Localization of Spin Labels in Oat Leaf Protoplasts

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ABSTRACT

An assay based on light-mediated oxidation was used to determine whether specific spin labels were partitioned throughout the protoplast or retained in the plasma membrane of Avena sativa L. cv. Garry and Park. Many classes of spin label were tested, including phospholipids, fatty acid, fatty acid methyl ester, maleimide, iodoacetamide, short chain hydrocarbon, androstane, 2,2,6,6-tetramethyl-4-aminopiperidinoxy (TEMPAMINE) and 2,2,6,6-tetramethylpiperidinoxy (TEMPO). All except the phosphotidylcholine spin label were found to partition throughout the cell. The phosphotidylcholine spin label may have been selectively retained in the plasma membrane.

Changes in the plasma membranes of higher plants have been proposed as the basis of many cellular phenomena. Examples are development of cold tolerance, salt tolerance, and senescence. Also, changes in membrane structure may result from exposure to hormones, pathogens, radiation, and pressure. Usually, membrane structure has been studied with fixed tissues or with isolated membranes, precluding direct measurement of membrane changes in living cells.

Spin labels have been used to study the plasma membrane of living cells. However, to selectively probe the plasma membrane of an intact protoplast, the probe must be reporting only from the plasma membrane. This condition can be met if the probe is only present in the plasma membrane or is spectroscopically inactive everywhere except in the plasma membrane. Kaplan et al. (14) claimed that by adding K2Fe(CN)6 (a spin label-activating agent) to a labeled cell suspension, only the spin-label in the plasma membrane gave an ESR signal. The assumptions that spin labels cannot move quickly between membranes and that different membranes would create different spin label spectra are also used to support the contention that fatty acid spin labels are good probes for the plasma membrane (2–4, 7, 10, 13, 24, 25). However, some investigators dispute these assumptions (12, 16), arguing that most spin labels report an average from every membrane in the cell.

The objectives of this report were to: (a) evaluate whether or not fatty acid spin labels are selective for the plasma membrane of plant protoplasts; (b) develop criteria for ascertaining the selectivity of a spin label for the plasma membrane; and (c) test various classes of spin label and identify those which may have selectivity for the plasma membrane.

MATERIALS AND METHODS

Avena sativa L. cv. Garry and Park were grown in the laboratory at 22°C in vermiculite irrigated with White's nutrient solution (8). Fluorescent and incandescent bulbs provided a 16-h photoperiod. Primary leaves were harvested from 1- to 2-week-old seedlings.

Protoplasts were isolated by peeling away the lower epidermis of the leaves with forceps and floating the leaves (peeled surface down) on a solution composed of 0.5% Cellulysin (Calbiochem-Behring Corp.) and 0.6 m sorbitol, adjusted to pH 5.6 with KOH. The preparation was incubated at 28°C for 3 h in the light, then was swirled gently to release protoplasts. The protoplasts were filtered through a layer of Miracloth and the suspension was centrifuged at 40g for 10 min. The supernatant was removed and the pellet was washed (by centrifugation) in a suspension medium containing 0.6 m sorbitol and 10 mm CaCl2 (pH 5.7). Protoplasts were lysed by forcing through a 26-gauge syringe needle at room temperature into a test tube in an ice bath. The lysate was centrifuged at 2,500g for 2 min. The lysate supernatant was decanted and the chloroplasts were resuspended and washed in a solution containing 0.6 m sorbitol, 10 mm CaCl2, and 10 mm PIPES2 (pH 6.8). The lysate supernatant was centrifuged at 75,000g for 90 min. A white pellet was obtained, which will be referred to as nongreen membranes.

The following spin labels were purchased from Syva Associates, Palo Alto, CA: 12(12,3); I(1,14); Me-I(5,10); maleimide probe; iodoacetamide probe; TEMPO; cholestane probe; and androstane probe. PC(5,10) was obtained from Serdary Research Laboratories, London, Ontario, Canada. The short-chain hydrocarbon probes 2N8 and 5N10 were gifts from J. Raison, Macquarie University, North Ryde, Australia. Charles Caldwell, Michigan State University, kindly provided the TEMPAMINE.

