A Comparison of the Effects of Chilling on Thylakoid Electron Transfer in Pea (*Pisum sativum* L.) and Cucumber (*Cucumis sativus* L.)

THOMAS C. PEELE & AUBREY W. NAYLOR

Department of Botany, Duke University, Durham, North Carolina 27706

Received for publication April 21, 1987 and in revised form September 21, 1987

**ABSTRACT**

Experiments comparing the photosynthetic responses of a chilling-resistant species (*Pisum sativum* L. cv Alaska) and a chilling-sensitive species (*Cucumis sativus* L. cv Ashley) have shown that cucumber photosynthesis is adversely affected by chilling temperatures in the light, while pea photosynthesis is not inhibited by chilling in the light. To further investigate the site of the differential response of these two species to chilling stress, thylakoid membranes were isolated under various conditions and rates of photosynthetic electron transfer were determined. Preliminary experiments revealed that the integrity of cucumber thylakoids from 25°C-grown plants was affected by the isolation temperature; cucumber thylakoids isolated at 5°C in 400 millimolar NaCl were uncoupled, while thylakoids isolated at room temperature in 400 millimolar NaCl were coupled, as determined by addition of gramicidin. The concentration of NaCl in the homogenization buffer was found to be a critical factor in the uncoupling of cucumber thylakoids at 5°C. In contrast, pea thylakoid membranes were not influenced by isolation temperatures or NaCl concentrations. In a second set of experiments, thylakoid membranes were isolated from pea and cucumber plants at successive intervals during a whole-plant light period chilling stress (5°C). During whole-plant chilling, thylakoids isolated from cucumber membranes were uncoupled while those from pea were isolated during a whole-plant chilling treatment. The difference in integrity of thylakoid membrane coupling following chilling in the light demonstrates a fundamental difference in photosynthetic function between these two species that may have some bearing on why pea is a chilling-resistant plant and cucumber is a chilling-sensitive plant.

**MATERIALS AND METHODS**

Pea (*Pisum sativum* L. cv Alaska) and cucumber (*Cucumis sativus* L. cv Ashley) were grown in the Duke University Phyto-otron. Control conditions were 25°C day/18°C night, 16 hr photoperiod, and 350 μmol m⁻² s⁻¹ irradiances.

For chilling treatment, two hours after the beginning of the light period the chamber temperature was dropped to 5°C day/night. Just fully expanded leaves of pea and cucumber were removed at various times before and during the chilling treatment and used immediately for thylakoid isolation.

Approximately 3 g of leaves were sectioned into small pieces with a razor blade and placed in 200 ml of homogenization buffer which contained 0.4 M NaCl, 1.0 mM EDTA, 2.0 mg/ml BSA and 20 mM HEPES, pH 7.5. The tissue was homogenized using a Polytron grinder at high speed for 5 s. The resulting slurry was poured through four layers of cheesecloth, and the filtrate was centrifuged at 2000 g to remove cellular debris. The length of time for this centrifugation was kept as short as possible by turning off the centrifuge timer as soon as 2000 g was reached. Thylakoid membranes were then pelleted from the supernatant by centrifugation at 5000 g for 4 min. The pellet was resuspended and washed once in a buffer containing 0.15 M NaCl and 20 mM HEPES, pH 7.5. The final pellet was resuspended to a concentration of approximately 1 mg chlorophyll/ml of resuspension buffer. The temperature for the thylakoid isolation procedure was 4°C, except for the initial homogenization step which was performed at 25°C as described in "Results."

Electron transfer rates from H₂O to MV²⁻ were determined by measuring O₂ uptake using a Clark-type oxygen electrode. The chamber volume of the water-jacketed electrode was 2.0 ml, and temperature was maintained at 25°C. The assay buffer contained 0.1 M sorbitol, 50 mM KCl, 5.0 mM MgCl₂, 50 mM HEPES (pH 7.8), 30 μM MV, and thylakoids containing 25 to 50 μg of Chl. Gramicidin (0.2 μM) was used to uncouple electron transfer from the generation of a pH gradient. A projector lamp was used to electron transfer, rates of electron transfer were assayed in thylakoid membranes isolated at different times during the course of a chilling treatment. Chilling-sensitive cucumber and chilling-resistant pea were the two species compared in this series of experiments. The results show that chilling in the light does not immediately inhibit the potential for electron transfer in either of the species tested. Rates of electron transfer in cucumber were even more rapid than normal; these increased rates were generated because chilling in the light uncoupled electron transfer from photophosphorylation in the thylakoid membranes.

---

1 Supported by National Science Foundation grants PCM-8404911 to A. W. N. and BSR 83-14925 to the Duke University Phyto-otron.

