponding to the fluid lipid bilayer and the other to motionally restricted lipids interacting with the integral membrane proteins. Comparison of the spectra from the same spin label in thylakoid membranes from different plants shows that the overall lipid fluidity in the membranes decreases with chilling sensitivity. Spectral subtraction has been used to quantify the fraction of the membrane lipids in contact with integral membrane proteins. Thylakoid membranes of cucumber, a typical chilling-sensitive plant, have been found to have a higher proportion of motionally restricted lipids and a different lipid selectivity for lipid-protein interaction, as compared with those of pea, a typical chilling-resistant plant. This correlation with chilling sensitivity holds generally for the different plants studied. It seems likely that the chilling sensitivity in thylakoid membranes is not determined by lipid fluidity alone, but also by the lipid-protein interactions which could affect protein function in a more direct manner.

Plants can be divided broadly into chilling resistant and chilling-sensitive categories (Quinn and Williams, 1985). Chloroplasts, which contain the most extensive membrane systems, have long been speculated to be the primary sites of chilling injury in plants. A generally observed phenomenon is that the thylakoids of chilling-sensitive plants have a higher proportion of saturation in their lipid chains, as compared with those of chilling-resistant plants (Murata, 1983; Orr and Raison, 1987). Similar tendencies have also been found in plants grown at different temperatures (see e.g. Miller et al., 1988). It has been proposed that chilling injury in plants is initiated by a thermally induced transition in the structure or phase state of some of the lipids which constitute the bilayers of the cell membranes (Murata and Yamaya, 1984; Raison and Orr, 1986). However, it has also been argued that a simple correlation of chilling sensitivity with fatty acid content and phase behavior of individual lipid classes, e.g. phosphatidylglycerol, is inappropriate, since fluidity of the whole membrane cannot be simply predicted from the saturated fatty acid contents and phase transition temperatures of the component polar lipids (Orr and Raison, 1987). Lipid mobility and overall membrane fluidity depends markedly on lipid-lipid and lipid-protein interactions in the membranes. The presence of membrane proteins can also modify the lipid phase behavior. For instance, the total lipid extracts from thylakoid membranes form nonbilayer structures in addition to normal bilayer lamellae, when dispersed in aqueous solution at physiological temperatures, whereas such structures are not normally present in the native membranes, i.e. when lipids and proteins are both present (see e.g. Quinn and Williams, 1985; Gounaris and Barber, 1983; Murphy, 1986).

Both the molecular mobility in lipid extracts and the overall fluidity of thylakoid membranes have been investigated by spectroscopic methods, using either fluorescent or spin-labeled fatty acid probes (Arnon et al., 1983; Waggner et al., 1985; Raison and Orr, 1986). However, relatively little work has been done on lipid-protein interactions (cf. Li et al., 1989), especially in relation to chilling sensitivity.

In the present work, we have investigated the lipid mobility and lipid-protein interactions in thylakoid membranes from plants of different chilling sensitivity. Spin-labeled monogalactosyl diacylglycerol and phosphatidylglycerol, in thylakoid membranes, are found to show a lower overall lipid mobility and a higher proportion of motionally restricted lipids with increasing chilling sensitivity of the plant. The fraction of motionally restricted lipid was found to be qualitatively in line with the protein/lipid ratios of the different thylakoid membranes.

MATERIALS AND METHODS

Spin-labeled phosphatidylglycerol, 14-PGSL, was prepared from the corresponding spin-labeled stearic acid and egg lyso phosphatidylcholine, which was subsequently transphosphatidylated to yield phosphatidylglycerol. The synthetic procedures are described by Marsh and Watts (1982). Spin-labeled monogalactosyl diacylglycerol, 12-MGDGSL, was a gift from Dr. Ikku Nishida, National Institute for Basic Biology, Okazaki, Japan, and was prepared as described by Nishida and Yamada (1985). This spin label contains a spin-labeled

1 The abbreviations used are: 14-PGSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-N-oxide)stearoyl]-sn-glycero-3-phosphoglycerol; 12-MGDGSL, 1-Oleoyl-2-[12-(4,4-dimethyloxazolidine-N-oxide)stearoyl]-sn-glycero-3-galactose; PS2, photosystem 2; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; ESR, electron spin resonance; MGDG, 1,2-diacyl-sn-glycero-3-galactoside; PG, 1,2-diacyl-sn-glycero-3-phosphoglycerol.