Protoplasts (approximately 2 mg Chl/ml) or chloroplasts (approximately 4 mg Chl/ml) in thick slurry suspensions were labeled by adding 0.2 ml to a test tube containing a dry film of the selected spin-probe and gently rotating for 5 min at 23°C. The films for labeling chloroplasts and protoplasts were derived from 10 or 20 μl, respectively, of 30 mm spin label in ethanol, blown dry with air. Final spin label concentrations were approximately 2 mm.

2 Abbreviations: PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); I(12,3), 2-[3-carboxypropyl]-4,4-dimethyl-2-tridecyl-3-oxazolidin-3'-yloxy; I(1,14), 2-[4-(carboxytridecyl)]-2-ethyl-4,4-dimethyl-3-oxazolidin-3'-yloxy; Me-I(5,10), 2-[10-carboxydecyl]-2-hexyl-4,4-dimethyl-3-oxazolidin-3'-yloxy methyl ester; maleimide probe, 4-maleimido-2,2,6,6-tetramethylpiperidinoxy; iodoacetamide probe, 4-(2-iodoacetamide)-2,2,6,6-tetramethylpiperidinoxy; TEMPO, 2,2,6,6-tetramethylpiperidinoxy; TEMPAMINE, 2,2,6,6-tetramethyl-4-aminopiperidinoxy; cholestane probe, 4',4'-dimethylthylsipro [5α-cholestano-3,2'-oxazolidin]-3'-yloxy; androstane probe, 17β-hydroxy-4',4'-dimethylthylsipro [5α-androstano-3,2'-oxazolidin]-3'-yloxy; 2N8, 2-methyl-2-hexyl-4,4-dimethyl-3-oxazolidin-3'-yloxy; PC(5,10), distearoylphosphotidylcholine labeled at carbon 12 in the β-chain; ESR, electron spin resonance; P700+, oxidized state of PSI reaction center.

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Incorporation of probe PC(5,10) was difficult. First, dispersions were prepared by adding 0.5 ml suspension medium to a tube containing a dry film (25 μl of a 2.25-mM chloroform solution, blown dry with air) of the probe. The suspension was shaken and then sonicated with either a bath or tip-type sonicator. The dispersed spin label was mixed with 0.5 ml of protoplasts and incubated for 30 min at 23°C; free label was then washed away by centrifugation. Next, the samples were pipetted into Varian low temperature quartz cuvettes which were placed in the Dewar and scanned.

Samples were irradiated in the ESR cavity with a Hanovia 1000 W Xe-Hg arc lamp. Light was filtered through 5 cm of H₂O or through 5 cm H₂O plus a 739 nm interference filter, giving light intensities at the cavity of 2.7 x 10⁶ and 2.7 x 10⁷ ergs cm⁻² s⁻¹, respectively. A shutter was used to turn the light on and off. ESR spectra were recorded with a Varian model E-112 X-band spectrometer. Spectrometer settings for use with TEMPAMINE were: scan range, 100 G; field set, 3,260 G; time constant, 0.064 s; scan time, 4 min; modulation amplitude, 0.5 G; modulation frequency, 100 KHz; receiver gain, 1.6 x 10²; microwave power, 5 mw; microwave frequency, 9.1431 GHz. Spectrometer settings for TEMPO were the same as in Figure 5 and settings for I(1,14), Me-I(5,10), 5N10, and the androstane and cholestane probes were as in Figure 1. The sample temperature was controlled by a Varian variable temperature controller and was monitored by an Omega model 250 thermostouple positioned within the cuvette.

Relative ESR signal intensities were determined by dividing the peak-to-peak height of the mid-field line by the amplifier gain. Lineshape was analyzed in terms of the maximum hyperfine splitting parameter, 2T₁ (22), order parameter, S (22), rotational correlation time, τ (14), or partitioning parameter, F (23).

