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In situ evidence that chilling in the light does not cause uncoupling of photophosphorylation or detachment of coupling factor in chilling-sensitive plants

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Abstract

The potential involvement of impaired photophosphorylation in the chilling sensitivity of photosynthesis in warm climate plant species has been a topic of investigation for more than two decades. With recent advances in the analysis of photosynthetic energy transduction in intact leaves, experiments are now possible that either address or avoid important uncertainties in the significance and interpretation of earlier in vitro work. Nevertheless, different laboratories using different techniques to analyze the effects of chilling in the light on photophosphorylation in intact cucumber (*Cucumis sativus*) leaves have come to very different conclusions regarding the role of impaired ATP formation capacity in the inhibition of net photosynthesis. In order to evaluate these discrepancies and bring this issue to a final resolution, in this investigation, we have made a detailed analysis of the decay of the flash-induced electrochromic shift and changes in chlorophyll fluorescence yield in cucumber leaves before, during and after a 5 h light-chill at chill temperatures of between 4 and 10 °C. We feel that our findings address the major discrepancies in both data and interpretation as well as provide convincing evidence that photophosphorylation is not disrupted in cucumber leaves during or after light and chilling exposure. It follows that impaired photophosphorylation is not a contributing element to the inhibition of net photosynthesis that is widely observed in warm climate plants as a result of chilling in the light.

Abbreviations: CF – chloroplast coupling factor or CF_1CF_o-ATP synthase; ΔA_{518} – flash-induced electrochromic absorbance change measured at 518 nm; DCCD – N,N′-dicyclohexylcarbodiimide; $\Delta\mu_H^+$ – transmembrane electrochemical potential of hydrogen ions; $\Delta\psi$ – the electrical charge component of $\Delta\mu_H^+$; ΔpH – the hydrogen ion concentration component of $\Delta\mu_H^+$; F_o and F_m – the yields of chlorophyll fluorescence from dark-adapted material when all Photosystem II centers are open (F_o) or closed (F_m); F_o' and F_m' – F_o and F_m measured in light-adapted material; F_s – steady-state chlorophyll fluorescence yield in light-adapted material; Q_A – primary quinone electron acceptor of Photosystem II; PPFD – photosynthetic photon flux density

Introduction

Net photosynthesis in chilling-sensitive plant species is swiftly and severely inhibited when exposure to chilling temperatures ($0 < T < 10$ °C) occurs in the light (e.g. Baker et al. 1988). Because of the central role that ATP plays in chloroplast metabolism, photophosphorylation has appropriately received considerable attention in the search for those steps in photosynthesis which may account for its low temperature sensitivity. A divergence of views regarding the effect of low temperature and light exposure on photophosphory-

lation in chilling-sensitive species has emerged from these research efforts. Until recently (Terashima et al. 1991b) it appeared that seemingly contradictory results in the literature could be adequately reconciled on the basis of in vitro versus in situ differences (Wise and Ort 1989; Ortiz-Lopez et al. 1990).

In the earliest work on the topic, Kislyuk and Vas'kovskii (1972) reported that, coincident with a large reduction in photosynthesis of intact cucumber cotyledons, chilling in the light inhibited both cyclic and non-cyclic photophosphorylation capacity in thylakoids isolated from the chill-treated cotyledon tissue. Garber (1977) extended the characterization of the in vitro effects of light chilling showing that cyclic photophosphorylation and proton uptake activity declined in proportion to decreased capacity of the cucumber thylakoids for trypsin-activated ATP hydrolysis. Peeler and Naylor (1988a,b) chilled whole cucumber plants in the light and found that CO_2 assimilation by intact leaves was severely inhibited and that uncoupler treatment of isolated thylakoids failed to further stimulate the rate of electron transport. They concluded, as had Kislyuk and Vas'kovskii (1972) and Garber (1977) before them, that the chilling and light treatment of cucumber plants uncoupled ATP formation from electron transport in isolated chloroplasts. Albeit somewhat indirect, these results implicated detachment of the catalytic portion of the coupling factor (CF_1) from the thylakoid membrane as the underlying basis for the light and chilling-induced inhibition of photophosphorylation.

Using direct procedures to define the involvement of CF_1 in the inhibition, Terashima and co-workers (1989a,b, 1991a) provided the strongest evidence that photophosphorylation can be significantly inhibited in thylakoids isolated from cucumber leaves chilled in the light. Although electron transfer activity was not significantly affected in thylakoids isolated from cucumber leaf discs immediately after they had been chilled in the light, ATP synthesis and proton uptake were undetectable. The addition of DCCD, which prevents ungated proton efflux through CF_o that occurs if CF_1 becomes detached, restored most of the proton uptake and ATP formation capacity. Analysis by gel electrophoresis showed that the α and β subunits of CF_1 were partially lost from the cucumber thylakoid membranes isolated following the chilling and light treatment.

Thus, there is reasonably strong accumulated evidence that, in cucumber thylakoids isolated from illuminated plants immediately after chilling, ATP formation can be uncoupled from electron transport due to detachment of CF_1 from the thylakoid membrane. However, there remained the important issue of whether the uncoupling induced by CF_1 release is the cause of the persistent, largely irreversible inhibition of photosynthesis in cucumber plants after chilling in the light. In earlier work from our laboratory (Wise and Ort 1989), we investigated this issue employing the flash-induced electrochromic change to monitor photophosphorylation competence in intact cucumber leaves. Thirty minutes following a light and chilling exposure sufficient to inhibit light- and CO_2-saturated photosynthesis by 50%, the relaxation kinetics of the flash-induced electrochromic change showed that the capability for rapid ATP formation was maintained. This study provided strong evidence that there is little if any uncoupling, or inactivation of coupling factor, in light-chilled cucumber plants that exhibit a large and persistent inhibition of net photosynthesis.

Subsequent work (Terashima et al. 1991a,b) suggested that one important factor in explaining the difference between the in vitro findings of Terashima et al. and our in vivo measurements may have been the different length of rewarming used in the two studies. Terashima and coworkers (1991a) extended their initial work on isolated thylakoid membranes finding that, consistent with our results from electrochromic change measurements on whole leaves, thylakoids isolated after leaves had been rewarmed for 30 min showed good coupling between electron transfer and ATP formation. On the bases of these observations, our laboratories published a joint Letter to the Editor in this journal (Wise et al. 1990) concerning two important consensus interpretations:

i) Chilling in the light induces a reversible condition in cucumber that permits the release of CF_1 during thylakoid purification but,

ii) The inhibition of net photosynthesis that persists after rewarming is not directly due to any effect of chilling and light on photophosphorylation competence.