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photosystems, particularly those of tomato and lettuce described in Li et al. (1989). Thylakoids were resuspended in a buffer containing 10 mM EDTA, 1 mM MgCl₂, 0.5 mM K₂HPO₄, 5 mM NaH₂PO₄, 2 mM sodium ascorbate, and 10 mM tricine, pH 8.3 (adjusted with KOH).

Pea seedlings (*Pisum sativum* L. var. Kelvedon Wonder) were grown in a controlled growth room at 20°C. Leaves were harvested after a 12-h period in the dark, homogenized in buffer containing 300 mM glucose, 60 mM NaH₂PO₄, 60 mM KH₂PO₄, 5 mM MgCl₂ 26 mM NaCl, pH 6.5 (adjusted with KOH). Pea thylakoids were isolated as described in Li et al. (1989). Thylakoids were resuspended in a buffer (Buffer R) containing 330 mM sorbitol, 2 mM EDTA, 5 mM MgCl₂, 1 mM MnCl₂, 50 mM Hepes, pH 7.6 (adjusted with KOH).

Tomato (*Lycopersicon esculentum*) leaves were harvested in the early morning from a local greenhouse in which the temperature was kept at 28°C during the day and 16°C during the night. Tomato thylakoids were isolated by the same method as that used for pea thylakoids, except that the pH of the homogenizing buffer was 7.0 instead of 6.5.

Cucumber (*Cucumis sativus*) was grown under 100% relative humidity in a growth room in which the temperature was controlled between 25-30°C. Leaves were harvested after a 12-h period of dark and 1 h of light. Cucumber thylakoids were isolated essentially according to the method of Walden and Leaver (1981). The buffer used for both homogenization of the leaves and resuspension of the thylakoids contained 330 mM sorbitol, 5 mM MgCl₂, 1 mM MnCl₂, 50 mM Tris, pH 7.0 (adjusted with HCl).

Thylakoids were kept in the dark at 4°C before use (less than 1 h). Chlorophyll a/b ratios of the thylakoids and those of acissor-minced leaves were measured by the method of Arnon (1949). Values for the thylakoid preparations were similar to those of the minced leaves (approximately 2.4 for lettuce, pea and cucumber, and 2.6 for tomato). Any preparation with an appreciably different value was discarded. Tomato thylakoid preparations with high starch contents were also discarded. The protein content of the thylakoid preparations was measured by the method of Markwell et al. (1981), and the lipid content was determined by gas chromatographic analysis as described in Murphy et al. (1989).

Total lipids (including chlorophyll) were extracted from pea thylakoids using a modification of the procedure of Waggner et al. (1985), as described in Li et al. (1989). Polar lipids were separated from chlorophyll and other nonpolar lipids by chromatography on silica gel as described by Li et al. (1980). All fractions eluting after the pigments and other nonpolar lipids were pooled as polar lipids. These correspond to monogalactosyl diacylglycerol and all other thylakoid lipids of higher polarity.

All thylakoid membranes were washed once before use with R buffer without the 1 mM MnCl₂. For spin labeling, thylakoid membranes comprising approximately 1-2 mg of polar lipids were suspended in 2-3 ml of 20 mM MES, 50 mM KCl, 5 mM MgCl₂, pH 6.5 (adjusted with KOH), and 10-20 μl of 1 mg/ml spin label solution in ethanol was added slowly. (The relative spin label concentration was ~1% w/w with respect to total polar lipid and the volume of ethanol was less than 1% of the total buffer volume.) The spin-labeled samples were then briefly vortexed and incubated for approximately 15 min prior to centrifugation and packing into glass capillaries (1 mm, inner diameter). Samples were covered with dark paper or aluminum foil during handling, to minimize light-induced ESR signals.