RESULTS

I(12,3) in the Dark. A typical ESR spectrum of oat leaf protoplasts labeled with I(12,3) is shown in Figure 1. This spin label probes membranes in the region just below the phospholipid head group. Since T₁ and Tᵢ in the hyperfine splitting pattern can both be distinguished, the motion of the fatty acid is a rapid rotation about the long molecular axis (S = 0.65). The hyperfine coupling constant (γ''D) was determined to be 15.46 G, indicating that the nitroxide is in a relatively nonpolar environment (18). The data suggest that the fatty acid spin label is intercalated between the lipids of the membrane with the long axis of the fatty acid parallel to the lipid acyl chains. At low cell density, free label 'liquid lines' appear in the spectra as a result of spin label partitioning into the aqueous phase, where it undergoes rapid, isotropic motion (data not shown).

The spin label signal intensity from protoplasts in the ESR cavity at 23°C gradually decayed (Fig. 1). This phenomenon has been observed in other cell types; the decrease in signal intensity is attributed to chemical reduction of the nitroxide to the corresponding hydroxylamine by respiratory metabolites (20). There was also an increase in the maximum hyperfine splitting parameter, 2T₁, and a broadening of the 2T₁ peaks. This apparent increase in membrane viscosity from a 2T₁ value of 56 to 62 G at constant temperature corresponded to the increase observed upon lowering the temperature from 23°C to 3°C (data not shown). 2T₁ became too broad to detect, suggesting that rotation about the long molecular axis was severely restricted. The rates of change of microviscosity and signal intensity were the same. Peak height reduction due to line broadening could not account for the observed decreases in signal intensity.

Different results were obtained when a series of samples from one preparation were examined (Fig. 2). The samples on the bench and in the ESR cavity were kept at 23°C. Each sample, once placed in the cavity, behaved as shown in Figure 1, i.e. there was an increase in 2T₁. The sample did not seem to change while it sat on the bench, whether it was held in a cuvette or in an open test tube. When a sample was removed from the cavity, the original signal was gradually restored (data not shown), suggesting that the environment in the cavity caused a change in the membrane viscosity. In every case, the hyperfine splitting (2TI) increased, as the signal intensity decreased.

Turning off the magnets and klystron eliminated the possibilities that the magnetic field or microwaves might be causing the change. Another possibility was that the N₂ gas constantly flushing the cavity was involved. However, flushing with air yielded the same result.

I(12,3) in the Light. The possibility that the lack of light in the cavity was responsible for the ESR signal changes was tested by irradiating the sample in the cavity with white light. Illumination caused a rapid increase in the signal intensity; when the light was turned off, there was a decrease (Fig. 3). The chemical reduction of the nitroxide in the dark appeared to be reversed when the protoplasts were irradiated. However, there was also a gradual reduction of the signal intensity in the light. The apparent increase
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in microviscosity in the dark also was reversed by light (data not shown). These results suggest that I(12,3) is penetrating through the cellular membranes to a site of light-mediated oxidation in chloroplasts.

To determine if the oxidation of the spin label upon illumination is the result of PSI activity, spin-labeled protoplasts were placed in the ESR cavity and allowed to undergo the dark-induced changes. They were then irradiated with far-red (729 nm) light. Far-red light reversed both the increase in microviscosity and the decrease in signal intensity just as did white light (data not shown). The rate of response to far-red light was less than the rate of response to white light, probably because of the greater intensity of the white light. The response to far-red light was not reversed with red light and required continuous irradiation to be sustained.

Ascorbate Reduction of I(12,3). The kinetics of spin label reduction by ascorbate are used to determine whether spin label is restricted to the surface of the cell (21). When oat leaf protoplasts were spin labeled with I(12,3), cooled to 0°C, and then exposed to ascorbate, the kinetic data for loss of the signal intensity were distinctly biphasic (Fig. 4). The time elapsed between initial exposure of the cells to ascorbate and the initiation of the second phase of signal loss (21 min), is identical to the half-life for penetration of ascorbate into electric eel membrane vesicles at 0°C (17). This suggests that two different populations of spin label are being sequentially reduced: the first may be in the outer half of the plasmalemma, whereas the second is in the inner half and in the intracellular membranes. When the temperature of the sample was allowed to increase after exposure to ascorbate, the break in the reduction kinetics was not observed. Irradiation of the protoplasts with white light at 23°C rapidly regenerated the ESR signal (data not shown). These results and those obtained with far-red irradiation indicate that fatty acid spin labels may not be selectively retained in the plasmalemma.