2 Current address: Department of Botany, University of Texas, Austin, TX 78713.

---

**Abbreviations:** MV, methyl viologen; DCCD, dicyclohexylcarbodi-imide; CF, chlorophyll coupling factor.

---

Photosynthesis has been shown to be very sensitive to environmental stresses such as heat, drought, and chilling (0–15°C) (12). Several workers have found that photosynthetic activity is inhibited by long periods of chilling in the dark (4, 8). Photosynthesis is inhibited more rapidly, however, by chilling in the light, and especially at high irradiances (10). The inhibition of photosynthesis caused by chilling temperatures during illumination has been attributed to photooxidative processes (12, 15). However, the mechanism of the photooxidative process has not yet been elucidated. In an effort to determine the effects of whole plant chilling in the light on photosynthetic
provide saturating white light (~1100 μmol m⁻² s⁻¹) during electron transfer determinations. Chl concentration was determined using the procedure of Arnon (1).

RESULTS

Pea thylakoid membranes isolated at 4°C from control (warm-grown) plants exhibited an increase in the rate of light-stimulated electron transfer in the presence of the ionophore gramicidin A, a channel-forming molecule that uncouples phosphorylation by dissipating the pH gradient (Table I). In contrast, cucumber thylakoid membranes isolated at 4°C from control plants did not show an increase in electron transfer rates upon addition of gramicidin. Rates of transfer by cucumber thylakoids were relatively high before addition of gramicidin, indicating that the thylakoids were uncoupled by the isolation procedure itself, before addition of a specific uncoupling agent.

In an attempt to obtain coupled thylakoids from cucumber, membranes were isolated in homogenization buffer at room temperature. It was reasoned that chilling sensitivity of cucumber might be caused by loss of integrity of the coupling factor in the cold. The data in Table I show that the warm isolation procedure did result in coupled cucumber thylakoid membranes, and that gramicidin could now act as an uncoupler to increase the rate of electron flow. Warm isolation of pea thylakoids did not affect either their rate of electron transfer, or their extent of coupling (Table I).

Because warm-isolated, coupled cucumber thylakoids could be stored on ice after isolation without becoming uncoupled, it was apparent that cold temperatures alone were not responsible for the observed uncoupling. A series of experiments were initiated to determine which aspect of the isolation procedure was sensitive to cold temperatures. The step responsible for cucumber thylakoid uncoupling at cold temperatures appeared to be the initial homogenization procedure. Coupled, isolated cucumber thylakoids were resuspended in either warm (room temperature) or cold (4°C) homogenization buffer, resubjected to Polytron homogenization and then pelleted as usual. The initial resuspension in either cold or warm homogenization buffer did not uncouple the thylakoids (100% increase in rate of electron transfer with addition of gramicidin), but if they were then homogenized in cold buffer, they became substantially uncoupled (24% increase in rate of electron transfer with addition of gramicidin).

Even though short-term resuspension of coupled cucumber thylakoids in cold isolation buffer (0.4 M) did not uncouple the thylakoids, longer periods of storage (1 h) did affect the extent of coupling. In addition, the concentration of NaCl in the cold storage buffer appeared to be a critical factor. Coupled cucumber thylakoids were stored for 1 h at 5°C in a graded series of NaCl solutions. Cucumber thylakoids became uncoupled when stored in NaCl concentrations of 0.4 M or greater (Fig. 1). When pea thylakoids were stored under identical conditions, electron transfer remained coupled even in the presence of 0.75 M NaCl (Fig. 1).

DCCD was added to cucumber thylakoids isolated at 4°C (and therefore uncoupled) in an effort to prove that the uncoupling

Table I. Rates of Electron Transfer from Pea and Cucumber Thylakoids Isolated Either on Ice or at Room Temperature

<table>
<thead>
<tr>
<th>Species</th>
<th>Cold Isolation</th>
<th>Warm Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MV + Gramicidin</td>
<td>MV + Gramicidin</td>
</tr>
<tr>
<td></td>
<td>μmol O₂ mg⁻¹ Chl hr⁻¹</td>
<td></td>
</tr>
<tr>
<td>Pea</td>
<td>51</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>205</td>
</tr>
<tr>
<td>Cucumber</td>
<td>206</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>128</td>
</tr>
</tbody>
</table>

resulted from a loss of CF₁. DCCD blocks the proton channel of CF₀, which slows electron transfer by allowing a large pH gradient to build up. The addition of DCCD (100 nmol/50 μg Chl) reduced electron flow in cold-uncoupled cucumber thylakoids, but subsequent addition of gramicidin did not cause rates of electron transfer to return to the initial uncoupled rate, as would have been expected if DCCD had simply blocked the movement of protons through CF₀. Possibly DCCD may have directly affected the rate of electron transfer by altering the reduction and oxidation of plastoquinone (13).