Nevertheless, even transient uncoupling, were it to occur in intact leaves, could represent the first step in a cascade of events leading to an inhibition of photosynthesis that persists even after coupling has been restored. Thus it is important to establish if CF_1 detachment occurs in vivo during prolonged exposure of chilling-sensitive plants to low temperature in the light or, alternatively, if the loss of CF_1 is caused by thylakoid isolation procedures. Recently Terashima et al. (1991b) published findings which they interpreted

as indicating that thylakoid membranes in cucumber leaves gradually become uncoupled when the leaves are held at low temperature in the light. Since we did not feel that a consistent picture was emerging from the overall body of pertinent experimental data, we believed it important to extend the investigation and hopefully bring this issue to a final resolution. Our intent was to address seemingly contradictory findings by taking a critical look at alternative non-invasive methods used to monitor in vivo energy coupling and ATP formation. We feel that our findings address the major discrepancies in both data and interpretation as well as provide convincing evidence that uncoupling does not occur in cucumber leaves during light and chilling exposure.

Materials and methods

Plant material and chilling conditions

Cucumber plants (*Cucumis sativus* L. cv Poinsett 76) were pot grown in a growth chamber on a 16 h/8 h, 25 °C/22 °C day/night cycle. The incident PPFD was between 370 and 450 μmol m^{-2} s^{-1}. The first or second nearly fully expanded true leaves, or leaf discs cut from the same, were used for all measurements.

We mimicked the chilling treatment procedure used by Terashima et al. (1991b), in which attached leaves or leaf discs were floated on water thermostatted at 4, 6.5 or 10 °C and illuminated for 5 h at an incident PPFD of 180 μmol m^{-2} s^{-1}. Where measurements were made during the light-chill period, the temperature of a portion of a single attached leaf was maintained at 4 °C by circulating cooled, moist air through the leaf chamber.

Measurement of in vivo flash-induced electrochromic absorption change (ΔA_{518})

Measurements of flash-induced ΔA_{518} kinetics were made using a laboratory-built apparatus similar to that described by Wise and Ort (1989). Data were acquired through a PC-based system using software written in-house. Between 5 and 10 traces, collected at 0.1 Hz (23 °C) or 0.025 Hz (4 °C), were routinely averaged for analysis of ΔA_{518} decay. The amplitude of the fast phase of the flash-induced ΔA_{518} formation was determined from an average of 40 to 60 traces collected at 0.1 Hz. Assuming the predicted exponential saturation of the reaction centers as a function of increasing flash intensity, it was calculated from light attenuation experiments with calibrated neutral density filters, that the xenon flashes were more than 98% saturating.

The relaxation of the ΔA_{518} is most often evaluated as the sum of two first order exponentials (e.g. Wise and Ort 1989) according to the equation

$$\Delta A_{518} = \Delta A_{518f} e^{-\tau f\, t} + \Delta A_{518s} e^{-\tau s t} \qquad (1)$$

where both t (time) and τ (relaxation time constant) are in ms and f (fast) and s (slow) denote the two components of the decay. However, the well documented $\Delta\mu_H{}^+$ thresholds for activation and deactivation of coupling factor can further complicate the analysis as can the lowering of this threshold by reduction of the γ-subunit of CF_1 by the thioredoxin system (Mills and Mitchell 1984; Junesch and Gräber 1987; Hangarter et al. 1987; Kramer and Crofts 1989). These additional features of coupling factor regulation explain why it is often not possible to adequately fit ΔA_{518} decay profiles to only two exponential decay phases (e.g. Kramer and Crofts 1989).

In our recent work on coupling factor regulation in leaves it has become apparent that the complexity of the ΔA_{518} decay profile is very dependent on the redox state of CF. When CF is in the fully reduced state, the slow component of the relaxation is exceedingly small and the overall decay can actually be fit to a single exponential term. As the population of coupling factors becomes partially oxidized three exponential terms are needed to represent the different redox and activation states of CF and to obtain a good fit to the decay data. The introduction of a third phase (τ_{middle}) has only a small effect on the calculated rate constant for the fast phase (τ_{fast}), but can have a significant effect on the calculated value of τ_{slow} (which is increased) as well as on the amplitude of the decay attributable to this phase (which is decreased).

Finally it should be noted that overlapping formation kinetics of the flash-induced ΔA_{518} can, under some situations, influence the analysis of both the flash-induced amplitude and relaxation kinetics. In attached leaves, formation of the flash-induced ΔA_{518} has an extremely rapid phase contributed by charge separation at the reaction centers of PS I and PS II as well as a slow component associated with the Q-cycle of the cytochrome b_6f complex. Since the electrogenic reactions of the Q-cycle and the turnover time of CF have similar time constants, the rate constant and flash-induced amplitude of the fast phase of the decay will be somewhat underestimated. While correction of this underestimation can be made by assuming that the

kinetics and amplitude of the slow phase of the ΔA_{518} remain constant, we have not done so here since the correction would be the same throughout and thus not revealing.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence measurements were made using a Walz PAM 100 fluorometer (Effeltrich, Germany[1]). Actinic illumination and light-saturating pulses were provided from halogen light sources filtered by Melles Griot (Irvine, CA) hot mirrors. Pulses (800 ms) of intense white light (6500 μmol m^{-2} s^{-1}) were used to fully reduce Q_A.

The various chlorophyll fluorescence parameters referred to in the text were calculated as follows:

$$qP = (F_m' - F_s)/(F_m' - F_o') \quad (2)$$

$$qN = (F_m - F_m')/(F_m - F_o') \quad (3)$$

$$F_v/F_m = (F_m - F_o)/F_m \quad (4)$$

$$\Delta F/F_m' = qP \times F_v'/F_m' \quad (5)$$

$$\Delta F_m/F_m' = (F_m - F_m')/F_m' \quad (6)$$

The term qP is a coefficient for photochemical quenching of chlorophyll fluorescence, (quenching from F_m attributable to PS II photochemistry) and is primarily dependent on the redox state of Q_A (Bradbury and Baker 1981; Quick and Horton 1984; van Kooten and Snel 1990). qN is a coefficient for non-photochemical quenching of chlorophyll fluorescence, i.e. quenching from F_m that is independent of the redox state of Q_A (Bradbury and Baker 1981). Very often, a large proportion of qN is associated with the formation of a ΔpH and is termed 'energy-dependent' or 'high-energy state' quenching (Briantais et al. 1979; Quick and Horton 1984). F_v/F_m is theoretically proportional to the maximum quantum yield of PS II (Butler 1980). $\Delta F/F_m'$, which is the product of F_v'/F_m' and qP, is theoretically proportional to the quantum yield of PS II photochemistry, and has been shown to be proportional to the quantum yield of CO_2 fixation under many circumstances (Genty et al. 1989). $\Delta F_m/F_m'$ is theoretically proportional to the rate constant for non-radiative decay within the pigment bed of PS II (Demmig et al. 1987; Ruban et al. 1993) and, from a mechanistic viewpoint, is closely related to qN.

