Effect of Temperature on Proton Efflux from Isolated Chloroplast Thylakoids

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ABSTRACT

Temperature-induced changes in the decay of the light-induced proton gradient of chloroplast thylakoids isolated from chilling-resistant and chilling-sensitive plants have been examined. In the presence of N-methylphenazonium methosulfate, the thylakoids isolated from chilling-resistant barley (cv. Kanby) and pea (cv. Alaska) and chilling-sensitive mung bean (cv. Berken) plants showed temperature-induced changes at approximately 8.6, 13.3, and 14.0°C, respectively. barley thylakoids assayed in the presence of sodium thiocyanate also showed a change at 8.6°C, whereas with no addition or upon the inclusion of both N-methylphenazonium methosulfate and sodium thiocyanate the change occurred at approximately 11.5°C. Temperature-induced changes in electron transport activities occurred at temperatures approximating those for proton efflux for each of the three plants. These results indicate that temperature has a general effect on thylakoid membranes and that chloroplast thylakoids from chilling-resistant and chilling-sensitive plants have their proton permeability properties affected similarly by temperature.

The growth and survival of many plants, chilling-sensitive species, are deleteriously affected by exposure to nonfreezing temperatures below approximately 10 to 15°C (for reviews see 7, 17, 18). In contrast, chilling-resistant species are capable of growth at temperatures slightly above 0°C. Mitochondrial membranes isolated from chilling-sensitive species showed an increase in Ea for respiratory enzymes (8) and a decrease in lipid fluidity detected by spin-label motion (19) at temperatures below those which affect the growth of the plants. Such changes were absent in mitochondrial membranes isolated from chilling-resistant species (8, 19). Primarily on the basis of these observations it was proposed that the primary event affecting the growth of chilling-sensitive species is a thermitically induced change in the lipid ordering of the various cellular membranes (7, 17, 18). The observed change in Ea was considered to be a consequence of the change in lipid ordering. With regard to chloroplast membranes, Shneyour et al. (20) reported that temperature-induced changes in Ea for NADP+ reduction occurred for chloroplasts isolated from chilling-sensitive plants but not for chloroplasts isolated from chilling-resistant plants. Subsequently, temperature-induced changes in Ea for a variety of photochemical assays have been reported to occur in chloroplasts isolated from both chilling-resistant and chilling-sen-

MATERIALS AND METHODS

Plant Material. Mung bean (Vigna radiata L. cv. Berken), barley (Hordeum vulgare L. cv. Kanby), and pea (Pisum sativum L. cv. Alaska) plants were grown in vermiculite in a growth chamber at 20 ± 1°C. Grow-lux fluorescent tubes and incandescent bulbs provided light of approximately 30 w/m² for 12 h/day. Leaves were harvested after 6, 7, and 11 days of growth of mung bean, barley, and pea seedlings, respectively.

Chloroplast Isolation. Chloroplasts were isolated (at 0–4°C) in a medium of 50 mM Sörensen's phosphate buffer (pH 7.4), 50 mM NaCl, and 0.5% (w/v) BSA. DT at 10 mM and 1% (w/v) Polyclar AT were also included in the medium used for isolating mung bean chloroplasts. Leaf segments were blended for four periods of 5 s each using speed setting three of a domestic seven speed blender (Oster Corp. Model 847). Chloroplasts were isolated from the homogenate as previously described (14) except for the composition of the medium used to wash the chloroplasts. The chloroplasts were first washed in a medium of 3 mM Hepes (pH 7.0), 300 mM sorbitol, and 3 mM MgCl₂. They were then washed and resuspended in a medium of 1 mM Hepes (pH 7.0), 100 mM sorbitol, and 1 mM MgCl₂.

