Evidence for protection by heat-shock proteins against photoinhibition during heat-shock

Gadi Schuster, Dena Even, Klaus Kloppstech¹ and Itzhak Ohad

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel and ¹Institut für Botanik, Universität Hannover, D-3000 Hannover 21, FRG

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The nuclear-coded 22 kd heat-shock protein (HSP-22) which is transported into the chloroplast and localized in the thylakoids was further characterized and found to be located in the grana lamellae (stacked thylakoids) as an extrinsic protein in the green alga *Chlamydomonas reinhardtii*. Inhibition of photosynthetic electron flow during heat-shock of *Chlamydomonas* cells was light-dependent, occurring at low-light intensities ($<100\text{ W/m}^2$) as compared with photoinhibition at 25°C ($>1000\text{ W/m}^2$). The site of the damage was localized at the photosystem II (PS II) reaction center. The damage was drastically increased when heat-shock treatment was carried out in the presence of the 80S ribosomal translation inhibitor, cycloheximide (CHI). Pre-incubation of *Chlamydomonas* cells at 42°C resulted in partial protection against photoinhibition during heat-shock, as compared with cells pre-incubated at 42°C in the presence of CHI which, therefore, did not translate the heat-shock proteins. Analysis of the thylakoid polypeptides' pattern by SDS-PAGE revealed that during heat-shock in the light, thylakoid proteins became aggregated proportionally to the light intensity. Heat-shock in the presence of CHI enhanced the aggregation process which, at low light intensities, was specific to the PS II reaction center D1-protein. The results suggest that the chloroplast HSPs prevent damage to the PS II reaction center during heat-shock in the light.

Key words: *Chlamydomonas*/D1-protein/heat-shock/photoinhibition/photosystem II reaction center

Introduction

Exposure of cells to temperatures several degrees above those of normal growth induces the expression of a set of nuclear and organelle genes coding for the synthesis of heat-shock proteins (Schlesinger *et al.*, 1982; Sinibaldi and Turpen, 1985; Lindquist, 1986; Kloppstech *et al.*, 1985, 1986). Synthesis of HSPs has been detected in every organism so far investigated, including higher plants (Kimpel and Key, 1985; Key *et al.*, 1985) and green algae (Kloppstech *et al.*, 1985, 1986; Kloppstech and Ohad, 1986). Although no specific function(s) has yet been established for these proteins, it is widely assumed that HSPs confer at least a transient protection against heat-induced damage (Key *et al.*, 1985; Schlesinger, 1986). The discovery that ubiquitin synthesis is enhanced during heat-shock suggested that specific proteo-

lysis might be involved in the heat-shock response (Munro and Pelham, 1985). In plants, heat-shock was reported to reduce photosynthetic activity due to specific damage to photosystem II (PS II) (Berry and Bjorkman, 1980; Nash *et al.*, 1985). Under normal conditions in the field, heat-shock is usually accompanied by water stress and high-light intensity, both of which may induce partial photoinhibition (Bitan, 1982; Key *et al.*, 1985). In heat-adapted plants, the threshold of heat-shock is shifted toward higher temperatures and the damage to PS II activity is significantly diminished (Key *et al.*, 1985; Suss and Yordanov, 1986).

In previous studies, it has been shown that a 22 kd nuclear-coded heat-shock protein (HSP-22) which is translated on cytoplasmic ribosomes, is transported into the chloroplasts and incorporated into the photosynthetic membranes (Kloppstech *et al.*, 1985). In addition to the HSP-22, nuclear-coded proteins of 25–29 kd were also detected in the thylakoids of soybean (Vierling *et al.*, 1986) and pea (Kloppstech *et al.*, in preparation). A 29 kd nuclear-coded HSP could also be detected in some experiments in *Chlamydomonas* (Kloppstech *et al.*, 1985). In pea, HSP-22 is synthesized as a precursor of 26 kd which is processed to a mature HSP while transported into the chloroplast (Kloppstech *et al.*, 1985; Vierling *et al.*, 1986).

