Acclimation of the Photosynthetic Machinery to High Temperature in *Chlamydomonas reinhardtii* Requires Synthesis de Novo of Proteins Encoded by the Nuclear and Chloroplast Genomes[1]

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The mechanism responsible for the enhancement of the thermal stability of the oxygen-evolving machinery of photosystem II during acclimation of *Chlamydomonas reinhardtii* to high temperatures such as 35°C remains unknown. When cells that had been grown at 20°C were transferred to 35°C, the thermal stability of the oxygen-evolving machinery increased and within 8 h it was equivalent to that in cells grown initially at 35°C. Such enhancement of thermal stability was prevented by cycloheximide and by lincomycin, suggesting that the synthesis de novo of proteins encoded by both the nuclear and the chloroplast genome was required for this process. No increase in thermal stability was observed when cells that had been grown at 35°C were exposed to heat shock at 41°C, optimum conditions for the induction of the synthesis of homologs of three heat shock proteins (Hsps), namely, Hsp60, Hsp70, and Hsp22. Moreover, no synthesis of these homologs of Hsps was induced at 35°C. Thus it appears likely that Hsps are not involved in the enhancement of the thermal stability of the oxygen-evolving machinery.

When photosynthetic organisms are exposed to heat, their photosynthetic machinery is irreversibly inactivated. However, when such organisms have become acclimated to high temperatures within the physiological range, their photosynthetic machinery exhibits enhanced thermal stability (Berry and Björkman, 1980). This phenomenon has been observed in several species of higher plants (Armond et al., 1978; Pearcy, 1978; Raison et al., 1982) and cyanobacteria (Fork et al., 1987; Nishiyama et al., 1993).

The oxygen-evolving machinery of the photosystem (PS) II complex is often the most susceptible to heat among the various components of the photosynthetic system (Katoh and San Pietro, 1967; Yamashita and Butler, 1968; Santarius, 1975; Mamedov et al., 1993). The release by heat of two of four Mn atoms from the catalytic site of the oxygen-evolving machinery results in complete inactivation of the oxygen-evolving machinery without any significant loss of proteins (Nash et al., 1985). Thus the thermal stability of the oxygen-evolving machinery appears to influence the thermal stability of the entire photosynthetic system.

Efforts have been made to define the factors that stabilize the PSII complex against heat-induced inactivation. It has been suggested that heat shock proteins (Hsps; Stapel et al., 1993; Eriksson and Clarke, 1996; Heckathorn et al., 1998), carotenoids that participate in the xanthophyll cycle (Havaux et al., 1996), and isoprene (Sharkey and Singsaas, 1995) might be involved in protection of the PSII complex against heat stress. However, it remains unclear whether these factors are involved in the enhancement of the thermal stability of the oxygen-evolving machinery at high temperatures that fall within the physiological range. The contribution of saturated membrane lipids to the thermal stability of the PSII complex has been excluded by studies in transgenic cyanobacteria and higher plants (Gombos et al., 1994; Wada et al., 1994; Moon et al., 1995).

In the cyanobacterium *Synechococcus* sp. PCC 7002, the oxygen-evolving machinery is stabilized against heat-induced inactivation by cytochrome (Cyt) c_{550} and PsbU, which are the extrinsic proteins of the PSII complex (Nishiyama et al., 1994, 1997). A mutant of *Synechococcus* with a defective *psbU* gene is not only unable to increase the thermal stability of its oxygen-evolving machinery, but it is also unable to develop the cellular thermotolerance to survive at higher temperatures upon acclimation to high temperatures (Nishiyama et al., 1999). These findings suggest that enhancement of the thermal stability of the oxygen-evolving machinery might be an important response during acclimation to high temperature in cells that are able to tolerate such temperatures.

In the present study we examined the enhancement of the thermal stability of the oxygen-evolving machinery of *Chlamydomonas reinhardtii* during acclima-

tion to high temperature, in particular, as it relates to the synthesis of proteins. We found that synthesis de novo of proteins encoded by both the nuclear and the chloroplast genome was required for enhancement of the thermal stability of the oxygen-evolving machinery and, moreover, that Hsps, which were induced by heat shock at 41°C, appeared not to play a role in this process.

