Properties of Plasma Membrane Isolated from Chilling-Sensitive Etiolated Seedlings of *Vigna radiata* L.1, 2

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ABSTRACT

Plasma membrane was isolated in a uniform population and with a high purity from chilling-sensitive etiolated young seedlings of *Vigna radiata* (mung bean) utilizing an aqueous two polymer phase separation system and subsequent sucrose density gradient. The isolated plasma membrane was associated with vanadate-sensitive and KNO3-insensitive ATPase. The ATPase has high specificities both for substrate and Mg2+ ion with optimum pH at 6.5. It was slightly stimulated by monovalent anions, especially Cl-. Proton ionophores such as gramicidin D and carbonyl cyanide p-trifluoromethoxyphenylhydrazone did not stimulate the enzyme activity. The ATPase is apparently latent and highly stimulated by the addition of detergents such as Triton X-100. A maximum stimulation was achieved by the addition of 0.02% Triton X-100. After treatment with proteinase K in an isotonic buffer solution, the enzyme activity was lost completely, whereas the peptides were specifically digested. Based on these facts, the isolated plasma membrane vesicles appear to be tightly sealed and in a right-side-out orientation. The plasma membrane ATPase had two inflection points at higher (18.9°C) and lower (6.7°C) temperatures on the Arrhenius plots of the activity. The lower inflection temperature apparently coincided with that of the anisotropy parameter of embedded 1,6-diphenyl-1,3,5-hexatriene, indicating that the membrane bound ATPase activity was affected by a phase transition of membrane lipids and/or temperature-dependent conformational changes in the enzyme molecules per se. Considering the fact that the plant material used here is highly sensitive to chilling temperatures and injured severely by exposure to temperatures below 5°C for a relatively short period, the thermotropic properties of membrane molecules are considered to be involved in the mechanism of chilling injury.

Despite numerous studies on chilling injury of plants in the past decade (6, 14, 15), the mechanism is still obscure. In our previous studies using highly chilling-sensitive, cultured cells derived from *Cornus stolonifera* L. (18, 30), which is a cold resistant woody plant species, a series of sequential ultrastructural changes was demonstrated to occur in the cells immediately after exposure to chilling, i.e. structural transformation of proplastids from elipsoid to stretched and constricted form, a dilation and a vesiculation of ER, an aggregation of inner membrane particles of tonoplasts, and finally a rupture of vacuoles. Along with these time-dependent sequential changes in cell structures, cell leakage of electrolytes and amino acids (29), and a diversion of electron flow to the alternate pathway (31) were observed as the physio-

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MATERIALS AND METHODS

Plant Materials. The seeds of *Vigna radiata* were imbibed and germinated at 26°C in darkness on a stainless steel mesh installed at the one-third depth of a square plastic box (20 × 30 × 16 cm). To this about 3 L of 1 mm CaSO4 solution made up in distilled H2O were added to the level of the seeds on the stainless steel mesh. After 4 d of germination, hypocotyls (about 6 cm in length) were excised and utilized for the experiments.

Preparation of Membranes. Hypocotyls (140 g fresh weight) were washed once in distilled H2O and prechilled at 10°C before use. The prechilled tissues were sliced into 180 ml of homogenizing medium prechilled at 0°C and were immediately homogenized with Polytron PT30 at the medium speed setting for 30 s. The medium used for homogenization contained 0.25 mM sucrose, 75 mM MOPS-KOH, 5 mM EGTA, 2 mM PMSF, 2 mM SHAM, 2.5 mM potassium metabisulfite, 1.5% PVP (mol wt 24,000), 0.5% BSA (defatted), 10 μg/ml BHT, pH 7.6. The slurry was passed through four layers of gauze and subjected to differential centrifugation at 12,500 g for 15 min and subsequently at 156,000 g for 20 min. The 12,500 to 156,000 g pellets were suspended in 0.25 mM sucrose-10 mM K-phosphate buffer (pH 7.8)