ESR spectra were recorded on a Bruker ER200 9 GHz spectrometer equipped with a nitrogen gas flow system for temperature control to within ±0.3°C. The sample capillaries were accommodated within a standard 4-mm quartz ESR tube which contained light silicone oil for thermal stability. Spectral subtraction was performed essentially as described by Marsh (1982).

**RESULTS**

The Mn⁺⁺ ions of the water-splitting complex of thylakoids give rise to an ESR signal which overlaps with the spin label spectrum. This background signal was removed from the lipid spin label spectra by spectral subtraction using unlabeled membrane samples. The light-induced ESR signals from the photosystems, particularly those of tomato and lettuce thylakoid samples, were similarly removed.

Representative ESR spectra of the 12-MGDGSL spin label in the different thylakoid membranes are shown in Fig. 1, and those of the 14-PGSL spin label are shown in Fig. 2. As in many other lipid-protein systems studied (see e.g. Marsh, 1983), the spectra of either spin label consist of two components, corresponding to lipid environments of different mo-
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Fig. 3. Spectral subtraction with the 12-MGDGSL spin label in cucumber thylakoid membranes at 36°C. a, fluid component difference spectrum, obtained by subtracting a motionally restricted spectrum from the total spectrum from the thylakoid membrane given in b. This component comprises 33% of the total integrated intensity (double integral) of spectrum b. b, experimental (two component) spectrum from thylakoid membranes. c, motionally restricted component difference spectrum, obtained by subtracting a pure lipid spectrum from spectrum b. This component comprises 67% of the total integrated intensity of spectrum b. Total scan width = 100 gauss.

Fig. 4. Spectral subtraction with the 14-PGSL spin label in cucumber thylakoid membranes at 18°C. a, fluid component difference spectrum, obtained by subtracting a motionally restricted spectrum from the total spectrum from the thylakoid membrane given in b. This component comprises 33% of the total integrated intensity of spectrum b. b, experimental (two component) spectrum from thylakoid membranes. c, motionally restricted component difference spectrum, obtained by subtracting a pure lipid spectrum from spectrum b. This component comprises 67% of the total integrated intensity of spectrum b. Total scan width = 100 gauss.

Table I

<table>
<thead>
<tr>
<th>Plant</th>
<th>L/Chl/P</th>
<th>12-MGDGSL</th>
<th>14-PGSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucumber</td>
<td>0.37/0.14/1</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>Tomato</td>
<td>0.30/0.13/1</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Pea</td>
<td>0.57/0.14/1</td>
<td>0.36</td>
<td>0.53</td>
</tr>
<tr>
<td>Lettuce</td>
<td>0.67/0.25/1</td>
<td>0.25</td>
<td>0.49</td>
</tr>
</tbody>
</table>

*L, lipid; Chl, chlorophyll; P, protein.

Previous results have also shown that there is little difference between the proportions of motionally restricted lipid recorded by labels at different positions in the chain of a given lipid (Pates and Marsh, 1987).

The differences in lineshapes of the fluid and motionally restricted components, and their temperature dependence, indicate the relative lipid mobilities in the thylakoid membranes of the plants with different chilling sensitivities.

