Influence of Light on the Heat Sensitivity of the Photosynthetic Apparatus in Isolated Spinach Chloroplasts

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ABSTRACT

The most heat-sensitive functions of chloroplasts in Spinacia oleracea L. including the stromal carboxylation reaction, the light-induced electrical field gradient across the thylakoid membrane, as well as the overall photosynthetic CO₂ fixation were less affected by heat if chloroplasts were heated in the light: 50% inactivation occurred around 35°C in the dark and around 40°C in the light. Relative low light intensities were sufficient to obtain optimal protection against heat. In contrast, the light-induced ΔpH across the thylakoid membrane, the photophosphorylation, and the photochemical activity of photosystem II which were less sensitive to heat in the dark (50% inactivation above 40°C) were not protected by light. Photosystem II even was destabilized somewhat by light.

The effect of light on the heat sensitivity of the water-splitting reaction was dependent on the pH in the medium. Protection by light only occurred at alkaline pH, in which case heat sensitivity was high (50% inactivation at 33°C in the dark and at 38°C in the light). Protection was prevented by uncouplers. At pH 6.8 when the heat sensitivity was low in any case (50% inactivation at 41°C in the dark), light had no further protecting effect.

Protection by light has been discussed in terms of light-induced transport of protons from the stroma to the thylakoid space and related ion fluxes.

The sensitivity of chloroplast membranes to high temperatures is well known: the PSII-water-splitting complex and the photophosphorylation reactions are suggested to be the most heat-sensitive systems of the membrane-bound photosynthetic apparatus (for a review, see Ref. 3). Recently, it has been demonstrated with intact chloroplasts that the process of light activation of the stromal RuBP-carboxylase is one of the most heat-sensitive reactions within the overall process of photosynthetic CO₂ fixation (23, 24). However, little is known about the interactive effects of extreme temperatures and other environmental factors such as light, which are often co-varying in nature. For example, until now in most investigations dealing with reversible or irreversible heat inactivation of photosynthetic activities, the heat treatments have been carried out in the dark, whereas in nature, peak leaf temperature usually will be reached under illumination. The few data available on the effect of light on intact leaf tissue and on isolated chloroplasts during exposure to high temperature stress are rather contradictory. There are some indications that in whole leaf tissue light increases the resistance of the photosynthetic apparatus against heat (12, 22). On the other hand, it was reported that in isolated pea chloroplasts the photochemical reactions seemed to be even more sensitive to heat if chloroplasts were heated in the light (e.g. Ref. 1). In a previous paper (26), it was shown that photosynthetic electron transport of isolated intact spinach chloroplasts was less sensitive to heat in the light, but this light-induced protection was completely abolished by an uncoupler.

In the present paper, the influence of light on the heat sensitivity of photosynthetic CO₂ fixation, the electrochemical gradient across the thylakoid membrane, the water-splitting reaction, and the photochemical activity of PSII has been studied with isolated intact and broken spinach chloroplasts; the role of the light-induced proton gradient across the thylakoid membrane in protection against heat stress will be discussed.

MATERIALS AND METHODS

Spinach (Spinacia oleracea L.) was grown in a growth chamber under the following conditions: 10-h photoperiod with 20°C day/15°C night, 70% RH, light intensity about 15,000 lux. Intact chloroplasts were isolated from spinach leaves as described in Ref. 9. To prepare thylakoids, the intact chloroplasts were osmotically shocked in 5 mM MgCl₂ (1 min), and the released membranes were washed and stored in a medium containing 330 mM sorbitol, 40 mM Hepes-NaOH (pH 7.0), 30 mM KCl, and 1 mM MgCl₂.

For heat treatment, intact chloroplasts were suspended in 330 mM sorbitol, 40 mM Hepes-NaOH (pH 7.5), 1 mM MnCl₂, 1 mM MgCl₂, and 2 mM EDTA. Thylakoids were suspended for heat treatment in a medium containing 330 mM sorbitol, 40 mM Hepes-NaOH (pH as indicated), 30 mM KCl, and 3 mM MgCl₂. A suspension of 0.2 ml was placed in a glass cuvette provided with a fiber optic to allow illumination with red light (heat-absorbing filters and RG 630 cut-off filter; Schott and Gen., Mainz). The sample was preincubated in the illuminated or darkened cuvette for 3 min at 20°C; then the cuvette was transferred into a water bath having temperatures as indicated. Usually, the sample reached the treatment temperature within 10 to 30 s. After 6 min in the dark or in the light, the sample was diluted with 1 to 2 ml reaction medium and measured at 20°C for photosynthetic activities.