Because cucumber thylakoid membranes were affected by cold isolation, all subsequent experiments described here utilized thylakoid membrane preparations that were isolated by first homogenizing the leaf tissue in room temperature isolation buffer (see "Materials and Methods"), and then quickly centrifuging at 4°C. The remainder of the isolation procedure was also performed at 4°C, which did not affect the coupling of the thylakoids.

The ability to obtain active and coupled thylakoid membranes from both pea and cucumber allowed comparative measurements of the effect of a whole-plant light and chilling treatment on rates of electron transfer. Isolated pea thylakoid membranes exhibited slight variations in the rates of both coupled and uncoupled electron transfer during the chilling treatment (Fig. 2). Throughout the experiment, the addition of gramicidin to the pea membranes was accompanied by an increase in the rate of electron transfer, indicating they were coupled in each instance.

Cucumber thylakoid membranes maintained electron transport rates at control levels or higher throughout the first day of chilling (Fig. 3). In contrast to pea thylakoids, all cucumber thylakoids isolated following initiation of the chilling treatment were uncoupled, even though initial homogenization of the tissue was carried out at room temperature. By the beginning of the second day of chilling, the rate of electron transfer had decreased to less than 40% of 25°C controls, and the isolated membranes remained uncoupled.

In additional experiments, whole plants were illuminated for 3 hr in the cold, then moved into a warm greenhouse for variable times of recovery. As shown in Figure 4, tissue that was rewarmed for 30 min still yielded uncoupled thylakloid membranes, showing that the membranes from rewarmed tissue remained uncoupled by the chilling treatment. However, thylakoids isolated from cucumber leaves after 2 or 3 hr of rewarmed were coupled once again (Fig. 4), indicating that the effect of three hours of chilling
MV was not rapidly inhibited by the chilling treatment, indicating that the potential for electron transfer exists in cucumber even after several hours of light and chilling conditions. The observed light and chilling-induced uncoupling of cucumber thylakoid membranes indicates that the components responsible for photophosphorylation are rapidly affected by this stress treatment. Pea thylakoids were not uncoupled by any temperatures or light conditions used in these experiments.

The observation that the isolation of coupled cucumber thylakoid membranes requires warm isolation temperatures may be an important consideration for the isolation of thylakoids from other species that are difficult to obtain in a coupled state. The exact mechanism of the uncoupling is currently unknown. The isolation temperature could affect the proton permeability of the thylakoid membranes. If this were the case, though, simply returning the membranes to 25°C should allow them to return to a state of lowered proton conductance. Based on the experiments reported here, it appears that either irreversible conformational changes occurred in some crucial component involved in the coupling of the thylakoid membrane, or some factor necessary for coupling was released during the isolation procedure. One likely candidate for this component or factor would be the chloroplast coupling factor.

The chloroplast coupling factor consists of two protein components, one of which (CF₂) is tightly associated with the thylakoid membrane, while the other (CF₁) resides on the stromal surface of the membrane, connected to CF₀ (for a review, see Merchant and Selman [9]). Low temperatures (0–4°C) have been shown to dissociate purified spinach CF₁ in the presence of salts (7), so low temperatures may generally affect the coupling factor of a number of plant species, including those, such as spinach, considered chilling tolerant. Santarius (14) has shown that spinach thylakoid membranes are uncoupled by temperatures below 0°C (in the absence of ice formation) in the presence of NaCl (250 mM to 1.0 M). Uncoupling was shown to be due to the dissociation of CF₁ from the thylakoid membranes. Assuming the same mechanism is functioning in these experiments, the NaCl-induced uncoupling of cucumber thylakoids after one hour of storage at 5°C suggests that the CF₁ component of cucumber dissociates at higher temperatures than the CF₁ of chilling-resistant spinach. Pea thylakoid coupling, like spinach, is not affected by higher concentrations of NaCl at 5°C, suggesting that the forces binding CF₀ and CF₁ of chilling-resistant species are stronger or in some way different from those of chilling-sensitive cucumber.

During whole-plant chilling stresses, pea thylakoid membranes were affected very little by the chilling stress imposed in this study. Pea is a chilling-resistant species, and does not display visible symptoms of photosynthetic damage during a light and chilling stress (11). Rates of electron transfer and the degree of membrane coupling in pea were both close to control values during the course of the chilling treatment.

Cucumber, on the other hand, is a chilling-sensitive species that is severely injured by exposure to cool temperatures and light (2, 5, 11, 16). Electron transfer rates in thylakoid membranes isolated from chilled cucumber leaves were much higher than coupled control rates. Further, the addition of the uncoupler gramicidin to thylakoids from chilled plants caused no increase in electron transfer rates, indicating that the membranes were already in an uncoupled state.