Using spectral subtraction and intersubtraction methods (for details see Marsh, 1982; Li et al., 1989), the two spectral components of both spin labels in cucumber thylakoid membranes have been quantitated (see Figs. 3 and 4). Comparison of the fluid and motionally restricted components for cucumber thylakoids can be made with those for pea thylakoids given in Fig. 5 of Li et al. (1989), and is discussed later. The results show that in cucumber thylakoid membranes the proportion of motionally restricted 12-MGDGSL and 14-PGSL is 67% of the total for both labels. This value is considerably greater than those found in the other three thylakoid membrane systems. For pea thylakoid membranes, for example, spectral subtraction yields values of 36 and 53% motionally restricted lipid for 12-MGDGSL and 14-PGSL, respectively. These values, f, for the fraction of motionally restricted 12-MGDGSL and 14-PGSL spin labels, in the different thylakoid membranes, are summarized along with the lipid/chlorophyll/protein ratios in Table I. The values reflect the qualitative trends that are clear from Figs. 1 and 2, namely that the proportion of motionally restricted lipid decreases in the order cucumber > tomato > pea > lettuce, particularly for the 12-MGDGSL label. Subtractions performed with the 14-PGSL label in pea thylakoid membranes showed that the proportion of motionally restricted lipid did not vary appreciably with temperature over the range 12–24°C, in agreement with previous results from other systems (see e.g. Ryba et al., 1987). The temperature dependence between the proportions of motionally restricted lipid recorded by labels at different positions in the chain of a given lipid (Pates and Marsh, 1987). The differences in lineshapes of the fluid and motionally restricted components, and their temperature dependence, indicate the relative lipid mobilities in the thylakoid membranes of the plants with different chilling sensitivities. The temperature dependence of the spectra from the 14-PGSL label in pea thylakoid membranes is given in Fig. 5a. The fluid component lineshape displays a much steeper temperature dependence than that of the motionally restricted component. This is expected, since the latter lies in the slow motion regime of nitroxide ESR spectroscopy for which the spectra are less sensitive to changes in mobility. The temperature dependence, therefore, indicates very clearly the difference in mobility between the fluid and motionally restricted lipid populations. The former corresponds to rotational correlation times in the nanosecond time range, whereas that for the latter corresponds to values closer to 10 ns (cf. Freed, 1976).

The temperature dependence of spectra from 14-PGSL in cucumber thylakoid membrane is given in Fig. 5b. It can be seen that the larger proportion of the motionally restricted component and the lower mobility of the fluid component relative to the spectra from pea thylakoids (Figs. 2 and 5a)

Capabilities in all the thylakoid membrane systems investigated.

One component, in the central region of the spectrum, corresponds to spin labels in a fluid lipid environment, and the other, which is visible in the outer wings (i.e. the low field and high field features) of the spectrum, corresponds to lipids whose motion is restricted by interaction with the integral membrane proteins. In general, the spectra indicate that both the overall lipid mobility and the proportion of lipids that are motionally restricted differ among the thylakoid membranes of the plants with different chilling sensitivity.

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FIG. 5. Temperature dependence of the ESR spectra of 14-PGSL in thylakoid membranes from pea (a) and cucumber (b). Total scan width = 100 gauss.

FIG. 6. Temperature dependence of the effective rotational correlation time, \( \tau_\text{eff} \), deduced from the fluid component in the spectra of the 14-PGSL phosphatidylglycerol spin label in pea thylakoid membranes (x) and in dispersions of the extracted thylakoid membrane lipids (+).

hold throughout the entire temperature range. There is a gradual increase in mobility of the fluid component with increasing temperature rather than abrupt changes in the chilling-sensitive range. Noticeably, however, the mobility of the fluid component is much reduced, relative to pea, in the chilling-sensitive range and is situated wholly within the slow motional regime of ESR spectroscopy.

The spectra of the fluid component at the higher temperatures correspond to rapid, near-isotropic rotation. Under these circumstances, the effective rotational correlation time can be approximated from the relative linewidths (or lineheights) using motional narrowing theory appropriate to isotropic motion (see e.g. Marsh, 1981) as follows,

\[
\tau_\text{eff} = 0.6 \times \frac{H(0)}{\left[ \sqrt{h_L/h_H} - \sqrt{h_H/h_L} \right]} \text{ ns} \quad (1)
\]