Treatment of attached leaves with DCCD

A 10–15 cm^2 area of both the abaxial and adaxial sides of an attached cucumber leaf were lightly abraded with 400 grit carborundum and washed with water. A solution of 400 μM DCCD, 5% (v/v) methanol and 1% (v/v) Tween 20 was applied to both surfaces following a 5 h treatment in the light (180 μmol m^{-2} s^{-1}) at either room temperature (control) or at 4 °C (chilled). The DCCD treatment on control leaves was for 30 min in the dark at room temperature. For chilled leaves, the DCCD treatment was carried out at 4 °C for 1 h under dim illumination (30 μmol m^{-2} s^{-1}).

Results

Measurements of ΔA_{518} in cucumber leaves before, during and after light and chilling exposure. The in situ activity of CF can be monitored in intact leaves by taking advantage of the contribution that the electric potential of the $\Delta\mu_H^+$ makes to flash-driven ATP formation (e.g. Morita et al. 1981; Wise and Ort 1989; Kramer and Crofts 1989). In photosynthetic membranes, the fate of the electric potential can be conveniently monitored through its effect on the absorption spectrum of a specialized group of pigments within the membrane. Membrane depolarizing proton efflux through the coupling factor complex associated with ATP synthesis results in an accelerated relaxation of the electric field-associated absorption change (Witt 1979). Loss of the naturally low conductance of the thylakoid bilayer to protons and other ions would also cause an accelerated decay, but then, the rate of decay would not respond to factors which control CF activity (such as $\Delta\mu_H^+$-dependent activation) and can be distinguished on that basis. This approach can be extended to monitor the reduction of coupling factor since reduction of CF γ-subunit significantly lowers the energetic threshold, and therefore the number of actinic flashes, necessary to activate CF and initiate ATP formation (Hangarter et al. 1987). The wavelength dependence of the flash-induced absorbance change in cucumber leaves has been published elsewhere (Wise and Ort 1989) and is dominated by the electrochromic band-

[1] Product and manufacturer names are necessary to report factually on the experiments performed, however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that many also be suitable.

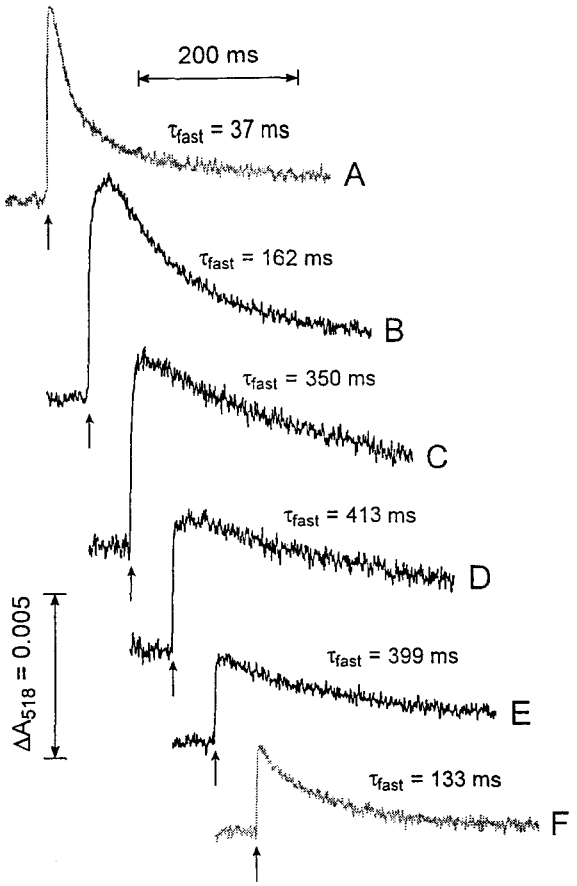

Fig. 1. The effect of low temperature exposure (4 °C) and simultaneous illumination (180 μmol m^{-2} s^{-1}) on the amplitude and relaxation kinetics of the flash-induced ΔA_{518} in intact cucumber leaves. Each trace is the average of 5 flashes repeated at 0.1 Hz (23 °C) or 0.25 Hz (4 °C). A slower flash frequency was used at low temperature to accommodate the slower rates of electron transfer and thus ensure complete recovery of photochemical activity between flashes. Leaf temperature was monitored with a thermocouple mounted on the leaf surface. The arrows indicate the position of the single turnover actinic flash. The relaxation rate constants (τ_{fast}) were calculated as detailed in 'Materials and methods'. Trace A shows the typical flash-induced ΔA_{518} change in a light-adapted leaf at 23 °C. Trace B was taken immediately after the leaf was cooled to 4 °C and traces C, D and E after the leaf had been at low temperature in light for 1.5, 3 and 5 h. Trace F was recorded after a 5 min dark recovery at 23 °C following the 5 h chill treatment. Note that traces A and F were made at 23 °C whereas traces B-E were made at 4 °C