In experiments in which the translation products of poly-(A)⁺ RNA obtained from heat-shocked pea plants were transported into isolated intact chloroplasts, it was found that only chloroplasts isolated from heat-shocked plants integrated the HSP-22 into the thylakoids (Kloppstech *et al.*, 1985). The ability of such chloroplasts to integrate HSPs into the thylakoids required a pretreatment of minimum 15 min at 38°C of the pea plants before isolation of chloroplasts (Kloppstech *et al.*, submitted).

In the present work, we report that HSP-22 and 29 are associated with the grana lamellae fraction of *Chlamydomonas* thylakoids. It is also demonstrated that heat-shock inactivates the PS II reaction center in a light-dependent process which induces aggregation of the thylakoid proteins as well. Inhibition of nuclear-coded HSPs during heat-shock drastically enhanced the light-dependent inactivation of PS II and the thylakoid proteins aggregation process. The results support the hypothesis (Schuster *et al.*, 1987) that chloroplast HSPs play a protective role against light-induced damage to the photosynthetic apparatus during heat-shock.

Results

Localization of the thylakoid HSPs in the grana lamellae as extrinsic membrane proteins

Chlamydomonas cells were heat-shocked at 42°C for 2 h in the presence of $^{35}\text{SO}_4^{2-}$ and the thylakoids were isolated and fractionated into grana and stroma lamellae. The results (Figure 1) show that HSP-22 and HSP-29 were present only in thylakoids from heat-shocked cells, appearing only in

Fig. 1. HSP-22 and HSP-29 are located in the grana lamellae. *Chlamydomonas* cells were labeled with $^{35}\text{SO}_4^{2-}$ for 2 h at 42 or 25°C in low-light (7 W/m^2). Thylakoids (M) (ratio chlorophyll-*a/b*, 2.5) were fractionated by treatment with digitonin and differential centrifugation, as described in Materials and methods. The chlorophyll-*a/b* ratios were 2.1 and 7.0 in the 10 k fraction (enriched in grana lamellae) and 144 k fraction (enriched in stroma lamellae) respectively. G, Coomassie Brilliant blue R stained gel. A, autoradiogram.

the grana-enriched fraction. In addition to HSP-22 and 29, a heat-shock protein of about 75–80 kd can also be observed in the membrane preparation used in this experiment. Nuclear-coded HSPs in this mol. wt range have been reported before in *Chlamydomonas* (Kloppstech *et al.*, 1985). These HSPs are not associated with the chloroplast membranes, therefore we assume that the presence of this protein in the membrane fraction in this case is an artifact which might have been caused by the conditions used to fractionate the chloroplast membranes into grana and stroma lamellae fractions. Treatment of thylakoids containing radiolabeled HSPs with 1 M urea, 1 M NaCl or 0.8 M Tris buffer released the HSPs into the soluble fraction, together with the 29, 23 and 18 kd membrane extrinsic proteins involved in the water oxidation process (Schuster *et al.*, 1987; Even *et al.*, in preparation). Based on these data we conclude that the HSPs are membrane extrinsic proteins localized in the grana region of the thylakoids.

Inhibition of photosynthetic electron flow and loss of variable fluorescence during heat-shock

Oxygen evolution was not significantly inhibited in cells exposed to heat-shock at 42°C for 3 h in the dark. Heat-shock as above at low-light intensity ($7\text{--}100 \text{ W/m}^2$) resulted in inhibition of electron flow and reduction of variable fluorescence $(F_m - F_0)/F_0$, indicating photoinhibition damage to PS II. The ratio $(F_m - F_s)/F_0$ decreased as well, indicating loss of overall photosynthetic activity (Figure 2). Measurements of variable fluorescence during heat-shock

Fig. 2. Effect of heat-shock on variable fluorescence and photosynthetic electron flow of *Chlamydomonas* cells. **Left panel:** changes in variable fluorescence $[(F_m - F_0)/F_0]$ and electron flow $[(F_m - F_s)/F_0]$ in cells incubated for 4 h at low-light intensity (7 W/m^2) at 25, 42 or 42°C with addition of CHI. **Right panel:** loss of variable fluorescence as a function of heat-shock duration at 5 W/m^2 light intensity, with or without addition of CHI. The levels of F_0 , F_s and F_m obtained in the presence of the PS II inhibitor Diuron ($5 \times 10^{-6} \text{ M}$) are indicated in the upper part of the left panel. For additional details see Materials and methods.