1 Abbreviations: MOPS, 3-(N-morpholino)propanesulfonic acid; SHAM, salicylhydroxamic acid; PMSF, phenylmethylsulfon fluoride; BHT, butylated hydroxytoluene; PEG 4000, polyethylene glycol (mol wt 3,340); NPA, napthylphthalamic acid; DPH, 1,6-di-phenyl-1,3,5-hexatriene; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; DCCD, N,N'-dicyclohexylcarbodiimide; DIDS, 4,4-diisothiocyano-2,2'-stilbene disulfonic acid; DES, diethylstilbestrol; PTA, phosphotungstic acid; DOC, deoxycholate.
and centrifuged for 20 min at 156,000g for 20 min. The washed pellets were subjected to an aqueous two-polymer phase partition system as reported previously (21, 32). The phase partition system consisted of 5.6% (w/w) each of PEG 4000 and Dextran T500 made up in 0.25 M sucrose-10 mM K-phosphate buffer, pH 7.8, and various concentrations of NaCl (0–30 mM).

Sucrose Density Gradient. The plasma membrane-enriched fraction partitioned into the PEG-enriched upper phase of the phase partition system was diluted with 0.25 M sucrose, 5 mM MOPS-KOH, 1 mM EDTA, 10 mM KCl, 0.2 mM PMSF, 10 μg/ml BHT, and 1 mM DTT (pH 7.3) and pelleted by centrifugation at 156,000g for 20 min. The pellet was resuspended in the 7 ml of the same buffer system as used above and loaded on a linear sucrose gradient (30 ml, 15–45%, w/w) made up in 5 mM MOPS-KOH, 1 mM EDTA, 10 mM KCl, 0.2 mM PMSF, and 10 μg/ml BHT (pH 7.3). After centrifugation at 85,000g for 15 h at 2°C on a Hitachi SW-1 rotor, 1.2 ml aliquots were collected from the top of the gradient.

Enzyme Assay. Activities of Mg2+-ATPase, antitoxin A-insensitive NADH-Cyt c reductase, and Cyt c oxidase and phosphatase were assayed as previously reported (21, 32). UDPase activity was assayed according to the method of Nagahashi and Kane (16). Glucan synthetase I activity was determined by the transfer of radioactivity from [3H]UDP-glucose into heat denatured materials. The assay method was essentially the same as the method described by Hall and Moore (7). A naphthylphthalamic acid-binding test was performed as reported elsewhere (21) by using the 3H-labeled compound.

One-Dimensional SDS-PAGE. The membrane pellets were solubilized in 50 mM Tris-HCl (pH 6.8), 3% SDS, 0.5 mM m-sorbitol, 50 mM DTT, and 0.001% bromophenol blue with boiling for 3 min. The discontinuous SDS buffer system of Laemml (11) was used for the SDS slab-PAGE. After electrophoresis, the slab gels were stained with Coomasie blue according to Fairbanks et al. (5). For the determination of glycopeptides, the concanavalin A-peroxidase method was used (23).

Electron Microscopy. Intact tissue sections and membrane materials were fixed in 3% buffered glutaraldehyde followed by washing in buffer solution, postfixed in 2% OsO4 solution, dehydrated in an increasing concentration of ethanol, and finally in n-butylglycidyl ether as previously reported (21). The dehydrated specimens were embedded in Spurr's (20) epoxy resin. The sections were stained with either saturated uranyl acetate and Reynold's lead solution (19) or periodic-PTA-CrO3 (17). The stained sections were viewed with a JEM 100C electron microscope.

Fluorescent Polarization Measurement. The fluorescent hydrocarbon DPH was used as a probe to monitor the fluorescent polarization properties of isolated membranes and the liposomes prepared from the extracted lipids (25). The final concentration of DPH was 10 μM/100 μg protein. The steady state fluorescent polarization was measured at various temperatures in an Elscent microviscometer MV-1a. The anisotropy parameters calculated from the P values were taken as relative index for membrane fluidity and the logarithms were plotted against reciprocals of absolute temperatures to detect a thermotropic transition of membranes (25).