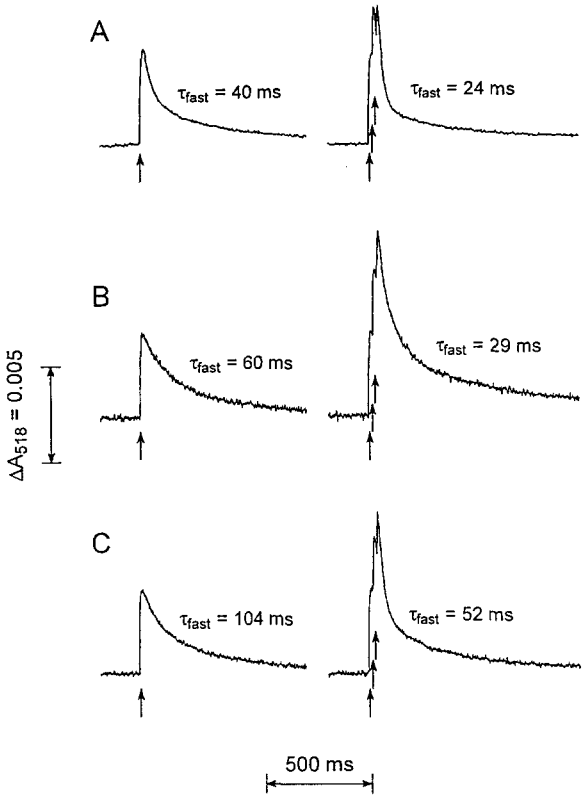

Fig. 2. The dependence of ΔA_{518} relaxation kinetics on the amplitude of the membrane potential before and after chilling of intact cucumber leaves. This experiment was performed in a similar fashion to that described in Fig. 1 and compares the effects of cumulative energization from 3 sequential flashes (spaced 10 ms apart) to a single flash on ΔA_{518} amplitude and relaxation kinetics. The traces in part A were performed with light-adapted control leaves. The traces in part B were performed on leaves that had been chilled at 4 °C at an irradiance of 180 μmol m^{-2} s^{-1} for 5 h and then dark adapted at 23 °C for 5 min. In part C, the 23 °C dark adaptation period was extended to 30 min.

shift showing an absorption change maximum at 518 nm.

In order to investigate the effect of light and low temperature on ATP formation competence in cucumber, we examined the effect of light-chill conditions on ΔA_{518} relaxation kinetics in an intact cucumber leaf before, during and after a 5 h exposure to 4 °C and 180 μmol m^{-2} s^{-1}. Trace A in Fig. 1 shows the typical flash-induced ΔA_{518} change in a light-adapted leaf at room temperature. The predominate, rapidly relaxing component (τ_{fast} = 37 ms) is attributable to H^+ efflux through CF associated with ATP formation. The expected temperature dependence of ATP formation is seen in the substantially slower relaxation (trace B, τ_{fast} = 162 ms) that immediately accompanies a decrease in leaf temperature from 23 °C to 4 °C. Other, non-thermodynamic, effects of low temperature on ATP formation are evident as the length of the light and chill treatment is extended.

After a 1.5 h exposure (trace C), relaxation of the ΔA_{518} is extremely slow. This effect of prolonged light chilling on photosynthetic ATP formation in cucumber leaves (i.e. traces C–E), can be at least partly attributed to lower membrane energization (Wise and Ort 1989). The lowered level of energization is revealed in the decreasing amplitude of the ΔA_{518} (e.g. compare trace E to trace B) which resulted from progressive inhibition of flash-driven electron and proton transfer by the light chilling treatment (discussed later). It is almost certain that other factors involving the activation state of the CF pool are also involved in determining the rate of ATP formation during the light chilling treatment. However, what is important for the central focus of this study is that there is clearly nothing within these data to suggest an uncoupling of photophosphorylation during the low temperature treatment (i.e. the decay rates become slower, not accelerated). Similar measurements on leaf discs produced comparable results (not shown).

The data presented in Fig. 2 extend the experiments of Fig. 1 demonstrating that not only did the thylakoid membranes retain low conductance to protons and other ions during the low temperature and light treatment but that they also retain nearly full ATP formation competence. In these experiments, 3 closely-spaced flashes were used to overcome the diminishing effects of the chilling exposure on membrane energization and thereby isolate any effects which the chilling treatment had directly on CF function. Post light-chill measurements were made after dark-adapting the leaf discs for either 5 (traces B) or 30 (traces C) min at 23 °C. Whereas comparison of traces A and F in Fig. 1 indicated a substantial inhibition of coupling factor activity following chilling, when comparisons were made under comparable levels of membrane energization (traces A and B, Fig. 2) the difference was essentially non-existent. After 30 min dark adaptation (trace C), CF_1 has largely reoxidized, causing the flash-induced ΔA_{518} relaxation to become slower (see discussion of Fig. 3 below). The most important point to be made from these data is that τ_{fast} remained dependent on the amplitude of ΔA_{518} following the light-chill indicating that, not only did CF_1 remain attached to thylakoid, it also retained high net ATP synthesis capacity.

As mentioned earlier, thioredoxin-mediated reduction of the CF_1 γ-subunit lowers the energetic threshold for CF activation and in so doing results in an accelerated flash-induced ΔA_{518} decay by increasing the proportion of activated CF in the total pool (e.g. Kramer et al. 1990; Wise and Ort 1989). The experiment summarized by Fig. 3 was designed to investigate whether this light-dependent reduction of CF_1 was affected by a 5 h light-chill. In this case, leaf discs were floated on water at 10 °C (Fig. 3A), 6.5 °C (Fig. 3B) or 4 °C (Fig. 3C) and illuminated for 5 h at 180 μmol m^{-2} s^{-1}. Following the light-chill (after 2.5 min dark-adaptation at 23 °C) τ_{fast} was determined from the decay of ΔA_{518}, following a saturating single-turnover flash. The disc was then illuminated by 300 μmol m^{-2} s^{-1} for 30 s which is normally sufficient to cause full reduction of the CF pool (Kramer et al. 1990). After dark-adapting the leaf disc for a further 2.5 min, τ_{fast} was re-measured. This process was repeated on the same leaf discs 30 min after the end of the light-chill