Table I. Localization of the damage to photosynthetic activity during heat-shock in the light at photosystem II reaction center

Treatment	Activities			
	$\text{H}_2\text{O} \rightarrow \text{MV}$ (whole chain electron flow)	$\text{H}_2\text{O} \rightarrow \text{DCIP}$ (PS II)	DPC $\rightarrow$ DCIP (PS II)	DCIP/ ASC $\rightarrow$ MV (PS I)
Control	60	80	80	110
Heat-shock	27	42	46	110
Heat-shock + CHI	5	13	13	190

Thylakoids were isolated from cells incubated at 42°C for 2 h at 7 W/m^2 light intensity and thereafter 2 h at 80 W/m^2 with or without addition of CHI. The activities are given in $\mu\text{mol/mg}$ chlorophyll/hour. Control cells were incubated at 25°C. Thylakoids from cells incubated at 42°C in the dark gave similar results to the control cells.

at low-light intensity showed a significant reduction during the first hour, followed by a transient recovery during the next 2–3 h (Figure 2b) which coincides with the time scale of transcription and translation of HSP-22 during the heat-shock process (Kloppstech *et al.*, 1985). This recovery process was inhibited by cycloheximide (CHI).

Thylakoids isolated from cells heat-shocked at a light intensity harmless at 25°C lost PS II activity measured as a reduction of 2,6-dichlorophenol-indophenol (DCIP), using either H_2O or 1,5-diphenylcarbazide (DPC) as electron donors, while PS I activity was actually enhanced, as observed before in pea chloroplasts (Thomas *et al.*, 1986) (Table I). The light-induced damage under heat-shock conditions was further enhanced by the addition of CHI (Table I). These results demonstrate that the inhibition of electron flow by heat-shock of cells exposed to low-light is localized at the reaction center of PS II.

To further examine the enhancement of PS II inactivation during heat-shock by inhibition of HSP synthesis, cells were exposed to 40°C for 2 h in the dark, in the absence or

Fig. 3. Effect of dark pre-incubation of cells at 40°C on the loss of photosynthetic activity during heat-shock in the light. Cells were pre-incubated in the dark at 25°C (●) or at 40°C without (○) or with (△) addition of CHI and then transferred to 40°C at a light intensity of 250 W/m².

presence of CHI. The cells were then illuminated for 1 h by 250 W/m² white light and the degree of photoinhibition was monitored as loss of variable fluorescence. Pre-incubation at 40°C in the dark protected the cells from photoinhibition during heat-shock at 250 W/m², as compared with cells in which HSPs were not synthesized due to pre-incubation at 40°C in the presence of CHI, or at 25°C (Figure 3).

Change in the electrophoretic mobility of thylakoid proteins during heat-shock in light

The light-induced aggregation of the thylakoid proteins during heat-shock could be detected by autoradiography of ³⁵S-labeled, SDS-PAGE resolved polypeptides (Figure 4). The aggregation of the thylakoid polypeptides was enhanced by increasing the light intensity during heat-shock. After 90 min of heat-shock at 250 W/m², all the thylakoid polypeptides became aggregated into a large mol. wt mass which could no longer penetrate the 5% acrylamide stacking gel (Figure 4). The process of thylakoid polypeptides aggregation was clearly due to the synergistic effect of light and heat-shock, since exposure of *Chlamydomonas* cells to 10-fold higher light intensities at 25°C resulted in the specific loss of the radiolabeled polypeptides at 32–33 kd, including the D1-protein (Figure 4), without aggregation of other thylakoid proteins, as also reported before by Kyle *et al.* (1984b).