RESULTS

Plant plasma membranes have been isolated from wide range of plant materials by using an aqueous two-polymer phase system containing NaCl (21, 27, 32). In the present study, the 12,500 to 156,000g pellets prepared from hypocotyls of etiolated young seedlings of V. radiata were subjected to the phase partition system consisting of 5.6% (w/w) each of Dextran T500 and PEG 4,000 made up in 0.25 M sucrose-10 mM K-phosphate buffer, pH 7.8, and various concentrations of NaCl. As shown in Figure 1, with increasing NaCl concentration in the phase system, ER, mitochondria, and Golgi membranes were preferentially partitioned into the Dextran-enriched lower phase as assessed by the activities of marker enzymes; antitoxin A-insensitive NADH-Cyt c reductase, Cyt c oxidase, glucan synthetase I, and UDPase, respectively. Yellow-colored membrane materials, presumably plastid envelopes, were effectively partitioned into the lower phase even at the lower concentration of NaCl (10 mM). The control Mg2+-ATPase activity (assayed in the absence of Triton X-100), however, decreased proportionally with increasing in NaCl concentration. On the other hand, the majority of the Triton X-100-stimulated Mg2+-ATPase activity (assayed in the presence of 0.016% Triton X-100) was recovered in the PEG-enriched upper phase even at the higher NaCl concentration; over 60% of the total activity in the 12,500 to 156,000g pellet was recovered in the upper phase at the NaCl concentration of 30 mM. From these results, it appears that plasma membrane can be effectively separated from the crude membranes by using the phase partition system containing 30 mM NaCl.

There existed, however, less consistency in the partition behavior between the Triton X-100-stimulated Mg2+-ATPase and the NPA-binding capacity which is another useful marker for plasma membranes (13). This discrepancy would be due to a heterogeneity of plasma membrane vesicles differing in the capacity of their NPA-binding sites and in their partitioning behavior, especially, at the relatively high concentrations of NaCl. One of the plasma membrane populations would be enriched in the NPA-binding sites and is considered to be partitioned into the lower phase with increasing NaCl concentration.

As shown in Figure 2, the specific activity of the Triton X-
glucan synthetase inhibited (17) at tions shown), suggesting tonoplast of crose activity pelleted plasma membrane-enriched fraction obtained after partitioning of microsomal fraction in the presence of 10 mM NaCl, the plasma membrane-enriched upper phase was pelleted and resuspended in 7 ml of 0.25 m sucrose, 5 mM MOPS/KOH, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml BHT, 10 mM KCl, and 1 mM DTT, pH 7.3, and then loaded on 30 ml of a linear sucrose gradient (15-45%, w/w) made up in the same buffer system as above. After centrifugation at 85,000 g for 15 h at 2°C, 1.2 ml aliquots were fractionated from the top of the gradient. ATPase activities were assayed in the presence of 0.02% Triton X-100 with (O) or without (Δ) addition of 100 μM vanadate. ATPase (Δ); Protein content (X). Enzyme assays were done as described in the text.

The lighter tonoplast and Golgi membranes and the heavier plasma membranes were banded on the sample/32% and the 32/43% interfaces, respectively.

Table 1 characterizes each separate membrane from both interfaces. More than 70% of the total UDPase activity applied was recovered in the lighter membrane fraction (sample/32% interface), while over 90% of the total NPA-binding activity was recovered in the heavier membrane fraction (32/43% interface). The ATPase associated with the lighter membranes was inhibited by KNO3, but less inhibited by vanadate. On the other hand, the ATPase associated with the 32/43% interfacial fraction was highly sensitive to vanadate (about 80% inhibition by 100 μM vanadate), but was insensitive to KNO3. A remarkable difference was also noted in the stimulation of ATPases by the addition of 0.02% Triton X-100, i.e. 4.5-fold stimulation in plasma membrane ATPase and 1.5-fold stimulation in ATPases of tonoplast and Golgi-enriched membrane fraction (sample/32% interface). From these results, plasma membranes are effectively separated from the contaminated tonoplasts and Golgi membranes after the sucrose density gradient of the plasma membrane-enriched upper phase in the phase partition system contained 10 mM NaCl.