The photosynthetic CO₂ fixation of intact chloroplasts and the initial activity of the RuBP-carboxylase after osmotic rupture of pretreated chloroplasts were measured as in Ref. 24.

Absorption changes at 518 nm were determined as followed: samples of intact chloroplasts were pretreated as outlined above and then divided into two parts: 100 µl was used to measure photosynthetic CO₂ fixation; 100 µl was osmotically shocked in 0.7 ml distilled H₂O to release thylakoid membranes. After 30 s, 0.6 ml of a medium containing 660 mM sorbitol, 80 mM Hepes-NaOH (pH 7.5), and 60 mM KCl was added; then, the sample (20–30 µg Chl/ml final concentration) was measured for light-induced absorption changes. The absorption of the weak measuring beam, produced by a monochromator (Bausch and Lomb)
was recorded with a photomultiplier (EMI 9558 B) protected from the actinic light by glass filters (Corning 9782, 9780; Schott BG 18). The intensity of the red actinic light (Schott RG 630 and heat-absorbing filters) was 200 W/m². The optical path was 0.5 cm.

9-Aminoacridine fluorescence has been measured as described earlier (23). In order to avoid any interference with the ΔpH across the chloroplast envelope (8), pretreated intact chloroplasts were transferred to a hypotonic reaction medium containing 15 mM N,N’-bis(2-hydroxyethyl)glycine-NaOH (pH 8.0), 15 mM KCl, and 5 μM 9-aminoacridine. Phenazine methosulfate was present in the medium (7 μM) to mediate a cyclic electron transport around PSI. The intensity of the red actinic light (9 W/m²) was about half-saturating with respect to fluorescence quenching.

PSII-dependent reduction of DCIP was induced by red light (50% saturation) and measured as described in Ref. 18.

Chl fluorescence induction curves have been measured with a spectrofluorimeter (Farrand Mark I) equipped with a shutter (opening time about 0.5 ms). The intensity of the actinic light (440–480 nm) was 8 W/m²; the fluorescence was measured at 680 nm. After the pretreatment, the thylakoids were transferred into a medium containing 330 mM sorbitol, 40 mM Hepes-NaOH (pH 7.5), 30 mM KCl, and 5 mM MgCl₂ and stored in the dark at 5°C for at least 30 min. Then the sample (10 μg Chl/ml) was measured for fluorescence. The addition of ferri cyanide to the reaction medium quenched the variable fluorescence but did not influence the initial fluorescence, indicating that the PSII reaction centers were open before measuring.

RESULTS

Mild heating around 35°C which strongly inactivates the overall photosynthetic CO₂ fixation in intact chloroplasts does not affect photophosphorylation and photosynthetic electron transport from H₂O to NADP (23). Evidence exists that this inhibition is due to a decrease in the stromal carboxylation reaction; it has been suggested that high temperatures interfere with the activation of the RuBP-carboxylase (23, 24). This inhibition of CO₂ fixation is partially prevented if chloroplasts were heated in the light (Fig. 1). Illumination during the heat treatment (red light, 40 W/m²) increased T₉₀ from 35°C to 40°C. Figure 2, upper part, indicates an optimal light intensity for the light-induced protection at about 50 w/m²; however, 10 to 30 w/m² already were sufficient to obtain a substantial effect. Under similar experimental conditions, the photosynthetic CO₂ fixation reached its maximum at about 250 w/m², whereas the light-induced ΔpH across the thylakoid membrane was saturated at 20 to 30 w/m² (not shown), suggesting that the light-induced protection shown in Figure 2 was related to the light-induced ΔpH. However, at higher light intensities, the protection became diminished.

In isolated chloroplasts, the RuBP-carboxylase was found to be in a relatively inactive state in the dark and in a more active state in the light (e.g. 10). As reported earlier (24), the heat-induced inhibition of the overall photosynthetic CO₂ fixation is closely related to an inhibition of this light-dependent enzyme activation, suggesting that a slowdown in the stromal carboxylation reaction could be a primary cause for the inhibition of the overall CO₂ fixation. In the experiment shown here (Fig. 3), the RuBP-carboxylase activation was completely inhibited after a pretreatment at 40°C in the dark (a and b); this inhibition was partially prevented if the chloroplasts were heated in the light (e).