It has been previously demonstrated that light induces a rapid turnover of the D1-protein in plants (Mattoo *et al.*, 1984), as well as in green algae (Kyle *et al.*, 1984b). Exposure of *Chlamydomonas* cells to extreme light intensity (~1500–2500 W/m²) results in loss of PS II activity and degradation of the D1-protein (Kyle *et al.*, 1984b). Since specific loss of PS II activity is induced by low light (≤100 W/m²) during heat-shock, it was interesting to assess whether one could demonstrate a correlation between the light-induced loss of PS II reaction center activity and specific loss of reaction center proteins during heat-shock in the light. For this purpose, ³⁵S pulse-labeled cells were first exposed to heat-shock in the dark for 2 h to allow synthesis and accumulation of HSPs. The cells were then exposed to light at 100 W/m², while the heat-shock treatment continued for an additional hour. No changes in the thylakoid polypeptide

Fig. 4. Light-dependent aggregation of thylakoid proteins during heat-shock. *Chlamydomonas* cells, pre-labeled with ³⁵SO₄²⁻ (1 h labeling followed by 2 h chase), were incubated for 90 min at different light intensities at 42°C or 25°C as indicated at the bottom of the figure. Only the autoradiogram of SDS-PAGE resolved membrane polypeptides is shown. Note the appearance of radiolabeled aggregates at the top of the stacking gel.

pattern could be detected in this case by examination of the stained gel pattern (Figure 5). However, examination of the autoradiogram showed a specific reduction in the radioactivity of the D1-protein in samples of cells transferred to the light during the heat-shock. No such effect was detected in control samples from cells kept in the dark or exposed to the same light intensity at 25°C (Figure 5).

Since inhibition by CHI of cytoplasmic translation (and thus prevention of HSP synthesis) enhanced the light-dependent inactivation of PS II during heat-shock (Figures 2 and 3, Table I), the question arose whether a correlation exists between this phenomenon and the heat-shock effect on the D1-protein. To detect differences between the CHI and control heat-shock treatments, the light intensity used in this experiment was further reduced, as compared with that described in Figure 5.

³⁵S-Pre-labeled cells were first heat-shocked for 2 h at 7 W/m² light intensity, allowing HSPs to accumulate or preventing their synthesis by addition of CHI. The light intensity was then raised only to 50 W/m² (as compared with 100 W/m² in Figure 5) and the heat-shock was continued for an additional hour. Treatment with CHI (Figure 6) caused a specific loss of the D1-protein, with an apparent mol. wt of 32 kd and formation of protein aggregates which did not penetrate the stacking gel. The D1-protein could be detected by immunoblotting in these protein aggregates which were less pronounced in the samples which were not treated with

Fig. 5. Specific changes in the amount of D1-protein during heat-shock in the light. Cells pre-labeled with $^{35}\text{SO}_4^{-2}$ at 25°C were incubated for 2 h in the dark (D) at 40 or 25°C and then transferred to the light (100 W/m²) for 1 h (D→L). Thylakoids were isolated and solubilized in a solution containing 2% SDS and mercaptoethanol, heated to 90°C for 2 min and fractionated by SDS-PAGE. To, end of labeling with $^{35}\text{SO}_4^{-2}$; *, a radiolabeled polypeptide which appeared in the D → L samples in the 22 kd region; G, stained gel; A, autoradiogram.

CHI during the heat-shock (Figure 6). Effects similar to these caused by CHI were also obtained when anisomycin was used as an 80S ribosomes translation inhibitor (data not shown).

Discussion

We have shown before that the nuclear-coded HSPs 22 and 29 of *Chlamydomonas* as well as the HSPs 22 and 25 of pea are transported into the chloroplast and bound to chloroplast membranes (Kloppstech *et al.*, 1985). In this paper we extend our finding by showing that the two HSPs are preferentially located in the grana region of the thylakoid membranes. The distribution pattern of the *Chlamydomonas* HSPs is very similar to that of the D1-protein of the PS II reaction center known to be mostly localized in the grana lamellae (Kyle *et al.*, 1984a).