Assays. Electron transport and proton efflux measurements were performed using a Beckman Model 3500 pH meter and a fast response Beckman Futura combination pH electrode (No. 39505). The output of the pH meter was recorded on a Corning Model 840 chart recorder. Red actinic light (4 × 10³ µW/cm²) was obtained by filtering light from a 500-w slide projector through a Schott RG-630 filter and a 500-ml boiling flask which served as a heat filter and to focus the actinic light on the reaction vessel. A
sample compartment in a YSI Model 5301 O2 electrode assembly was used as the reaction vessel. The temperature of the reaction mixture was generally maintained to within ±0.05 °C during the course of each assay.

The reaction mixture for measurement of electron transport activities consisted of 1 mm Hepes (pH 7.0), 100 mm sorbitol, 1 mm MgCl₂, 167 μM potassium ferricyanide, 4 μg ml⁻¹ gramicidin D, and 8 μg ml⁻¹ Chl. The recorder tracings were calibrated by the addition of a known amount of HCl. Measurements of proton efflux were conducted in a medium composed of 1 mm Hepes (pH 7.0), 100 mm sorbitol, 1 mm MgCl₂, 40 μg ml⁻¹ Chl and, where indicated, 20 μM N-methylphenazonium methosulfate and 5 mM NaSCN. Reproducibility of electron transport and proton efflux measurements was generally better than ±2%.

The reaction mixture (minus chloroplasts) was preoquilibrated to the required temperature. The chloroplasts which had been stored at 0 °C were added to the reaction mixture and incubated for 2 min prior to assay. Measurements of proton efflux were conducted upon turning off the actinic light after a 2.5-min period of illumination of the reaction mixture. Determinations of lines of best fit through the data points in the Arrhenius plots presented were determined using a least-squares regression analysis (2). Chl was determined according to the method of Arnon (1).

RESULTS

Effect of Temperature on Electron Transport Activities of Chloroplast Thylakoids from Chilling-Resistant and Chilling-Sensitive Plants. Arrhenius plots of electron transport by isolated barley, pea and mung bean thylakoids are presented in Figure 1. Reaction rates were determined by measuring acidification of the reaction mixtures accompanying ferricyanide reduction. The uncoupler gramicidin D was included in the reaction mixtures as it has been shown previously that temperature-induced changes in electron transport are not observed at low temperatures in these plants when electron transport is rate-limited (12-14).

Figure 1A shows that a change in Ea of electron transport activity of barley thylakoids occurs at approximately 10.3 °C. (The temperature cited for a temperature-induced change is an approximation and is the temperature midway between the two straight-line segments.) This temperature is close to that at which several electron transport activities showed a temperature-induced change in Ea when thylakoids from a different variety of barley were utilized (14). Table I summarizes the Ea values above and below the change in slope and the temperatures at which a change in slope occurs for the various activities assayed. Figure 1B presents data showing that a change in Ea occurs at approximately 14.9 °C for electron transport by pea thylakoids. Several varieties of pea have been reported to show temperature-induced changes in electron transport activities at approximately this same temperature (12, 13). A change in Ea for electron transport by mung bean thylakoids occurs at approximately 12.9 °C (Fig. 1C). This temperature is somewhat lower than that at which thylakoids from a different variety of mung bean showed a temperature-induced change in Ea of electron transport activity (13).

The close correlation between the data presented and that previously reported (12-14) demonstrates the validity of the technique utilized to determine temperature-induced changes in electron transport activity.

Effect of Temperature on Proton Efflux from Chloroplast Thylakoids of Chilling-Resistant and Chilling-Sensitive Plants. Arrhenius plots showing the temperature dependence of proton efflux (measured as the reciprocal of half decay time) from isolated barley thylakoids are presented in Figures 2 and 3. When proton efflux is measured in the absence of PMS, an abrupt change in its temperature dependence occurs at approximately 11.5 °C (Fig. 2A). At temperatures below 11.5 °C, the rate of efflux is quite insensitive to any further decrease in temperature, whereas at higher temper-