RESULTS AND DISCUSSION

Enhanced Thermal Stability of the Oxygen-Evolving Machinery of *C. reinhardtii* at High Temperatures

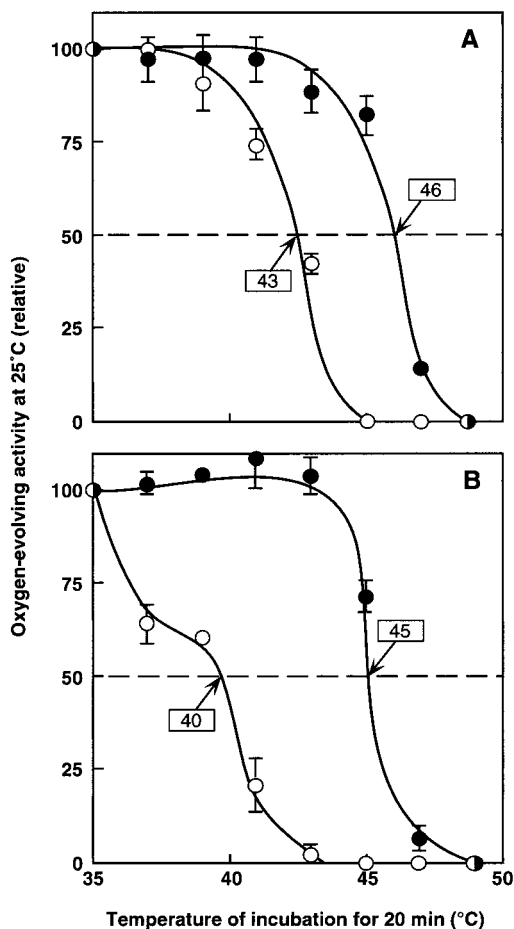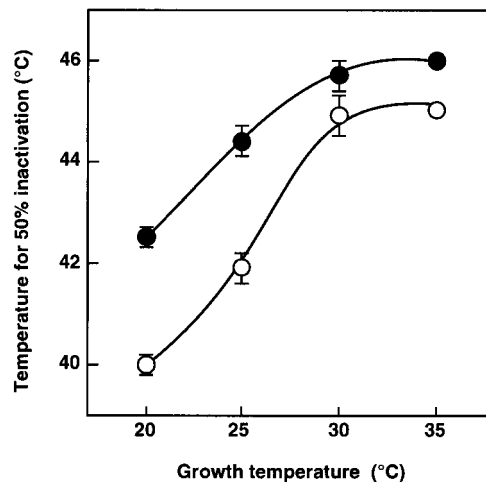Figure 1A shows the profiles of inactivation by heat

Figure 2. Relationships between growth temperature and the thermal stability of the oxygen-evolving machinery in cells grown photomixotrophically and photoautotrophically at various temperatures. Thermal stability of the oxygen-evolving machinery was examined as described in the legend to Figure 1. ●, Photomixotrophic growth; ○, photoautotrophic growth. Values are means ± SD (bars) of results from four independent experiments.

Figure 1. Effects of growth temperature on the thermal stability of the oxygen-evolving machinery in cells of *C. reinhardtii.* Cells that had been grown photomixotrophically (A) or photoautotrophically (B) at 20°C (○) or 35°C (●) were incubated at the indicated temperatures for 20 min in darkness. Then the evolution of oxygen was measured at 25°C in the presence of 2 mM BQ. The oxygen-evolving activities taken as 100% in cells grown photomixotrophically at 20°C and 35°C, were 233 and 256 μmol O_2 mg^{-1} chlorophyll (Chl) mL^{-1} h^{-1} respectively, and those in cells grown photoautotrophically at 20°C and 35°C were 196 and 223 μmol O_2 mg^{-1} Chl mL^{-1} h^{-1}, respectively. The numbers in boxes are temperatures (°C) for 50% inactivation. Values are means ± SD (bars) of results from four independent experiments.

of the oxygen-evolving machinery in cells that had been grown photomixotrophically at 20°C and 35°C. The growth temperature had a clear effect on the thermal stability of the oxygen-evolving machinery. The temperature for 50% inactivation (T_{50}) of the oxygen-evolving machinery in cells grown at 20°C was 43°C, whereas the T_{50} in cells grown at 35°C was 46°C. Thus the thermal stability of the oxygen-evolving machinery increased upon acclimation of cells to a high temperature.