Furthermore, as already shown with intact leaves (23, 25), the heat-induced inhibition of photosynthetic CO₂ fixation is accompanied by a decrease in the light-dependent electrical field gradient across the thylakoid membrane. This can be demonstrated by light-induced absorption transients at 518 nm (Fig. 4a) which are identified as the electrochromic response of pigments to the electrical field gradient across the thylakoid membrane generated by light-induced charge separation (for a review, see Ref. 11). Figure 5 shows the influence of light on the inactivation of the electrochromic change: T₉₀ was shifted from 35°C in the dark to 40.5°C in the light; the inactivation of ΔA was closely correlated with the inactivation of photosynthetic CO₂ fixation. A protecting effect of light on the electrical field gradient is demonstrated in Figure 4b, as well. This figure shows relaxation kinetics of the electrochromic change after a light flash. Under nonphosphorylating conditions, the relaxation rate of the electrochromic absorption change and of the electrical field gradient, respectively, mainly reflects the ionic conductivity of the thylakoid membrane (11). The increase in the relaxation rate after a 38°C treatment in the dark (curves 4) was completely prevented if the treatment occurred in the light (curve 5). Essentially the same results have been obtained when broken chloroplasts were heated in the dark or in the light (not shown). The experiments strongly suggest that light prevents a heat-induced increase in the ionic conductivity of the thylakoid membrane.

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Fig. 1. Photosynthetic CO₂ fixation (▪, □) and PGA reduction (▲, △) as a function of the temperature of a 6-min pretreatment in the dark (▪, ▲) or in the light (40 W/m²; □, △). CO₂ fixation was measured polarographically in a medium containing 2 mM NaHCO₃. PGA reduction was measured in a CO₂-free medium containing 2 mM PGA. The rates of CO₂ and PGA-dependent O₂ reduction after a treatment at 20°C (100%) were 94 and 75 nmol O₂/mg Chl-h, respectively. All measurements were at 20°C.

Fig. 2. The inactivation of photosynthetic CO₂ fixation after a 35°C pretreatment (upper part) and of PSII-dependent DCIP reduction after a 41°C treatment at pH 8.0 (lower part) as a function of the intensity of illumination during the heat pretreatment. Conditions are as outlined in Figures 1 and 6, respectively.
As compared with the electrical field gradient, the light-induced proton gradient across the thylakoid membrane measured with the 9-aminoacridine method (20) was less sensitive to heat (T_{0.5} about 43°C in the dark), and light had only little effect on T_{0.5} (see Fig. 5).

The photosynthetic O₂ evolving reaction is well known to be the most heat-sensitive step within the electron transport chain from H₂O to NADP (e.g. 6, 18, 27). Figure 6 demonstrates the inactivation of the photosynthetic reduction of DCIP depending on the pretreatment temperature, mainly reflecting the inactivation of the water-splitting reaction (e.g. 18). T_{0.5} was 33°C at pH 8.0 and 41°C at pH 6.8 when the thylakoids were heated in the dark, as already shown in Ref. 26. In the light, however, there was almost no pH dependence of T_{0.5} in the pH region studied: at pH 8.0 as well as at 6.8, T_{0.5} was about 38°C. This means that T_{0.5} increased by light at pH 8.0 and even somewhat decreased at pH 6.8. Very low light intensities (10 w/m²) were sufficient to obtain maximal stabilization at pH 8.0 (Fig. 2, lower part). In the presence of the uncoupler nigericin, the stabilizing effect of light was completely abolished, suggesting that the light-induced acidification of the intrathylakoid space leads to the observed stabilization. In some experiments, illumination during the heating even caused a small destabilization at pH 8.0 if the uncoupler ammonium chloride was present in the light (see also Ref. 26).