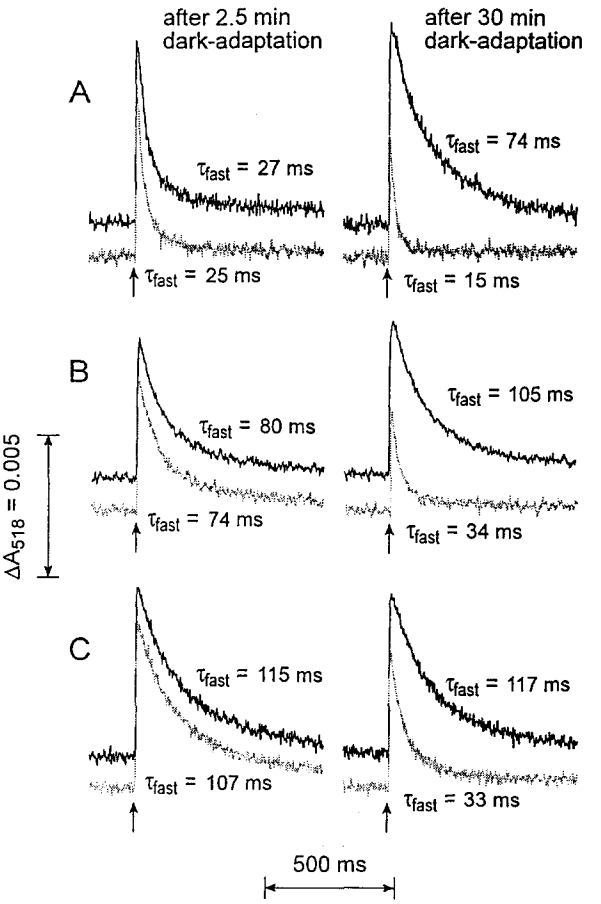

Fig. 3. The effect of the chilling treatments on light-dependent coupling factor reduction. Cucumber leaf discs were floated on water thermostatted at 10 °C (A), 6.5 °C (B) or 4 °C (C) for 5 h at an irradiance of 180 μmol m^{-2} s^{-1}. The flash-induced ΔA_{518} was measured after 2.5 and again after 30 min of dark adaptation at 23 °C (upper traces). The lower traces were recorded following a 30 s illumination at 300 μmol m^{-2} s^{-1} (which is normally sufficient full thioredoxin-dependent reduction of the CF pool) and a subsequent 2 min dark interval (to permit relaxation of the $\Delta\mu_H^+$ formed during the preillumination). Each trace is the average of 10 flashes given at 0.1 Hz. All measurements were made at 23 °C.

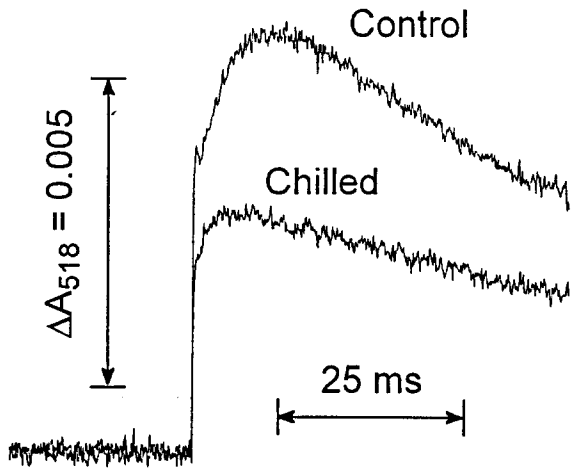

Fig. 4. Differential inhibition of a 5 h chilling treatment at 4 °C (C) and an irradiance of 25 μmol m^{-2} s^{-1} on the fast and slow components of flash-induced ΔA_{518} formation. Measurements were made at 23 °C following a 5 min dark adaptation. Each trace is the average of 10 flashes given at 0.1 Hz.

period (the leaf discs spent the intervening time in the dark at 23°C which is normally sufficient to allow complete oxidation of CF). Immediately following the 4 °C chilling treatment (i.e. following a 2.5 min rewarming period in the dark) the 30 s illumination was ineffective in inducing an acceleration of the flash-induced ΔA_{518} decay (Fig. 3c) possibly indicating that electron flow to thioredoxin or from thioredoxin to coupling factor was diverted due to the light-chill treatment. Within 30 min, this effect of chilling on coupling factor activation had all but fully reversed even though the > 50% inhibition of light and CO_2-saturated net photosynthesis would persist for many hours (data not shown). This figure further demonstrates that the treatment at 10 °C is benign (Fig. 3A) whereas treatment at 6.5 °C results in characteristics intermediate between control behavior and the relatively stronger effects of the 4 °C treatment. Thus, immediately following the chill treatment, there is a modest impairment of ATP formation capacity due, not to uncoupling, but rather to less efficient light activation of the chloroplast ATP synthase.

The formation of the flash-induced transmembrane electrical field, measured as the ΔA_{518}, normally has two distinct kinetic components. A very rapid component, not kinetically resolved in our measurements, arises from charge separation within the reaction centers of PS I and PS II. Figure 4 shows that slightly more than 40% of the ΔA_{518} amplitude (corrected for relaxation by extrapolation of the exponential decay to the time of the flash) in control cucumber leaves is contributed by a slow component. This slow phase of the ΔA_{518} formation has been shown to arise from electrogenic electron transfer through the cytochrome b_6f complex associated with operation of the Q-cycle (Crofts and Wraight 1983). After a 5 h exposure to 4 °C and 25 μmol quanta m^{-2} s^{-1} the contribution of the slow phase to the total ΔA_{518} amplitude was less than 25% indicative of a limitation in electron transfer beyond PS II. Nearly identical changes in the amplitude of both the fast and slow phases of the ΔA_{518} formation were obtained when the chill treatment was conducted under and irradiance of 180 μmol m^{-2} s^{-1}.

Measurements of chlorophyll fluorescence in cucumber leaves before, during and after light and chilling exposure

Most of the chlorophyll fluorescence emitted from leaves arises from chlorophyll a associated with PS II. The potential usefulness of chlorophyll fluorescence measurements as a monitor of photosynthetic activity was established by the work of Butler and co-workers (reviewed by Butler 1978) who showed that changes in the yield of chlorophyll fluorescence could be interpreted in the context of a simple model in which fluorescence competes with photochemistry and non-fluorescent decay processes for absorbed excitation energy. Thus, by measuring the fluorescent yield, it is possible to monitor changes that occur in the proportion of energy that goes to photochemistry (i.e. photosynthesis) or is dissipated by thermal deactivation processes. An important and relatively recent technical embellishment was the development of modulated fluorometers which greatly simplified the separation and quantitation of photochemical and non-photochemical fluorescence quenching processes. It is the latter of these that is of most interest in the current study since the largest component of non-photochemical quenching is usually energy-dependent quenching (qE) that is associated with the presence of a transmembrane ΔpH (Murata and Sugahara 1969; Briantais et al. 1979). Thus, if a dark-adapted leaf is exposed to an actinic light, the fluorescence emission will rise quickly to a peak level, which will be lower than F_m if the actinic light is not bright enough to fully reduce Q_A of the PS II pool. Thereafter, the fluorescence emission slowly declines as non-photochemical quenching forms. The amount of non-photochemical quenching can be quan-

Table 1. The effect of chilling at different temperatures on chlorophyll fluorescence parameters in cucumber leaf discs. F_o and F_m were measured at a 5 min dark adaptation period. F_o', F_m' and F_s were measured following 12 min illumination at 300 μmol m^{-2} s^{-1}. The calculation of the fluorescence ratios is discussed in 'Materials and methods'