where \( H(0) \) is the linewidth of the central hyperfine line (in gauss) and \( h_L, h_H, \) and \( h_{\perp} \) are the heights of the low field, central, and high field hyperfine lines, respectively. The temperature dependence of these values deduced from the fluid component in the spectra of 14-PGSL in thylakoid membranes from pea and in dispersions of the corresponding extracted membrane lipids is given in Fig. 6. The effective rotational correlation times are seen to decrease with increasing temperature, corresponding to increasing lipid fluidity in the membrane. At the lower temperatures, the rotational correlation times become slower and begin to enter the slow motional regime, probably with accompanying anisotropy of the motion. For this reason, the correlation times in Fig. 6 are best considered as effective values, but nonetheless serve very well for a qualitative analysis. Comparison of the effective correlation times of the fluid component in membranes and in the extracted membrane lipids shows that the consequence of lipid-protein interactions is an increase in the effective correlation time, i.e. a decrease in the lipid fluidity. (Similar effects have been seen in reconstituted lipid/protein systems (Knowles et al., 1979).) At the higher temperatures, the effective correlation times are under 1 ns and therefore lie in the fast motional regime, and the motion is close to isotropic (cf. Schreier et al., 1978).

The fluid component difference spectra of 14-PGSL in thylakoid membranes from cucumber, pea, and lettuce are compared in Fig. 7. The higher lipid mobility in the chilling-resistant thylakoid membranes (Figs. 7, b and c) than in the chilling-sensitive membranes (Fig. 7a) is clearly seen from the difference in linewidths between these two sets of spectra. By comparison, the spectra of lipid extracts from cucumber, pea and lettuce thylakoids shown in Fig. 8 are closely similar. It can be seen that for lettuce which has the highest lipid/
A number of different studies have shown a correlation between chilling sensitivity of various plants and the fatty acid composition of their acyl lipids, particularly that of phosphatidylglycerol (e.g. Murata and Yamaya, 1984; Roughan, 1986; Toriyama et al., 1986). Whereas the lipid chain composition will directly affect the overall lipid mobility in the thylakoid membrane, as seen from the ESR spectra of the fluid spin label component, it is unlikely that this will have such a pronounced effect on the lipid-protein interactions as is found here. Results with other lipid-protein systems (see e.g. Marsh, 1985), suggest that the latter will be determined principally by the protein/lipid ratio and the protein composition in the membrane. It thus seems probable that lipid-protein interactions, in addition to overall membrane fluidity, play an important role in the chilling sensitivity of plant thylakoid membranes. Therefore this former aspect is now analyzed in more detail.

The fraction of motionally restricted lipid, \( f \), is expected to be related to the surface area of the integral protein complexes in the thylakoid membrane. Assuming mean molecular masses of 0.9 and 623 kDa for the lipid and protein + chlorophyll complexes, respectively (Murphy, 1986), the mean lipid/protein mole ratios, \( n_{\ell} \), calculated from the compositional data given in Table I are: 225, 185, 345, and 375:1 for the thylakoid membranes of cucumber, tomato, pea, and lettuce, respectively. Taking the values of \( f \) for 12-MGDGSL from Table I would then imply that, overall, approximately 150, 100, 125, and 95 lipids per protein are motionally restricted in the thylakoid membranes of cucumber, tomato, pea, and lettuce, respectively. This estimate ignores lipid selectivity, which is justified to a first approximation, since MGDG is one of the majority lipid components of thylakoids and also displays the lesser selectivity. The similarity between these estimates of the mean number of motionally restricted lipids suggests that the principal reason for the differences in the fraction of the lipids which are motionally restricted lies in the different protein/lipid ratios of the thylakoid membranes of the plants of different chilling sensitivity.

The composition of the motionally restricted lipid population associated with the membrane proteins is reflected by the selectivity found between the 12-MGDGSL and 14-PGSL spin labels. If the fraction of the total membrane lipids that is of type L is designated by \( F_L \), the average number of motionally restricted lipids of type L can be approximated by

\[
\eta_L = f_L n_{\ell}
\]

where \( \eta_L \) is the fraction of motionally restricted lipids of type L. These data for the different thylakoid membranes are given in Table II, where values of \( F_{\text{MGDG}} = 0.42 \) and \( F_{\text{PG}} = 0.11 \) are taken from Chapman et al. (1983). Even after this correction for the differences in protein/lipid ratio, it is found that relatively more MGDG molecules than PG molecules are motionally restricted in pea and lettuce thylakoid membranes than in those of cucumber. The thermodynamic implications of the lipid selectivity may be expressed in terms of the relative association constants which