Cellular Distribution of I(12,3). Protoplasts which were precooled to 0°C were resistant to mechanical lysis. Therefore, pro-

<table>
<thead>
<tr>
<th>Sample</th>
<th>2T1 Light</th>
<th>2T1 Dark</th>
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<tbody>
<tr>
<td>Protoplasts</td>
<td>56.4</td>
<td>62.0</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>56.8</td>
<td>57.5</td>
</tr>
<tr>
<td>Nongreen membranes</td>
<td>63.5</td>
<td>63.5</td>
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</tbody>
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FIG. 4. Effect of ascorbate on spin label signal intensity from I(12,3)-labeled protoplasts. After labeling, the sample was cooled to 0°C, exposed to 10 mM ascorbate, and repeatedly scanned in the dark. A break in the line is seen at 21 min after addition of ascorbate (indicated by arrow).

Table 1. I(12,3) Spin Label Motion (Microviscosity) in Protoplast, Chloroplast, and Nongreen Membranes in the Light and Dark at 23°C
Localization of Spin Labels

Plants may be the site of light-mediated oxidation of spin label within protoplasts (6). Therefore, oxidation of spin label was used as an assay of spin label location. Any probes which were oxidized in the light were presumed to be distributed throughout the cell (i.e. in the chloroplasts).

I(1,14) and Me-I(5,10) in the Light and Dark. The spin labels I(1,14) and Me-I(5,10) probe the central region of the hydrocarbon interior of membranes. The motion of these probes was observed to be much greater than I(12,3), indicating that the fluidity of the bilayer is greater in the hydrocarbon interior. This is consistent with studies of model bilayer membranes and with microbial and animal membranes (11). The effect of dark incubation followed by irradiation with white or far-red light on the motion and redox states of these probes was similar to the results obtained with I(12,3) (data not shown). Therefore, these probes were probably distributed throughout the cell.

Iodoacetamide and Maleimide Probes in the Light and Dark. Spin label derivatives of iodoacetamide and maleimide are used to covalently label proteins by reacting with amino and thiol groups. These labels, when mixed with protoplasts, gave spectra characteristic of rapid, isotropic motion (Fig. 5). However, the motion was partially restricted as shown by rotational correlation times which were longer in a slurry of protoplasts (22 × 10⁻¹¹ s) than in the suspension medium alone (5 × 10⁻¹¹ s). The isotropic hyperfine coupling constant (a₀ = 16.25 G) indicated that the labels were in a polar environment. When protoplasts labeled with either the iodoacetamide or the maleimide spin label were incubated in the dark, the rotational correlation times increased (Fig. 5) and the signal intensity decreased. Irradiation with far-red light reversed this trend, indicating that the probes had penetrated the cells.

TEMPAMINE and TEMPO in the Light and Dark. A spin label which is restricted to the aqueous phase and does not bind proteins, TEMPO, was reduced by protoplasts in the dark. When the sample was irradiated, however, there was no effect on the spin label signal. Addition of 1 mm K₃Fe(CN)₆ to the dark-reduced sample increased the signal intensity 3-fold, showing that the probe had only been reduced, not destroyed. In contrast, TEMPO (which lacks the amino group) was oxidized in the light by protoplasts.

Isolated chloroplasts are able to react with TEMPAMINE in the light (6), so protoplasts have a potential site of interaction.

The possibility that TEMPAMINE was excluded from the cytoplasm is unlikely; 80 mm K₃Fe(CN)₆ eliminated only 92% of the ESR signal (via exchange-broadening [5]) from a freshly labeled sample of protoplasts. Presumably, the remaining 8% of the signal comes from the cell interior. We cannot explain why protoplasts fail to react with this probe in the light. Perhaps the site of interaction in isolated chloroplasts is unavailable in chloroplasts in situ.