Conditions	Chlorophyll fluorescence parameter								
	F_o	F_m	F_o'	F_m'	F_s	qP	F_v/F_m	$\Delta F_m/F_m'$	$\Delta F/F_m'$
Control (5 min dark)	0.82	4.4	0.77	3.40	1.25	0.82	0.81	0.29	0.63
Control (30 min dark)	0.89	4.45	0.82	3.50	1.30	0.82	0.80	0.27	0.63
4 °C chill (5 min dark)	1.63	3.66	1.57	2.76	2.65	0.09	0.55	0.33	0.04
4 °C chill (30 min dark)	1.46	3.60	1.54	1.80	1.70	0.38	0.59	1.00	0.06
6.5 °C chill (5 min dark)	1.32	3.76	1.22	2.20	1.67	0.54	0.65	0.71	0.24
6.5 °C chill (30 min dark)	1.22	4.38	1.11	2.20	1.68	0.48	0.72	0.99	0.24
10 °C chill (5 min dark)	1.03	3.35	0.94	2.55	1.15	0.89	0.69	0.31	0.55
10 °C chill (30 min dark)	1.05	4.07	0.89	2.80	1.10	0.82	0.74	0.45	0.61

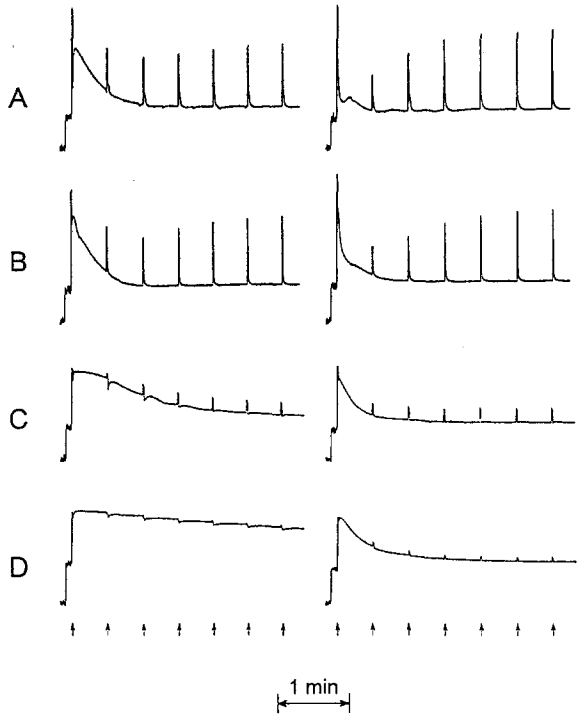

Fig. 5. The effect of different chilling temperatures and different dark recovery periods on qE formation in cucumber leafs discs. The various chilling treatments were performed has described in Fig. 3 at 10 °C (A), 6.5 °C (B) or 4 °C (D) and compared to control leaf discs which had been illuminated (180 μmol m^{-2} s^{-1}) for 5 h at 23 °C. Fluorescence measurements were made at 23 °C following a 5 min dark adaptation and again after 30 min dark adaptation. The arrows indicate the timing of the saturating pulses given to measure F_m and F_m'. The actinic light (300 μmol m^{-2} s^{-1}) was switched on concurrently with the first flash. The fluorescence parameters calculated from these experiments are compiled in Table 1.

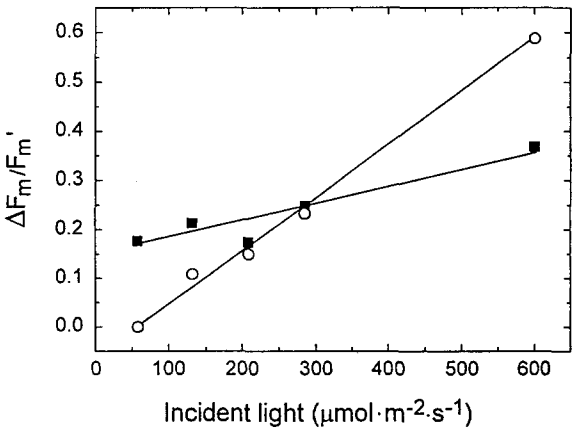

Fig. 6. The relationship between the intensity of the actinic illumination and the formation of non-photochemical fluorescence quenching ($\Delta F/F_m'$) before ■ and after ○ a 5 h chilling treatment at 4 °C and an irradiance of 180 μmol m^{-2} s^{-1}. The calculation of $\Delta F/F_m'$ was made after 12 min of actinic illumination to allow the level of non-photochemical quenching in the chill-treated plant to achieve its maximum level.

titated by using brief pulses of very intense light to fully reduce Q_A and comparing this fluorescence emission level (F_m') to F_m of the dark-adapted leaf. Although the formation of non-photochemical quenching is prevented by uncoupling, it is clear that its relationship to the ΔpH is indirect since the transmembrane pH difference is established on a much more rapid time scale than is nonphotochemical fluorescence quenching (see Rees et al. 1992 for recent review).

The traces in Fig. 5 compare the first 3 min of chlorophyll fluorescence induction curves from leaf discs which have been chilled at 10 (B), 6.5 (C) or 4

°C (D) for 5 h at an irradiance of 180 μmol m^{-2} s^{-1} with control leaves illuminated at the same intensity for 5 h at 23 °C (A). Following 5 min of dark adaptation at 23 °C, it is clear that there was a progressively severe inhibition of the formation rate of non-photochemical quenching with decreasing chilling treatment temperature. In fact, no non-photochemical quenching was evident after a 5 min dark adaptation following the 4 °C chilling treatment but, as the data ($\Delta F_m/F_m'$) in Table 1 reveals, the level of non-photochemical quenching after 12 min illumination was almost the same for the control and the 4 °C chilled leaf discs. These data are seemingly inconsistent with the observations of Terashima et al. (1991b) in which the level qE was reported to be substantially lower than the control following a nearly identical light and chilling treatment. The data presented in Fig. 6 show $\Delta F_m/F_m'$ measured from cucumber leaf discs after 12 min illumination at different irradiances. The leaf discs had either been dark-adapted for 5 min or chilled for 5 h at 4 °C at 180 μmol quanta m^{-2} s^{-1} followed by 5 min dark-adaptation. It is clear that the level of non-photochemical quenching in the chill-treated plant is strongly dependent on the illumination intensity used for the flourescence determination. Figure 5 also shows that after only 30 min at 23 °C, there was a significant recovery in the ability of the chill-treated leaves to form qN (e.g. Fig. 5D). (In all cases, the non-photochemical quenching of chlorophyll fluorescence was fully relaxed within 5 min following illumination indicating that this quenching can be attributed to qE).