Recently, it has been shown that a brief exposure of isolated spinach thylakoids to an elevated temperature induces re-distribution of PS II between the grana and stroma lamellae. This process is light-independent and occurs without loss of photosynthetic electron flow, measured at saturating light intensity (Sundby and Anderson, 1985). The re-distribution of PS II within the photosynthetic membranes might also occur *in vivo* and, as suggested by these authors, could represent a short-term regulatory mechanism protecting PS II against heat-shock damage. As opposed to that, the mechanism involving participation of HSPs in preventing the inactivation of reaction center II during heat-shock, as shown here, operates *in vivo* on a long-term schedule and might be related to the pre-adaptation phenomenon.

A general protective effect of HSPs has been demonstrated

Fig. 6. Changes in the electrophoretic mobility of the D1-protein following heat-shock in the presence of CHI. Cells pre-labeled with $^{35}\text{SO}_4^{-2}$ were incubated for 2 h at 7 W/m² light intensity, followed by 1 h at 50 W/m² at: (1) 25°C; (2) 42°C; (3) 42°C with addition of CHI; and (4) 25°C with addition of CHI. Thylakoids were isolated and fractionated by SDS-PAGE; G, stained gel; A, autoradiogram; I.B., immunoblot using anti-D1-protein antibodies. Arrow, appearance of protein aggregates of high apparent mol. wt including the D1-protein.

already by several groups in showing that heat-adapted organisms including plants can be more resistant to a second heat treatment than the non-adapted controls, but the mechanism of protection was not understood (Lindquist, 1986; Key *et al.*, 1985; Suss and Yordanov, 1986).

The data presented in this paper offer, for the first time, an approach to the study of the molecular basis of the protective function of HSPs by showing that protein synthesis of 80S ribosomes is essential to obtain protection against the light-induced damage of electron flow under heat-shock conditions. The observation that during an ongoing heat-shock a partial recovery of PS II function can be obtained supports this view. Even more convincing is the finding that a heat-pretreatment in the dark leads to a protection of PS II against light damage during a second heat-shock in the light. The protective effect can again be prevented by inhibition of protein synthesis on 80S ribosomes during the first heat-shock.

Another clue for the understanding of the damaging effect of the heat-shock is offered by our observation that thylakoid membrane proteins start to be aggregated even under low-light intensities which under heat-shock conditions are sufficient to induce photoinhibition. However, under heat-shock conditions the D1-protein is not removed from the membranes by the degradation process which operates at normal temperature in the light (Mattoo *et al.*, 1984; Kyle *et al.*, 1984b,c). As it has been shown using Western blotting the D1-protein appears cross-linked to other proteins and moves with a much higher apparent mol. wt whereas in the dark controls we do not observe such cross-linking. The mechanism of the light-dependent damage by free radicals to the phytochemical reaction center of PS II (Kyle *et al.*, 1984b, 1985) can be the same in both normal temperatures and heat-shock conditions. The ensuing degradation of the D1-protein

seems to be affected under heat-shock conditions. We assume that heat treatment in the light leads to an over-production of radicals (possibly oxygen and/or semiquinone radicals) which may induce the cross-linking of proteins. Cross-linking of proteins and phospholipids by free radicals has been shown to occur in many biological systems (Wolff *et al.*, 1986; Davies, 1987). Therefore the differences between heat-shock and control conditions could be explained assuming that under photoinhibitory conditions at ambient temperature the degradation of the damaged D1-protein is faster than cross-linking whereas under high temperatures the degradation system is slowed or inhibited thus allowing cross-linking to dominate.

Cross-linking has also been observed when *Chlamydomonas* cells have been exposed to photoinhibitory conditions at 0°C (Schuster *et al.*, in preparation). Preliminary experiments in which *Chlamydomonas* cells have been allowed to recover photosynthetic activity after heat-shock showed that this process is much slower (24 h) as compared with recovery from photoinhibition at 25°C, which is completed in 3–4 h (Ohad *et al.*, 1984). This could be explained if one considers that recovery of photosynthetic activity may require not only *de novo* synthesis of PS II proteins, but also degradation of the large protein aggregates, a process that might be more complex and time-consuming. It should be noted that the synthesis of D1-protein is not inhibited during heat-shock (Kloppstech *et al.*, 1985; Suss and Yordanov, 1986).