Figure 4 shows the electron micrographs of the sample/32% and 32/43% interfacial membrane fractions. The former fraction contained various sized smooth membrane vesicles (Ia) and they were not stained with PTA-CrO3 (Ib). The latter fraction (IIa, IIb), on the other hand, was highly enriched in uniformly sized smooth membrane vesicles most of which were positively stained by PTA-CrO3, indicating a high enrichment of plasma membranes.

As presented in Figure 5, a distinct difference was observed between the two membrane fractions in the SDS-PAGE patterns.
of polypeptides as revealed by either Coomassie blue staining or concanavalin A-peroxidase staining by which glucose or mannose type glycopeptides are specifically stained. The enrichment of tonoplast in the sample/32% interface is also clearly supported by the similarity of polypeptide or glycoprotein composition between the sample/32% interface (lanes A-3, B-3) and the purified tonoplast prepared from the same plant materials (28) as used in the present study. A difference was also remarkable in the polypeptide compositions between the lower phase (endo-membranes) and the upper phase (the plasma membrane) in the phase partition.

Figure 6 shows a pH-activity profile of plasma membrane ATPase in the presence or absence of Triton X-100. The optimal pH was located around 6.5 in the absence of the detergent. The activity was slightly stimulated by the addition of 50 mM KCl without any effect on the pH-activity profile. Triton X-100 (0.016%) stimulated the ATPase, especially around pH 6.5 and 7.0. The KCl stimulation, however, was abolished around the optimal pH and salt was observed to be inhibitory at a higher pH. From the pH-activity profile, KCl appeared to shift the pH profile by about 0.5 unit toward acidic region in the presence of the detergent.

Table II shows the effects of various inhibitors on the plasma membrane ATPase. The ATPase was highly sensitive to vanadate and moderately inhibited by DCCD and DES (data not shown). It was also slightly inhibited by DIDS (30 μM) which is an inhibitor for anion transport channels in plasma membranes (4, 9). Azide and KNO3 exhibited no inhibitory effect. The plasma membrane ATPase showed a high substrate specificity for ATP which could not be replaced by CTP, GTP, and ITP (Table III). However, relatively high activity was observed for UTP, UDP, and IDP. The activity for the latter two substrates must be due to a slight contamination by Golgi membranes. The activity for UTP is unknown as yet. Tables IV and V indicate effects of monovalent and divalent ions on plasma membrane ATPase. The plasma membrane ATPase showed a high specificity to Mg2+ and was slightly stimulated by monovalent salts such as KCl, NaCl, NH4Cl, and choline-Cl, but the stimulation was less in K2SO4, KI, and KNO3. These stimulations, however, were completely abolished by the addition of Triton X-100. Proton ionophores such as gramicidin D and FCCP did not affect the plasma membrane ATPase (data not shown).

The plasma membrane ATPase was stimulated by both non-ionic and anionic detergents. Figure 7 shows the effect of detergent concentrations on the ATPase activity. A maximum activity was obtained at the concentration of 0.025% in both detergents. Above 0.025%, the ATPase activity was depressed to some degree. The stimulation was relatively high in Triton X-100 compared with sodium DOC. The observed latency in the plasma membrane ATPase may indicate that the plasma membranes have been isolated in tightly sealed vesicles with a right-side-out orientation and retained the permeability barrier against Mg2+ and ATP. In suggesting this notion, the ATPase activity was less sensitive to proteinase K treatment even at higher concentrations where some populations of polypeptides were selectively digested (S. Yoshida, unpublished data). This result may suggest that the isolated plasma membrane vesicles are tightly sealed and oriented right-side-out during preparation, preventing the transmembrane traverse of the added protease and the direct contact with the ATPase located on the cytoplasmic surface of the plasma membranes.