Table 1 compiles a variety of chlorophyll fluorescence parameters, (measured after 12 min of illumination) that reveal several different effects of the light-chill treatments.

i) qP was substantially lower following a light-chill at 6.5 °C or 4 °C indicating that Q_A was correspondingly more reduced in these leaves. This perturbation of the PS II redox state recovers fairly slowly and is only partially reversed within 30 min.

ii) F_v/F_m is lowered significantly by the 5 h light-chill at 6.5 °C and 4 °C, but recovers less within a 30 min dark-adaptation than does qP. Varying the light intensity between 25 and 500 μmol m^{-2} s^{-1} during the chill strongly influenced the F_v/F_m value. There was virtually no change in this value when the 5 h chill was carried out at 25 μmol m^{-2} s^{-1} whereas this low temperature treatment at 500 μmol m^{-2} s^{-1} resulted in an F_v/F_m value which was consistently below 0.5.

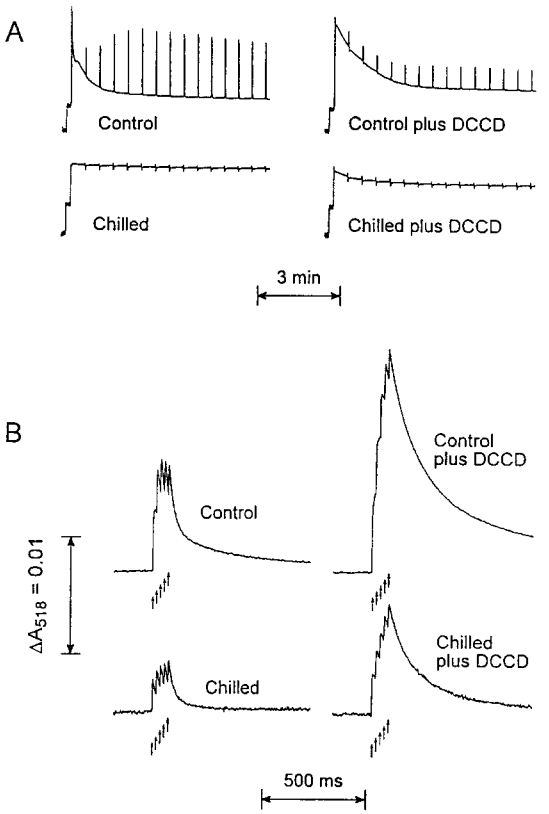

Fig. 7. The effect of DCCD on the ΔA_{518} and on qE formation in cucumber leaves before and after a chilling treatment. DCCD was introduced by abrading the leaf surface as described in 'Materials and methods'. In Fig. 7A, fluorescence induction was measured as described for Fig. 5 before and after a 5 h chilling treatment at 4 °C at an irradiance of 180 μmol m^{-2} s^{-1}. The flash-induced ΔA_{518} was measured on the same leaves. This experiment was conducted as described for Fig. 2.

iii) $\Delta F/F_m'$, a measure of the functional quantum efficiency of PS II, was markedly reduced by the 4 °C and 6.5 °C light-chills. There was minimal recovery of this parameter after 30 min dark-adaptation.

The effect of DCCD on the ΔA_{518} and on qE formation in cucumber leaves before and after light and chilling exposure

The water soluble carbodiimide DCCD binds covalently to a subunit of CF_0 thereby preventing efflux of protons and inhibiting ATP formation (Uribe 1972; Pick and Racker 1979). Following the detachment of CF_1 from the thylakoid membrane, DCCD can restore proton uptake (and even photophosphorylation if a sufficient coupling factor population remain intact) by blocking the rapid proton leakage through the 'open'

CF_o channels. Thus when Terashima et al. (1991b) observed a partial restoration of qE formation in chilled cucumber leaves by DCCD treatment, they interpreted this to mean that the carbodiimide had reversed the effect of chilling induced CF_1 release on proton accumulation. However, since it is well known that DCCD treatment substantially increases net proton accumulation even with coupling factor complexes fully intact due to the inhibition of ATP synthesis, their experiment is not actually interpretable since they did not investigate the effect of DCCD on control plants.

Figure 7A shows that the introduction of DCCD into chilled cucumber leaves does indeed enhance qE formation but the effect is small in comparison to the same experiment done with control leaves. This result is corroborated and shown even more dramatically in Fig. 7B where the effect of DCCD on ΔA_{518} formation was examined in both control and chill-treated leaves. In this experiment it can be seen that DCCD dramatically increases the cumulative ΔA_{518} from 5 sequential flashes (5 Hz) by slowing the rate of proton efflux; again the increase is much more dramatic in control leaves. Thus, when the experiment is done with the proper controls, it is clear that the results indicate a chill-induced limitation electron transfer (i.e. proton accumulation) and present no evidence for chill-induced CF_1 release.

Discussion

The ATP synthase of thylakoid membranes consists of two distinct parts. The CF_1 portion is extrinsic to the stromal face of the membrane, is composed of five different polypeptide subunits and contains the catalytic sites for ATP synthesis and hydrolysis. CF_o has four subunits, spans the membrane and lacks formal catalytic activity functioning instead to couple proton currents from the inner side of the thylakoid lumen through CF_1. The effect of CF_1 detachment on thylakoid membrane conductance has been investigated in considerable detail (e.g. Nelson and Eytan 1979; Lill et al. 1987; Lill and Junge 1989). Time-averaged single-channel conductance measurements revealed the presence of both high and low conductance CF_o channels in CF_1-depleted membranes (Lill and Junge 1989). The high conductance channels have an extraordinary proton conductance supporting a single channel current of 6×10^5 H^+ s^{-1} with a 100 mV driving force (Lill et al. 1987). That is, one high conductance CF_o per thylakoid vesicle is sufficient to fully uncouple ATP formation and comparable to potent ionophores such as gramicidin in this regard (Junge and Witt 1968). The sensitivity of the relaxation kinetics of the $\Delta A_{518}$is sufficient to easily detect not only the increased proton leakage through high conductance CF_o channels after CF_1 removal but also the flux through low conductance CF_o channels which is about two orders of magnitude lower (Lill and Junge 1989). Thus, it is expected that any significant CF_1 release induced in cucumber by chilling treatments would be readily detectable as an acceleration in the flash-induced ΔA_{518} relaxation.

The data presented within this study indicate that a 5 h light-chill at 10 °C or below in fact slowed the rate at which the flash-induced ΔA_{518} decayed compared to a light-adapted control leaf. Moreover, the rate of decay of ΔA_{518} remains sensitive to the size of the $\Delta\psi$ after a 5 h light-chill at 4 °C. This result would only be expected if ATP synthesis was the dominant process bringing about the dissipation of the flash-induced $\Delta\psi$. Although Terashima and co-workers (1991a) also observed a slowing of the ΔA_{518} decay following chilling treatment, they dismissed the ΔA_{518}result mistakenly concluding that the leakage of protons through CF_o after CF_1 detachment is a slow process and that flash-induced ΔA_{518} measurement was too insensitive to detect the H^+ leakage. As indicated above, the exquisite sensitivity of the ΔA_{518} as a integral transmembrane voltmeter has been verified in countless ways and is used extensively for this purpose in photosynthesis research.