Two earlier reports have suggested that photophosphorylation in cucumber thylakoids is rapidly inhibited by light and chilling (2, 5). In both reports, phosphorylation was inhibited more rapidly than electron transfer. Chilling in the dark had little subsequent effect on cucumber thylakoid photophosphorylation. Their results support the finding reported here that chilling in the light uncouples cucumber photosynthetic electron transfer.

DISCUSSION

The results of these experiments demonstrate two important points. First, the temperature at which cucumber thylakoid membranes are isolated can markedly affect the extent of coupling of their electron transfer chain. Second, electron flow from H₂O to

and light on cucumber thylakoid coupling was not permanent. Permanent uncoupling may require a longer exposure to stress.

A similar experiment was performed to determine the effect of chilling in the dark on cucumber thylakoid electron transfer. Cucumber plants were chilled in the dark at 5°C for 3 hr. The effect of dark rewarming on the coupling of thylakoid electron transfer following 3 h of dark chilling is shown in Figure 5. While dark chilling did cause uncoupling, coupling was restored within 10 min after the plants were returned to 25°C, demonstrating that the effect of chilling in the dark on coupling was much shorter lived than the effect of chilling in the light.

FIG. 2. The effect of whole-plant light and chilling on rates of electron transfer in isolated pea thylakoids. Electron transfer was from H₂O to MV, and was measured at 25°C in a Clark-type oxygen electrode. Thylakoids were uncoupled by addition of 0.2 μM gramicidin. Activities are expressed as a percentage of the uncoupled control rate, which was equal to 112 μmol O₂ mg⁻¹ Chl h⁻¹ in one experiment, and 89 μmol O₂ mg⁻¹ Chl h⁻¹ in another.

FIG. 3. The effect of whole-plant light and chilling on rates of electron transfer in isolated cucumber thylakoids. Electron transfer was from H₂O to MV, and was measured at 25°C in a Clark-type oxygen electrode. Thylakoids were uncoupled by addition of 0.2 μM gramicidin. Activities are expressed as a percentage of the uncoupled control rate, which was equal to 78 μmol O₂ mg⁻¹ Chl h⁻¹ in one experiment, and 170 μmol O₂ mg⁻¹ Chl h⁻¹ in another.
The uncoupling of cucumber thylakoid electron transfer from photophosphorylation by dark chilling appears to be in disagreement with earlier literature reports (2, 5). The speed with which thylakoid coupling returned during rewarming following 3 h of dark chilling may explain the difference in results. In earlier investigations, the thylakoids may have recoupled before photophosphorylation was measured. In contrast to dark chilling results, thylakoids isolated from cucumber plants chilled in the light remained fully uncoupled for at least 30 min after being removed from 5°C conditions.

The results presented here, along with the work of Garber (2) and Kislyuk and Vas’kovskii (5) lead to the conclusion that photophosphorylation in cucumber is markedly affected by chilling in the light. The situation may be analogous to the effect of temperature on the isolation procedure, where chilling temperatures, in concert with appropriate concentrations of NaCl in the isolation buffer, changed the thylakoid membranes so that they were uncoupled following isolation. However, chloroplast internal ion concentrations would have to be high to mimic the isolation conditions. While ion concentrations increase during illumination in the compartment containing the chloroplast coupling factor, the total concentration of ions within the chloroplast is not believed to reach 400 mM (3). In addition, the thylakoids uncoupled by chilling in the light required over 30 min of rewarming to return to a coupled state, while the uncoupling caused by a cold isolation procedure could be avoided by simply isolating at a higher temperature. Therefore, the uncoupling observed following chilling in the light may be the result of a different mechanism than uncoupling caused by cold isolation.

Uncoupling caused by chilling in the light could be the result of several different processes. The coupling factor itself could be quite sensitive to the photooxidative reactions that are believed to be responsible for damages caused by chilling in the light. In addition, the thylakoid membranes may be made permeable to protons by photooxidative damage during chilling in the light, which would effectively uncouple the membranes.

Uncoupling has previously been shown to increase the photoinhibitory effects of high light (6). Therefore, cucumber thylakoid uncoupling may contribute to the photosynthetic damage caused by chilling in the light, instead of simply being a result of photooxidative reactions.

In contrast to the uncoupling seen in cucumber, pea thylakoid membranes were not uncoupled by the conditions used in these experiments; namely low temperature isolation in high NaCl concentrations, or whole-plant chilling in the light. The difference in isolation stability at low temperature indicates a difference between the two species, specifically in their CF0-CF1 complexes. The difference in integrity of thylakoid membrane coupling following chilling in the light demonstrates a fundamental difference in a crucial photosynthetic function that may have some bearing on why pea is a chilling-resistant plant and cucumber a chilling-sensitive plant.

Acknowledgments—We appreciate the advice and support of Drs. J. N. Siedow and M. T. Peeler in preparing this manuscript.

LITERATURE CITED