To investigate the effect of temperatures on the plasma membrane ATPase, the activity was assayed simultaneously at different temperatures in a temperature gradient former and the data were arranged as Arrhenius plots. As indicated in Figure 8, two inflections were observed in the slope at 18.9 and 6.7°C. The presence of 0.016% Triton X-100 in the reaction system did not affect the activation energies above, between, and below these inflection points. However, the inflection points slightly shifted to higher temperatures, i.e., from 18.9 to 19.9°C and from 6.7 to 8.9°C, respectively.

The Arrhenius plots of anisotropy parameters of DPH incorporated into plasma membrane vesicles showed a break at 7.4°C (Fig. 9). This break point roughly corresponded to the lower
inflection point (6.7°C) on the Arrhenius plots of ATPase activity. Based on these results, it seems likely that the plasma membrane may undergo a thermotropic phase transition around 7°C, below which the etiolated seedlings suffer chilling injury.

DISCUSSION

In the present study, plasma membranes were isolated from chilling-sensitive, etiolated young seedlings of *V. radiata* L. by utilizing an aqueous two-polymer phase partitioning system containing 10 mM NaCl and by subsequent sucrose density gradient of the plasma membrane-enriched upper phase. The purity of the isolated plasma membranes was fairly high as assessed by the PTA-CrO₃ stain, NPA-binding capacity, and vanadate-sensitive Mg²⁺-ATPase.

Unlike other plant materials with which we have been concerned (21, 26, 32), the plasma membrane from *V. radiata* showed a different behavior in the phase partition at the higher concentration of NaCl. The specific activity of NPA-binding increased with increasing in NaCl concentration up to 15 mM, and then decreased above 15 mM NaCl, while the specific activity of Triton X-100-stimulated ATPase increased proportionally. In addition, the recovery of plasma membrane in the upper phase was relatively poor at the NaCl concentration of 30 mM. This might indicate that the plasma membrane vesicles consisted of heterogeneous populations, i.e. a membrane population enriched in the NPA-binding sites, which might have partitioned into the lower phase at the higher concentrations of NaCl and the other membrane population enriched in Triton X-100-stimulated ATPase, most of which might have been partitioned into the upper phase even at the higher NaCl concentrations. When phase partition was carried out in the presence of 10 mM NaCl, most yellow-colored membranes, ER, mitochondria, and a significant amount of Golgi-derived membrane (more than 75%) were preferentially partitioned into the lower phase, and thus plasma membranes enriched in both of the NPA-binding sites and ATPase were recovered in the whitish upper phase partly contaminated with Golgi membranes and tonoplasts. After sucrose density gradient centrifugation of the plasma membrane-enriched upper phase, the contaminating membranes were effectively separated on a 32% sucrose layer, and the plasma membrane with a high purity was concentrated on an interface of 32/43% sucrose layers.

The ATPase bound to the isolated plasma membrane showed a latent property being effectively stimulated by the addition of detergents such as Triton X-100 and DOC. Although the former was more effective than the latter, the maximum stimulation was observed at the concentrations of 0.02 to 0.025% in both detergents. At similar concentrations, rat liver rough microsomes have been reported to become reversibly permeable to macromolecules without any membrane disassembly (10). Based on these results it might be considered that the stimulation of the ATPase by the detergents is due to the increased permeability of plasma membrane for Mg²⁺-ATP so that the substrate becomes
accessibility to the enzyme located on the inside of the membrane. This was also supported by the fact that most of the ATPase activity remained intact after treatment of plasma membrane with proteinase K at the concentrations where a great deal of polypeptides were digested (S. Yoshida, unpublished data). From these results, it is conceivable that the plasma membranes have been isolated in sealed and right-side-out vesicles.

In general, plasma membrane vesicles with normal sidedness of a right-side-out are known to partition into PEG-enriched upper phase in an aqueous two-polymer phase partition system (8). According to Larsson et al. (12), the plasma membranes isolated from oat roots and cauliflower inflorescences by partition in dextran-polyethylene two-phase system were homogeneous with respect to the sidedness. Most of the vesicles were right-side-out and sealed as assessed by the latent Mg$^{2+}$-ATPase activity which was dramatically increased by introducing Triton X-100.