The observation that the ΔA_{518} decay is actually slower following the chill treatments is somewhat of a side issue to the main thrust of this study but nevertheless merits comment. Although this slower rate can be partly attributed to formation of a smaller flash-induced $\Delta\mu_H{}^+$ in the chill-treated leaves, the data in Fig. 2 indicate that other factors must also be involved. After 3 saturating flashes, τ_{fast} is slower immediately after the chill than before, despite formation of a larger ΔA_{518}. The likely explanation for the light chill-induced decrease in the rate of decay of ΔA_{518} is that CF_1 becomes more oxidized during the chill period. Reduction/oxidation of CF is mediated by thioredoxin (Mills and Mitchell 1984), and current estimates of the midpoint-potential for the γ-subunit of CF_1 place it close to equipotential with thioredoxin f, about −290 mV (Kramer et al. 1990; Hutchison 1993; K. Oxborough, R.S. Hutchison and D.R. Ort, unpublished). Consequently, this aspect of CF regulation is expected to be moderately sensitive to changes in the redox-state of thioredoxin (Ort

and Oxborough, 1992). Additionally, there is already strong evidence that similar light and chilling treatments interfere with the thioredoxin-mediated activation of chloroplast fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase in tomato, another chilling-sensitive plant species (Sassenrath et al. 1990).

It is also interesting to note that the decrease in the fast-phase of formation of the ΔA_{518} which occurs after a 5 h chill at 4 °C and a PPFD of 25 μmol m^{-2} s^{-1} (Fig. 4) is not accompanied by a decrease in F_v/F_m (data not shown). This implies that charge-separation at PS I is inhibited by the light-treatment, since an inhibition of charge-separation at PS II would almost inevitably decrease F_v/F_m through an increase in F_o. Indeed, at a PPFD of 600 μmol m^{-2} s^{-1} during the chill, further inhibition of the fast phase of formation of the ΔA_{518} occurred and was accompanied by a decrease in F_v/F_m (data not shown). Again, this observation is not directly related to the main thrust of this study since it does not provide any information relevant to the question of coupling, but an inhibition of charge separation at PS I could explain a number of parallel changes which occur during chilling in the light:

i) The decrease in qP (since oxidation of plastoquinol would be greatly inhibited).
ii) The decrease in the slow phase of formation of the ΔA_{518} (since the Q-cycle is driven by PS I).
iii) The apparent oxidation of CF (since fewer electrons would be transferred through the electron transport chain to thioredoxin).

The principle basis for the conclusion by Terashima and coworkers (1991a,b) that chilling treatments caused uncoupling in cucumber leaves due to CF_1 detachment, came from their analysis of nonphotochemical quenching of chlorophyll flourescence. As mentioned earlier, qE (associated with the formation of ΔpH) is usually the most important process contributing to qN at moderate to high light intensities. Terashima et al. (1991b) reported that the formation of qE was greatly inhibited by a 5 h light-chill at 5 °C and 100 μmol m^{-2} s^{-1}. They interpreted these data as evidence of an in situ CF_1 detachment that prevented ΔpH formation which in turn was reflected in reduced qE quenching. They reasoned that the extent of qE fluorescence quenching was a more sensitive monitor of $\Delta\mu_H^+$ formation than the ΔA_{518} measurements even though there is a wide range of published evidence indicating that qE quenching is both an indirect (e.g. Oxborough and Horton 1987; Ruban et al. 1992) and relatively low sensitivity monitor of thylakoid membrane energization. For example a 'threshold' ΔpH below which little or no qE is formed has been demonstrated in a number of studies (e.g. Briantais et al. 1979; Noctor et al. 1991). Secondly, we have shown that it is not actually the case that these chilling treatments prevent qE formation. For example, the data presented in Fig. 5 show that $\Delta F_m/F_m'$ measured after 12 min of irradiance at 300 μmol m^{-2} s^{-1} is lower for the control than immediately after a light-chill at 4, 6.5 or 10 °C. We found that the development of qE is slower and strongly light intensity dependent (Fig. 6) following the chilling treatments due at least in part to impaired electron/proton transport activity.

Among the experiments most misleading to Terashima et al. (1991b) was the stimulation of qE formation that they observed upon infiltrating a light-chilled leaf disc with DCCD. They reasoned that since DCCD is known to block the movement of protons through CF_o, the lower level of qE formed in the absence of DCCD must be due to the free flow of protons through CF_o from which CF_1 had become detached during the light-chill treatment. However, they failed to anticipate that DCCD would also inhibit the 'normal' dissipation of ΔpH by ATP synthesis in coupled membranes thereby stimulating qE formation. Consequently, they failed to conduct this essential control. We have shown that DCCD treatment stimulates the extent of both qE quenching and ΔA_{518} formation and that this stimulation is much greater in control than in chill-treated cucumber leaves (Fig. 7).

Finally, Terashima et al. (1991b) presented inhibition of light-scattering changes (decrease in apparent absorbance at 535 nm) as corroborating evidence that the chilling treatments discussed in this study induced CF_1 release in cucumber leaves. Although light-scattering changes have been attributed to the formation of ΔpH in numerous previous studies (e.g. Heber 1969; Krause 1973), as with qE formation, $\Delta\mu_H^+$ is a necessary but not sufficient condition for inducing chloroplast light-scattering changes. For example, the recent studies by Ruban et al. (1992) suggest that these changes in light scattering are more closely associated with the aggregation of LHC II than ΔpH formation per se showing that the aggregation of LHC II, the formation of qE and light-scattering changes are all inhibited by low concentrations of antimycin A without any effect on the formation of ΔpH. Since this technique is neither a sensitive nor reliable indicator of $\Delta\mu_H^+$ it cannot be relied upon to provide information about the effects of chilling treatments on chloroplast coupling.

In conclusion and in summary, Terashima et al. (1989, 1991a) have presented convincing evidence that thylakoid membranes isolated from cucumber leaves immediately following a light and chilling treatment are uncoupled due to the detachment of a portion of CF_1 from the thylakoid membranes. However, we have shown that their evidence that CF_1 detachment actually occurs in situ is flawed and, furthermore, that it in fact does not happen.

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