The rotational correlation time (τ) of TEMPAMINE inside the protoplasts was 7.35 × 10⁻¹¹ s. This is only slightly greater than that observed in the absence of 80 mm K₃Fe(CN)₆, 6.88 × 10⁻¹¹ s, or in wash medium alone, 6.05 × 10⁻¹¹ s. Other workers have made similar observations of the milieu inside chloroplasts (5), and find that the internal viscosity is approximately 10-fold greater than that of water. The much greater effect seen with thylakoids may be caused by the greater surface-to-volume ratio of membrane-to-internal aqueous phase; the membranes may be ordering the water (5).

2N8 in the Light and Dark. The spin label 2N8 partitions between the aqueous and hydrocarbon phases of membranes. The ESR signals from each of these two phases can be distinguished from each other. When the membrane becomes more fluid, the partition coefficient of the spin label changes such that the fraction of probe in the membrane increases. This is reflected by a change in the relative peak heights of the two ESR signals. A spectrum of 2N8-labeled protoplasts (Fig. 6) shows a high field line composed of components from the hydrocarbon (h) and polar (p) phases. The partition parameter, F, [F = h/(h + p)] increased when the labeled protoplasts were incubated in the dark (Fig. 6). There was a rapid decrease in F when the sample was irradiated. This effect is opposite to that observed with the other spin labels, where microviscosity increased in the dark and decreased in the light. However, the signal intensity followed the same pattern as the other spin labels by decreasing in the dark and increasing in the light (data not shown).

5N10 in the Light and Dark. A similar spin label, 5N10, partitioned completely into the membrane phase. Using the rotary...
tional correlation time rather than $F$ as an indicator of microviscosity, we observed that changes in both signal intensity and microviscosity reported by 5N10 followed the same pattern as the fatty acid probes.

**Androstane in the Light and Dark.** A spin-labeled analog of androstane was incorporated into protoplasts. Irradiation with white light caused an immediate increase in the signal intensity (data not shown), indicating that sterols are able to penetrate to the site of light-mediated oxidation. We were unable to incorporate the cholestane spin label into protoplasts.

**PC(5,10) in the Light and Dark.** Attempts to incorporate PC(5,10) into oat leaf protoplasts were successful (Fig. 7). The ESR spectrum showed that the label is rotating about the long molecular axis of the hydrocarbon chain. The label is not in a micelle or spin label vesicle since this would bring the nitroxides into such close proximity with each other that the resultant spin-spin interactions would obscure the hyperfine structure seen in the ESR spectrum. The intensity of the signal gradually decayed with time (Fig. 7). When the sample was irradiated with white light a transient $P700^+$ signal appeared, which caused the mid-field line to increase. However, the signal intensity due to the nitroxide continued to decrease. The rotational correlation time prior to irradiation ($5.03 \times 10^{-8}$ s) was not significantly different after irradiation ($4.47 \times 10^{-8}$ s).

**Cellular Distribution of PC(5,10).** PC(5,10)-labeled protoplasts were lysed and the chloroplasts and nongreen membrane fractions were assayed for the label. Approximately 50% of the label was in each of the two fractions. Since the chloroplast pellet was 10-fold larger than the nongreen membrane pellet, the nongreen membranes were enriched about 10-fold in PC(5,10). The spin label in the chloroplast pellet was probably contained in plasmalemma which pelleted with the chloroplasts (19). These results indicate that PC(5,10) may be retained in the plasmalemma.

**Ascorbate Reduction of PC(5,10).** The reduction of spin label by ascorbate was not useful in ascertaining the location of PC(5,10). Ascorbate caused no significant reduction of PC(5,10) in protoplasts at 0°C over a 1-h period (data not shown).

**DISCUSSION**

The light-mediated oxidation of a spin label by protoplasts appears to be a good indication that the spin label has penetrated to the cell interior. Oxidation seems to take place within chloroplast membranes but only if the protoplasts are intact; isolated chloroplasts reduce spin label in the light (6).