Since the pH-activity profile and the monovalent-ion stimulation of plasma membrane ATPase were significantly affected by Triton X-100 in the present study, the detergent may also have a side effect other than the substrate permeation.

The plasma membrane ATPase of etiolated seedlings of *V. radiata* showed high specificities for Mg$^{2+}$ ion and ATP. The relatively high hydrolytic activities for UDP and IDP are presumably due to the slight contamination by Golgi-derived mem-

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**Fig. 5.** Slab SDS-PAGE of various membrane fractions. The gels after electrophoresis were stained either with Coomassie blue (left) or concanavalin A-peroxidase stain (right). Lanes 1, 2, and 3 refer, respectively, to membranes partitioned into the dextran-enriched lower phase after a phase partitioning in the presence of 10 mM NaCl, to the 32/43% interfacial fraction (plasma membrane-enriched), and to the sample load/32% interfacial fraction (tonoplast and Golgi-enriched) after a discontinuous sucrose gradient of the upper phase. In each lane, 30 μg protein were applied.
Fig. 6. The pH-activity profiles of plasma membrane ATPase. ATPase assay mixtures contained 3 mM ATP (Tris-salt), 3 mM MgSO₄, and 25 mM Tris-Mes in varied pH with presence (○) or absence (●) of 50 mM KC1. Dotted lines (lower part), no detergent was added; solid line (upper part), 0.02% Triton X-100 was added into the reaction mixtures. The reaction was performed at 30°C for 30 min.

Table II. Effect of Inhibitors on Plasma Membrane ATPase

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<th>μmol/h·mg protein % control</th>
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<tr>
<td>Sodium vanadate 50 μM</td>
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<tr>
<td>NaN₃ (5 mM)</td>
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<tr>
<td>KNO₂ (50 mM)</td>
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<tr>
<td>Sodium molybdate 1 mm</td>
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<tr>
<td>DCCD (50 μM)</td>
<td>7.40</td>
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<tr>
<td>DIDS (30 μM)</td>
<td>9.13</td>
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<td>Ethanol (0.1%)</td>
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Table III. Substrate Specificity of Plasma Membrane ATPase

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<td>ADP</td>
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<td>AMP</td>
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<td>CTP</td>
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<tr>
<td>GTP</td>
<td>3.99</td>
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<td>ITP</td>
<td>4.91</td>
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<tr>
<td>IDP</td>
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<td>UTP</td>
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<tr>
<td>UDP</td>
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<tr>
<td>p-Nitrophenylphosphate</td>
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Table IV. Effect of Monovalent Ions on Plasma Membrane ATPase

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<th>μmol/h·mg protein</th>
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Table V. Effect of Divalent Cations on Plasma Membrane ATPase

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<th>Divalent Cation</th>
<th>Activity</th>
<th>μmol/h·mg protein</th>
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<td>Mg²⁺</td>
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<td>Co²⁺</td>
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<tr>
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<td>Zn²⁺</td>
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<tr>
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<td>Hg²⁺</td>
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* ND, not determined.

Branaes. The Golgi-derived membranes in the etiolated seedlings of *V. radiata* were found to have extremely high activities for IDP and UDP in the presence of Triton X-100, several-fold higher than the other plant materials with which we have been concerned so far. The plasma membrane ATPase was stimulated slightly by monovalent ions, especially by Cl⁻. This seems to correlate with the fact that the ATPase is inhibited by DIDS, which is a specific inhibitor for anion channels of plasma membranes as reported (4, 9).