We also examined the profiles of inactivation by heat of the oxygen-evolving machinery in cells that had been grown photoautotrophically at 20°C and 35°C (Fig. 1B). The T_{50} of the oxygen-evolving machinery shifted from 40°C to 45°C when the growth temperature was increased from 20°C to 35°C. Thus the thermal stability of the oxygen-evolving machinery increased when cells were grown either photomixotrophically (Fig. 1A) or photoautotrophically (Fig. 1B).

Figure 2 shows the relationship between the growth temperature and T_{50} for cells that had been grown photomixotrophically. The thermal stability of the oxygen-evolving machinery increased, exhibiting an almost linear relationship to growth temperatures, as the temperature was raised from 20°C to 30°C. A similar result was observed when cells were grown photoautotrophically.

Such relationships between the thermal stability of the oxygen-evolving machinery and the temperature of growth have also been observed in several species of higher plants (Armond et al., 1978; Pearcy, 1978; Raison et al., 1982) and cyanobacteria (Lehel et al., 1993; Nishiyama et al., 1993). Thus it is likely that the capacity for acclimation of the photosynthetic machin-

ery to high temperature is a universal property of eukaryotic and prokaryotic photosynthetic organisms.

Acclimation to High Temperature Requires the Synthesis of Proteins de Novo

We examined changes in the thermal stability of the oxygen-evolving machinery after cells that had been grown initially at 20°C were transferred to 35°C and then we examined the effects of specific inhibitors of protein synthesis on these changes (Fig. 3). Thermal stability was assayed in terms of the oxygen-evolving activity that remained after incubation of cells at 45°C for 20 min (black circles). This treatment completely inactivated the oxygen-evolving machinery of cells that had been grown at 20°C (Fig. 3A). After transfer of cells to 35°C, the thermal stability of the oxygen-evolving machinery increased and within 8 h it was equivalent to the thermal stability of cells that had been grown initially at 35°C (Fig. 3A). When cells were not incubated at 45°C for 20 min, the oxygen-evolving activity remained fairly constant. However, a transient increase in the thermal stability of the oxygen-evolving machinery was observed immediately after transfer of cells to 35°C (Fig. 3A, white circles).

The increase in thermal stability was completely prevented by cycloheximide at 8 μg mL^{-1} (Fig. 3B). This drug inhibits the synthesis of proteins that are encoded by the nuclear genome. The increase was also prevented almost completely by lincomycin at 150 μg mL^{-1} (Fig. 3C). Lincomycin inhibits the synthesis of proteins that are encoded by the chloroplast genome. Similar inhibition was observed in the presence of chloramphenicol at 100 μg mL^{-1} (data not shown). This drug also inhibits the synthesis of proteins that are encoded by the chloroplast genome. None of these inhibitors significantly affected the oxygen-evolving activity, when cells were not incubated at 45°C for 20 min (Fig. 3, B and C, white circles). Therefore it seems unlikely that these inhibitors directly inactivated the oxygen-evolving machinery, at least within 12 h under our conditions. Together, the results suggest that proteins synthesized de novo from the nuclear and the chloroplast genome are necessary for enhancement of the thermal stability of the oxygen-evolving machinery.

Enhancement of Thermal Stability in Darkness

Light is often involved in the regulation of the photosynthetic machinery in response to environmental changes. To clarify whether light might be involved in the acclimation of the photosynthetic machinery to high temperature of 35°C, we compared the changes in the thermal stability of the oxygen-evolving machinery in the light and in darkness after cells that had been grown at 20°C were transferred to 35°C. There were no significant differences between results obtained in the light and in darkness (Fig. 4). Thus the acclimation of the photosynthetic machinery to high temperature occurred independently of light. It seems likely that the thermal stability of the oxygen-evolving machinery during acclimation to high temperatures might be regulated solely by temperature.