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Arrhenius plot for electron transport by isolated chloroplast membranes from barley (A), pea (B), and mung bean (C). Assays were conducted by measuring acidification of the reaction mixtures accompanying ferricyanide reduction in the presence of the uncoupler gramicidin D.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Addition</th>
<th>Temperature Range</th>
<th>Ea</th>
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<tbody>
<tr>
<td>A. Electron transport</td>
<td></td>
<td></td>
<td>C</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>Pea</td>
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<td></td>
<td>&lt;14.9</td>
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<tr>
<td>Mung Bean</td>
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<td></td>
<td>&lt;12.9</td>
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<tr>
<td>B. Proton efflux</td>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Barley</td>
<td>PMS</td>
<td></td>
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</tr>
<tr>
<td>Pea</td>
<td>PMS, NaSCN</td>
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*Electron transport was measured as acidification of the reaction mixture during ferricyanide reduction in the presence of the uncoupler gramicidin D. Proton efflux was measured in the basic reaction mixture to which 20 μM PMS and 5 mM NaSCN were added as indicated.
Fig. 2. Arrhenius plot of the rate constant for proton efflux from barley thylakoids. Assays were conducted in the absence (A) or presence (B) of PMS.

Fig. 3. Arrhenius plot of the effect of NaSCN on the rate constant for proton efflux from barley thylakoids. Assays were conducted in the absence (A) or presence (B) of PMS.

Fig. 4. Arrhenius plot of the rate constant for proton efflux from pea (A) and mung bean (B) thylakoids. Assays were conducted in the presence of PMS.

Fig. 2A; Table I). In both instances the change in Ea for efflux occurs at a temperature relatively close to where a respective change in Ea occurs for electron transport (Table I; 12, 13). Compared to barley the efflux from pea and mung bean thylakoids shows a greater temperature dependence at temperatures below where the change in Ea occurs (Table I).

**DISCUSSION**

The data presented clearly demonstrate the existence of temperature-induced changes in proton efflux from isolated chloroplast thylakoids (Figs. 2–4). These changes, which occur in the range of chilling temperatures, are exhibited by thylakoids isolated from both chilling-resistant, barley and pea, and chilling-sensitive, mung bean, plants. These temperature-induced changes in proton efflux occur at temperatures approximating those at which temperature-induced changes in electron transport occur (Table I; 12–14). This close correlation supports the previous conclusion that temperature has a generalized effect on chloroplast thylakoid membranes (12).

The data presented are taken to signify that temperature has a marked effect on the permeability of chloroplast thylakoid membranes. The apparent activation energy values for temperatures below the change in slope indicate that the light-induced proton gradient is being dissipated by diffusion; this is particularly evident for isolated barley thylakoids, in which case the properties of the permeability barrier are very insensitive to any further reduction in temperature (Table I). At present, there is no reason to presume that the proton gradient is dissipated by a mechanism other than diffusion at temperatures above the change in slope. This is in contrast to the suggestion by Yamamoto and Nishimura (24) that the efflux of protons involves both diffusion and electron transport in the dark. Their proposal was based on the observation that a temperature-induced change in proton efflux from spinach thylakoids occurred at approximately 20°C in the absence but not in the presence of PMS. It was suggested that PMS may eliminate the temperature-induced change by either accelerating electron transport through the rate-limiting step or by circumventing the rate-limiting step. In the present report, however, a temperature-induced change in proton efflux is observed to occur in both the presence and absence of PMS (Fig. 2). As shown for barley thylakoids, PMS appears to have little effect on proton efflux although the temperature-induced change occurs at a somewhat lower temperature in its absence compared to in its absence (cf. Fig. 2B to 2A). The temperature-induced change also occurs at a lower temperature in the presence of the permeant anion SCN⁻ (Fig. 3A). However, in the presence of both PMS and SCN⁻, the

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change occurs at the same temperature as when both are absent (cf. Fig. 3B to 2A). It seems that in the presence of either PMS or SCN" that the temperature response of the thylakoid membranes is somewhat altered, but when both are present their individual effects are canceled out. Further investigation is required to determine the significance of these observations. Yamamoto and Nishimura (23) also observed an additional temperature-induced change in proton efflux from spinach thylakoids at approximately 8 C which is qualitatively very similar to those reported here. On the basis of experiments utilizing either 10% glycerol (which shifted the break to a higher temperature) or 5 mM SCN" (which eliminated the break), it was concluded that this temperature-induced change was due to a change in the amount or properties of structured water at the surface of the membrane, rather than being brought about by a temperature-induced change in membrane properties (23). Although the effect of these reagents has not been studied in detail, it is apparent that the chaotropic reagent SCN" at a concentration of 5 mM has no significant effect on proton efflux from barley thylakoids (Figs. 2 and 3; Table 1).