The evidence obtained in this paper suggests that either one or both of the chloroplast HSP-22 and HSP-29 confer protection against damage by photoinhibition at low-light intensities during heat-shock. Whether this is achieved by prevention of radical formation at PS II level or by the protection of the turnover mechanism of D1-protein is now under investigation.

Materials and methods

Cultivation of cells and heat-shock treatment

Chlamydomonas reinhardtii Y-1 cells were grown at 25°C in a mineral medium containing acetate as a carbon source (Ohad *et al.*, 1967). For heat-shock treatment, cells at the end of log phase of growth were harvested by centrifugation and resuspended in fresh growth medium to a density of 10^7 cells/ml (non-dividing conditions). The cells were incubated in a perspex clear water bath, kept at 25, 40 or 42°C $\pm$ 0.1. Illumination was provided by fluorescent lamps (low-light intensities) or by a tungsten halogen lamp (high-light intensities).

Radiolabeling of cells and isolation of thylakoids

Cells were radiolabeled with $^{35}\text{SO}_4^{-2}$ (≥ 400 $\mu\text{Ci}/\mu\text{mol}$) in a sulfate-free growth medium as described before (Kloppstech *et al.*, 1985). The labeling details are given in the legends to the figures. CHI (2 $\mu\text{g}/\text{ml}$) was used as a specific 80S ribosomal protein synthesis inhibitor. Thylakoids were isolated from cell homogenates obtained by passing the cells through a French press operating at 2000 psi in a buffer containing 50 mM tricine, pH 8.0, 10 mM NaCl and 5 mM MgCl_2 . The unbroken cells were pelleted by centrifugation at 300 g and thylakoids were sedimented at 10 000 g for 10 min. Fractionation of thylakoids into grana and stroma lamellae (stacked and unstacked regions) by digitonin treatment and differential centrifugation were performed as described by Kyle *et al.* (1984a).

Photochemical assays, fluorescence measurement and protein analysis

Photochemical reduction of 10 μM DCIP using either water or 10 μM DPC as electron donors was measured spectrophotometrically (Schuster *et al.*, 1985). PS I activity was measured polarographically as oxygen uptake in a reaction using DCIP (0.1 mM) reduced with ascorbate (ASC, 2.5 mM) as the electron donor and 0.1 mM methylviologen (MV) as the electron acceptor (Schuster *et al.*, 1985).

Measurements of variable fluorescence were performed using whole cells suspended in growth medium at a final concentration equivalent to 3–5 μg chlorophyll/ml.

Excitation light was provided by a DC projector, equipped with a Corning 4-96 blue filter and a 2×10^{-3} s electronic shutter (Uniblitz). The emitted fluorescence was detected by a photodiode protected by a red cut-off filter (Schott, 675 nm), and the signal was amplified and recorded via an A-to-D converter by an IBM-PC-60 computer. The stored information could be displayed on an NEC monochrome display monitor model JB-141 DP 2B or printed using a rapid dot printer (Facit). The computer was programmed to operate the shutter and sample the voltage changes at various rates so as to obtain information on the initial fast fluorescence transients (0–10 ms from shutter opening) as well as the following slow transients (10 ms–3 s) using a total of 640 time points. The program included automatic measurements of the intrinsic fluorescence (F_0), steady-state fluorescence (F_s) and maximal fluorescence (F_m) levels and calculation of the $(F_m - F_0)/F_0$ parameter (variable fluorescence) which indicates the relative amount of active reaction centers, and $(F_m - F_s)/F_0$ which is a measure of the energy utilized for photosynthetic electron flow and correlates with oxygen evolution or CO_2 fixation (Krause and Weiss, 1984).

Separation of thylakoid polypeptides by SDS-PAGE, electrotransfer of the proteins to nitrocellulose paper and decoration with specific antibodies to the D1-protein and [^{125}I]protein A (Amersham, 40 mCi/mg) were performed as described by Schuster *et al.* (1985).

Radiolabeled proteins were detected by fluorography (Kloppstech *et al.*, 1985). Chlorophyll concentrations were determined in 80% acetone solutions (Arnon, 1949).

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