<table>
<thead>
<tr>
<th>Plant</th>
<th>12-MGDGSL</th>
<th>14-PGSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucumber</td>
<td>63 ( \eta_L )</td>
<td>17 ( K_{L}(L)/K_{L}(MGDG) )</td>
</tr>
<tr>
<td>Tomato</td>
<td>43 ( \eta_L )</td>
<td>20 ( K_{L}(L)/K_{L}(MGDG) )</td>
</tr>
<tr>
<td>Pea</td>
<td>52 ( \eta_L )</td>
<td>20 ( K_{L}(L)/K_{L}(MGDG) )</td>
</tr>
<tr>
<td>Lettuce</td>
<td>39 ( \eta_L )</td>
<td>20 ( K_{L}(L)/K_{L}(MGDG) )</td>
</tr>
</tbody>
</table>

DISCUSSION

The above results indicate that the thylakoid membranes of plants with different chilling sensitivities exhibit different lipid-protein interactions, as well as having different overall lipid mobility. Both the overall fluidity of the membranes and the size of the fluid lipid population is found to lie in the order: cucumber (growth temperature 25-30 °C) < tomato (growth temperature 16-28 °C) < pea seedling (growth temperature 20 °C) < lettuce (field grown). In general, the mobility of the fluid lipid component increases in the order: cucumber < tomato < pea < lettuce, in parallel with the degree of chilling resistance.

**FIG. 5.** ESR spectra of the 14-PGSL phosphatidylglycerol spin label in aqueous dispersions of lipid extracts of: a, cucumber; b, pea; and c, lettuce thylakoids. Total scan width = 100 gauss; \( T = 18 \) °C.
are given (see e.g. Marsh, 1985) as follows,

\[ K_r(L) = \left( n_r/N_r - 1 \right) \times \left[ f_r/(1 - f_r) \right] \]

(2)

where \( N_r \) is the mean number of motionally restricted lipids surrounding the protein complexes. Hence for the lipids L and MGDG, in a given membrane, the ratio of the relative association constants is given as follows.

\[ K_r(L)/K_r(MGDG) = [f_r/(1 - f_r)]/[f_MGDG/(1 - f_MGDG)] \]

(3)

These ratios are given in Table II and are independent of the values of \( n_r \) and \( N_r \). The results clearly reflect the selectivity of PG relative to MGDG in pea and lettuce thylakoid membranes and the lack of selectivity in the case of cucumber. Such differences almost certainly arise from differences in protein composition, or in the sequence and structure of equivalent functional protein complexes, of the thylakoid membranes from the chilling-resistant and chilling-sensitive plants.

The results of these studies suggest possible ways in which lipid-protein interactions may affect chilling sensitivity in plant thylakoid membranes. Firstly, the motionally restricted lipids surrounding the membrane proteins provide the interface between the fluid bilayer regions of the membranes and the functional protein units in the membrane. Therefore, this lipid population mediates the effects which changes in membrane fluidity may have on the efficiency of photosynthesis. Secondly, the overall lipid mobility of the membranes depends on the lipid-protein interactions to an extent which is determined by the lipid/protein ratio. Thirdly, the proportion of lipids that are motionally restricted, which differs between the various membranes, may also affect the chilling sensitivity. When the fluid lipid population is smaller, it is conceivable that it may not possess sufficient buffering capacity to mediate against the effects of changes in temperature. Finally, differences in lipid selectivity may play a significant role. A functional association of phosphatidylglycerol with the light harvesting chlorophyll-protein complexes of the thylakoid membrane has been suggested (Trémolières et al., 1981; Rémy et al., 1982). It is possible that specific lipids might stabilize particular protein complexes against changes in temperature or lipid fluidity.

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