The effect of temperature on the permeability of thylakoids has been studied indirectly by determining the temperature-dependence of the decay of light-induced absorption changes at 515 or 520 nm. Graeber and Witt (3) observed a temperature-induced decrease in Ea at temperatures below approximately 22 C for the decay of the flash-induced A change at 515 nm of spinach chloroplast thylakoids. This change was interpreted as arising from a change in the mechanism of ion permeation due either directly or indirectly to a phase transition of the membrane lipids.

Yamamoto and Nishimura studied the decay of the 515 nm change of spinach thylakoids after continuous illumination (22). The decay showed first order kinetics above 15 C, whereas below this temperature it was composed of fast and slow components. A decrease in Ea occurred at temperatures below approximately 11 C for the slow component, whereas the fast decay component showed a constant Ea. Evidence was presented indicating that the slow component is due to a structural change of the thylakoid membranes (22). This structural change presumably may be related to the altered proton efflux kinetics observed below approximately 18 C for spinach thylakoids (24). The results of the studies by Graeber and Witt (3), and Yamamoto and Nishimura (22) are in agreement with numerous other studies which indicate that thylakoids which have shown the occurrence of temperature-induced changes in electron transport activities (4, 9, 12), proton efflux (24), photophosphorylation (9), decay of atenine fluorescence (6), and delayed light emission (5). The above results indicate that temperature has a generalized effect on the properties of thylakoids isolated from chilling-resistant spinach plants.

Murata and Fork (10) have investigated the decay of the 520 nm absorption change of leaves from *Tidestroma oblongifolia*, chilling-sensitive tomato and bean, and chilling-resistant lettuce and spinach plants. The decay kinetics for leaves of *T. oblongifolia* were monophasic at temperatures above 10 C, whereas below this temperature fast and slow components existed. Analysis of the decay kinetics for leaves of tomato, bean, lettuce, and spinach showed the presence of only the slow component. Arrhenius plots of the dark decay of the slow component showed changes in Ea at approximately 5, 10, and 15 C for leaves of *T. oblongifolia*, tomato, and bean, respectively. In contrast, no temperature-induced changes in Ea were observed for leaves of lettuce and spinach. Fork and Murata concluded that a phase change occurred in the membranes of *T. oblongifolia*, tomato, and bean, which was regulating the dissipation of the ion gradient established across the thylakoid membranes during illumination. Whereas the change in 520 nm decay kinetics for *T. oblongifolia* is related to a phase change of the membrane lipids as indicated by fluorescence data (10), data obtained by this technique have been presented indicating no phase change in tomato (11) and bean (5, 11) as well as in lettuce (11) and spinach (5, 11). Although ion flux across thylakoid membranes is undoubtedly different *in vivo* from that *in vitro*, it is puzzling that the decay of the 520 nm absorption change of spinach leaves does not show the temperature-induced changes which are observed in the decay of the 515 nm change of isolated spinach thylakoids (3, 22).

It is apparent from the above discussion that temperature-induced changes occur in the permeability and activities of thylakoid membranes. Although discrepancies exist which need to be resolved, the available data do not indicate that there is any fundamental difference between the temperature response of thylakoid membranes isolated from chilling-sensitive and from chilling-resistant plants. Further work is required to establish the relationship of temperature-induced changes in chloroplast membranes to the phenomena of chilling-sensitivity and resistance and to determine the causal agent of the temperature-induced changes.

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