Chl fluorescence has been proposed to be a useful intrinsic probe for testing the energy transfer within the PSII pigment apparatus and the photochemical activity of the PSII reaction centers (for a review, see Ref. 15). Heat damage of the pigment apparatus is reflected by characteristic changes in light-induced fluorescence transients (4, 13, 17, 21), and in fluorescence yield (4, 14, 22). Two parameters have been obtained from induction curves shown in Figure 7 (upper part): fluorescence yield (F₀) and the slope of the variable fluorescence (F_v). The F₀ level at the beginning of the illumination should represent mainly the weak fluorescence of light-harvesting and PSII-antenna pigments when all reaction centers are open. In terms of the quencher theory (7), the slow rise in F_v is due to the accumulation of the reduced primary acceptor Q after the reduction of the plastoquinone pool. The inhibition of F_v most likely represents the inhibition of the water-splitting reaction, as is demonstrated by the observation that F_v recovered up to 40°C if NH₄OH was present in the reaction medium. NH₄OH acts as an artificial electron donor, substituting for the water-splitting reaction (2). Therefore, it is not surprising that the inactivation of F_v (minus NH₄OH) after heating in the dark or in the light (Fig. 7, lower part) resembles the inactivation of the photosynthetic DCIP-reduction (see Fig. 6). In the presence

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Fig. 3. Initial activity of the RuBP-carboxylase of isolated chloroplasts which were pretreated for 6 min at 20°C in the dark (a), at 40°C in the dark (b), and at 40°C in the light (40 w/m² red light; c). After pretreatment, the chloroplasts were incubated for 6 min at 20°C either in the dark (●) or in the light (○); then, RuBP-carboxylase activity was measured at 20°C immediately after chloroplast rupture.

Fig. 4. a, Light-induced absorption changes at 518 nm. Intact chloroplasts were preincubated in the dark at 20°C (curve 1) or at 35°C (curve 2); then, they were osmotically broken and measured at 20°C for light-induced absorption changes. Downward arrow, light on; upward arrow, light off. b, Relaxation kinetics of the absorption change at 518 nm induced by a 1-ms light flash. The intact chloroplasts were pretreated at 20°C in the dark (curve 3), at 38°C in the dark (curve 4), and at 38°C in the light (curve 5); then they were osmotically broken and treated with five flashes before measuring at 20°C. Each curve represents four sets of measurements. Illumination during pretreatment at 20°C had no effect on the absorption change signal.

Fig. 5. Photosynthetic CO₂ fixation (●, ○), light-induced absorption changes at 518 nm (■, □), and light-induced quenching of 9-aminoacridine (9-AA) fluorescence (▲, △) as a function of the temperature of a 6-min pretreatment. The pretreatment has been carried out with intact chloroplasts either in the dark (●, ■, ▲) or in the light (○, □, △). Before measuring absorption and fluorescence changes, the intact chloroplasts were osmotically broken. Determination of ΔA was as indicated in Figure 4a. All measurements were at 20°C.

Fig. 6. Dependence of DCIP reduction of isolated thylakoids on the temperature of a pretreatment in the dark (●, ■) or in the light (○, □, △). The pH of the medium during the pretreatment was 8.0 (●, ○, △) or 6.8 (■, □, △). Pretreatment in the light in the presence of 0.3 μM nigericin DCIP reduction was measured at 20°C; The pH of the reaction medium was 7.5.

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FIG. 7. Upper part, original traces of the Chl fluorescence induction. Six-minute pretreatment of thylakoids was as follows: curve 1, 20°C in the dark; curve 2, 20°C in red light (40 w/m²); curve 3, 48°C in the dark; curve 4, 39°C in the dark; curve 5, 39°C in the dark, measured in the presence of 20 mM NH₄OH. Lower part, the slope of Fₛ as a function of the temperature of a pretreatment in the dark (●, ○) or in the light (□, □). Thylakoids treated in the light in the presence of 0.3 μM nigericin. (■, ▲). Measurements in presence of 20 mM NH₄OH.

of NH₄OH, Fₜ, was inactivated at higher temperatures (T₀.₅ at 44°C in the dark), and illumination did not cause protection but rather a small destabilization (Fig. 7, lower part). This heat-induced decrease in Fₛ in the presence of NH₄OH most likely reflects a decrease in the photochemical activity of PSII reaction centers, probably due to a damage of the PSII pigment system. A heat-induced rise in F₀ yield (Fig. 8) occurred almost in parallel with this decrease in Fₛ (plus NH₄OH). Such a heat-induced rise in F₀ has been discussed in terms of perturbation of the PSII pigment system and related inhibition of energy transfer to the reaction centers (21). Illumination during heating in the absence or presence of uncouplers had no significant effect on the F₀ → T curves (Fig. 8). Only the amplitude of the heat-induced rise in F₀ was somewhat lowered. Essentially the same results were obtained with intact chloroplasts (results not shown).