Protoplasts labeled with TEMPO and the spin label analogs of maleimide, iodoacetamide, androstane, octane, decane, fatty acids, and fatty acid esters were able to oxidize these probes in the light. TEMPAMINE and the phospholipid spin label PC(5,10) were not oxidized. The results indicate that most spin labels partition throughout the membranes of protoplasts; only the phospholipid analog may have selectively labeled the plasmalemma. Further evidence is required before this can be known with certainty.

The ascorbate reduction assay also indicated that the fatty acid probe I(12,3) was distributed throughout the cell since reduction was biphasic. However, ascorbate caused no significant reduction of PC(5,10). The results with PC(5,10) are consistent with those of Rousselet et al. (21) who found that 1 h was required to completely reduce PC(10,3) in the outer leaflet of erythrocyte membranes, but that 4 h were required for comparable reduction of PC(7,6). PC(m,n) spin labels are probably more resistant to ascorbate reduction than are I(m,n) spin labels because PC(m,n) labels have greater stability in the membrane; I(m,n) spin labels may orient in bilayers such that their nitroxyl and carboxyl groups are at the membrane surface (9) facilitating interaction with ascorbate.

The amount of I(12,3) found in the chloroplasts and nongreen membranes was roughly proportional to the amount of membrane material. Since neither membrane fraction was enriched with the probe, its distribution was uniform. However, the nongreen membranes were enriched with PC(5,10), leading to the suggestion that PC(5,10) may be selectively retained in the plasmalemma.

We believe that the apparent changes in microviscosity of protoplast membranes in the light and dark are artifacts. The observed ESR signal is a composite of all the spin label molecules which are producing signals. In addition, the majority of the membranes in an oat leaf protoplast are thylakoid. Therefore, the bulk of the spin label population (i.e. the major contributor to the ESR signal) should be in the thylakoids with the remainder in the nongreen membranes. The spin label population in the thylakoids is probably reduced to the hydroxylamine in the dark and reoxidized to the nitroxide in the light. The spin label residing in the nongreen membranes was probably reduced and oxidized at a much slower rate. Table 1 shows that, with regard to the $2T_1$ measure of microviscosity, the nongreen membranes were rigid and unaffected by light, the chloroplast membranes were fluid and unaffected by light, and the protoplast membranes varied in fluidity between the values of the chloroplast and nongreen membranes, depending upon light conditions. These findings suggest that the net ESR signal was dominated during irradiation by the population of spin label located in the thylakoid membrane. In the dark, this population was chemically reduced so that the signal was dominated by the population in the nongreen membranes. The apparent increases and decreases of microviscosity were the result of the changes in the respective contribution these two spin populations made to the net ESR signal.

Apparent changes in microviscosity as determined by measuring the rotational correlation time, $\tau$, and the partitioning parameter, $F$, can be explained by spectral disproportionation of the ESR signal during a period of increasing or decreasing signal intensity. As the ESR signal is scanned from low- to high-field, each peak represents a signal intensity at a different time. Thus, $\tau$ decreases.
and $F$ increases as signal intensity decreases because the $h$, and $h$ peaks will be scanned sooner than the $h$, and $p$ peaks; this skews the ratios. Spectral disproportionation did not appear to affect $2T_{1}$ values.

At high gains, an ESR signal from unbound Mn$^{2+}$ and P700$^{+}$ (1) can distort the nitroxide line shape (data not shown). These endogenous ESR signals may be confused with ESR signals from spin labels; scans of nonlabeled protoplasts can be used to determine whether or not such distortion is occurring.

Although spin labeling can be one of the most informative methods for studying membrane structure, a lack of knowledge concerning the location of the probe can lead to erroneous conclusions. When studying cells, investigators should establish which membrane(s) the spin label is reporting from before drawing conclusions about the behavior of certain membranes in response to various treatments. We have shown that reports concerning the specificity of spin labels for the plasma membrane of animal cells may not be accurate indicators of location in plant protoplasts. We believe that spin label location should be established independently for every case. Light-mediated oxidation of spin label by chlorophyllous cells may be useful for this purpose.

LITERATURE CITED