Evidence to show the existence of a H⁺-pumping ATPase on plasma membranes as well as on tonoplasts has now accumulated for plants (1–3, 22). The plasma membrane ATPase of *V. radiata* was sensitive to DCCD. However, no stimulation was observed in FCCP and gramicidin D. This fact may indicate either that the plasma membrane vesicles have lost the permeability barrier for H⁺ during the preparation or that the plasma membranes have been isolated in right-side-out vesicles where the ATP substrate site is inaccessible. This needs to be studied in future.

The dark-grown young seedlings of *V. radiata* are highly chilling sensitive, suffering injury after exposure to temperatures lower than 5°C. During the chilling treatments under low temperatures, electrolytes were observed to leak out of the tissues,
PLASMA MEMBRANE OF *VIGNA HYPOCOTYL*

**Fig. 7.** Effect of detergents on plasma membrane ATPase activity. ATPase assay was performed in a reaction mixture contained 3 mm ATP (Na-salt), 3 mm MgSO₄, 25 mm Tris-Mes, pH 6.5, 50 mm KCl, and various concentration of detergents. The reaction was carried out at 30°C for 20 min. Triton X-100 (O), sodium DOC (C).

**Fig. 8.** Arrhenius plots of plasma membrane ATPase activity. ATPase assay mixture contained 3 mm ATP (Tris-salt), 3 mm MgSO₄, 25 mm Tris-Mes, pH 6.5, and 50 mm KCl. The reaction was performed simultaneously at various temperatures in a temperature gradient former in which a linear temperature gradient was formed between 0 and 30°C at 2°C intervals. The slopes of the curves were obtained by fitting regression to points derived from the averages of two replications and selecting those giving the best fit. Where discontinuities were suspected, regressions were fitted to all combinations above and below the apparent breaks and the partition with the minimum sum of square was selected. Lines of best fit were then drawn for each partition and the break point was estimated from the intersection of the regression.

and the degree of the leakage was closely associated with the degree of cell injury (S Etani, S Yoshida, unpublished data). This might indicate that a loss of physiological function of the plasma membrane as a result of chilling is involved in the mechanism of the cell injury.

With respect to the temperature dependency of the plasma membrane ATPase, an inflection has been determined on the Arrhenius plots. In orchard grass (S Yoshida, unpublished data), winter rye (M Uemura, unpublished data), Jerusalem artichoke tubers (M Ishikawa, S Yoshida, unpublished data), which are all cold-resistant species, inflections were observed around 15 to 17°C, regardless of season. These inflections, however, were apparently not coincident with a phase transition in the lipids as assessed by fluorescent polarization study using DPH. As has been suggested by Wright et al. (24), the temperature-dependency of the enzyme might be ascribable to the intrinsic properties of the enzyme molecules and/or the properties of the boundary lipids. In the present study, plasma membrane ATPase of *V. radiata* showed inflections at higher (18.9°C) and lower (6.7°C) temperatures (Fig. 8) and the latter was apparently coincident with the break point on the Arrhenius plots of anisotropy parameter of DPH incorporated into the membrane (Fig. 9).

Fatty acids of plasma membrane phospholipids were less unsaturated (data not shown) than those of cold hardy plant species (26, 27). In liposomes prepared from the total lipid extracts or the separated phospholipids, however, no inflection was detected in the Arrhenius plots of the anisotropy parameters (data not shown). Nearly the same situation is reported for plasma membranes isolated from cold hardy plants (25) and the liposomes prepared from the extracted lipids (25). In the plasma membranes, the inflection on the Arrhenius plots of the anisotropy parameters of DPH occurred at a freezing temperature where the cells sustain frost injury (25). The inflection, however, was not dependent on the membrane lipids *per se*, but primarily on the membrane proteins (25).

Although there still remains uncertainty in the thermotropic behavior of the membrane molecules as far as a steady state measurement of the fluorescent polarization is concerned, it is of interest to note that the inflections of the Arrhenius slopes are well correlated with chilling injury and frost injury of plant cells, whereas the temperature range is quite different. Further detailed studies on the molecular basis of the thermotropic properties of plasma membranes are needed to elucidate the fundamental meaning of these inflections and thereby may provide useful information to explain the mechanisms.

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