DISCUSSION

As shown in the present study, the heat-induced inactivation of the overall photosynthetic CO₂ fixation and of the light-induced electrical gradient across the thylakoid membrane in isolated chloroplasts becomes essentially diminished if the heat treatment occurs in the light (Figs. 1, 3, 4, and 5). In previous papers (23, 24), it has been concluded that after mild heat treatment the stromal environment is altered in such a way that the RuBP-carboxylase is in a more inactive state, whereas the catalytic site of the enzyme protein itself remains intact. The primary effect of mild heat stress could be its action on the chloroplast membranes structure. The results in Figures 4 and 5 suggest that mild heating increases the permeability of the thylakoid membrane to ions. This might affect the ionic environment of the RuBP-carboxylase and, therefore, prevent its activation. Illumination during mild heat stress presumably prevents this heat-induced change in membrane properties and in RuBP-carboxylase environment: during illumination a proton gradient will be built up across the thylakoid membrane and might shift this membrane to a state less sensitive to heat stress. Further experiments may clarify this suggestion.

The light-induced proton gradient itself is much less sensitive to heat, and illumination has only little effect on its inactivation (Fig. 5). The inactivation of photosynthetic PGA reduction most likely representing the inactivation of photophosphorylation (23) is less affected by light as well (Fig. 1). Therefore, I concluded that illumination does not significantly interfere with the response of photosynthetic energy conservation to heat stress.

As shown previously, the T₀.₅ temperature of the water-splitting reaction increases with increasing concentration of metal cations and protons in the medium (13, 26); especially in the range of pH 7.0 to 8.5, there is an increase in T₀.₅ by more than 10°C upon increasing proton concentration. This has been interpreted in terms of salt- and pH-dependent changes in the repulsive ionic forces within the water-splitting complex (26). From this pH dependence, it becomes clear that the water-splitting complex which is situated on the inner face of the thylakoid membrane (for a review, see Ref. 5) is highly labile in a medium with alkaline pH, but becomes stabilized when the proton concentration in the interthylakoid space is increased in the light. Accordingly, the light-induced stabilization is abolished, if uncouplers are present.

With increasing proton concentration in the medium, the complex is much less labile and light-induced acidification of the interthylakoid space has no further stabilizing effect. The significance of the small light-induced decrease in T₀.₅ occurring at pH 6.8 is still unclear. From the results, it becomes clear that in intact cells the effect of light on the thermal stability of the water-splitting complex would depend on the pH of the cytoplasm.

PSII reaction centers are known to be less sensitive to heat treatment as compared with the water-splitting complex (6). The results shown in Figures 7 and 8 indicate that light does not prevent heat damage of PSII; rather, it becomes slightly more sensitive by illumination. In intact spinach leaves as well, light does not significantly alter the transition temperature of the heat-induced rise in F₀ (results not shown). This seems to contradict results from Schreiber and Berry (22) who reported that the transition temperature of the heat-induced rise in Chl fluorescence of leaves from Tidestromia and Atriplex increased with increasing light intensity during heating. This disagreement might result from differences in the experimental conditions; in the present experiments, chloroplasts (or leaves, not shown) were heated in the dark or in the light and then kept in the dark to allow reoxidation of
the acceptor Q before measuring. Schreiber and Berry, however, have followed the steady-state fluorescence during heating under different light intensities. On the other hand, it cannot be excluded that results obtained from different plant species may differ from each other.

It is concluded that the most heat-sensitive functions of chloroplasts including the overall photosynthetic CO₂ fixation are less affected by heat if heating occurs in the light. All of the protecting effects reported in this paper might be related to the light-induced transport of protons from the stroma to the intrathylakoid space and resulting changes in the ionic milieu within the chloroplast compartments. The physiological significance of a protection by light is obvious; peak leaf temperature usually will be reached in the light. Further studies have to clarify whether there is an interference between light-induced stabilization and pho

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LITERATURE CITED