Thermal Stability during De-Acclimation

We examined changes in the thermal stability of the oxygen-evolving machinery during de-acclimation by transferring cells that had been grown at 35°C to 20°C (Fig. 5). The thermal stability, measured after incubation at 45°C for 20 min, decreased only slightly over the course of 12 h. However, the oxygen-evolving activity decreased at the same rate upon similar in-
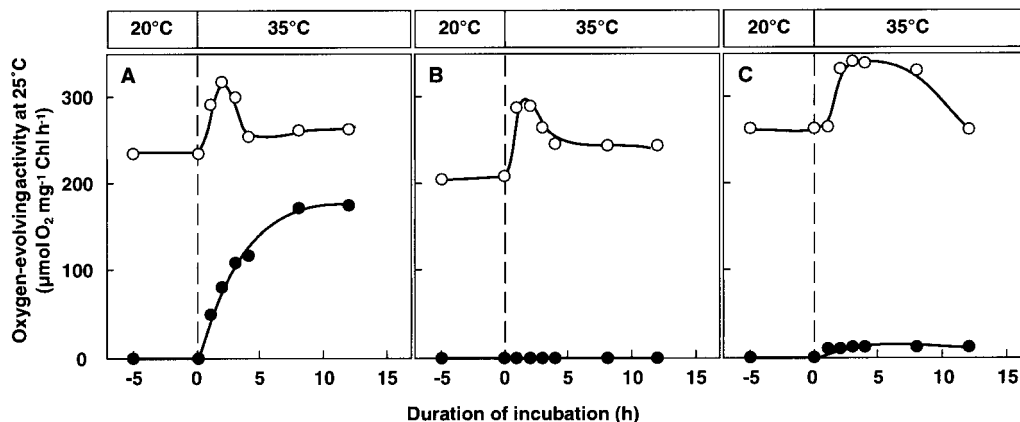

Figure 3. Effects of various inhibitors of protein synthesis on the enhancement of the thermal stability of the oxygen-evolving machinery. Cells that had been grown photomixotrophically at 20°C were transferred to 35°C and incubated in the absence of drugs (A), in the presence of 8 μg mL^{-1} cycloheximide (B), and in the presence of 150 μg mL^{-1} lincomycin (C). After the indicated periods of time, cells were incubated at 35°C (O) or 45°C (●) for 20 min in darkness and the oxygen-evolving activity was measured as described in the legend to Figure 1. Experiments were performed three times with independent cultures and essentially the same results were obtained in each case.

Figure 4. Enhancement of the thermal stability of the oxygen-evolving machinery in darkness. Cells that had been grown photomixotrophically at 20°C were transferred to 35°C and incubated in the light (white symbols) or in darkness (black symbols) for the indicated periods of time. Cells were then incubated at 35°C (squares) or 45°C (circles) for 20 min in darkness and the oxygen-evolving activity was measured under the same conditions as described in the legend to Figure 1. Experiments were performed three times with independent cultures and essentially the same results were obtained in each case.
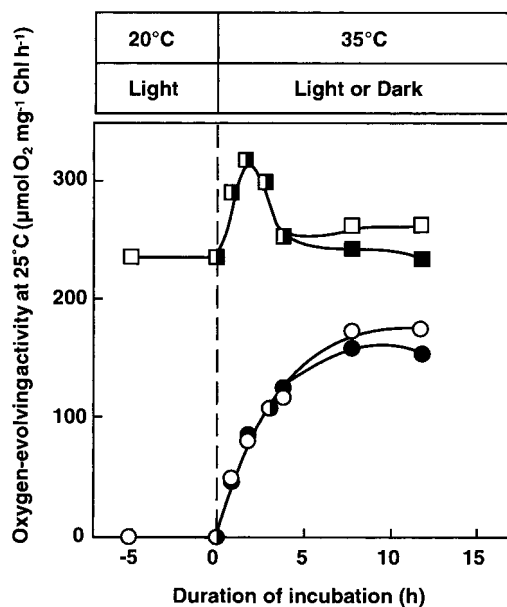
cubation of the de-acclimated cells at 35°C for 20 min. Thus the thermal stability of the oxygen-evolving machinery did not change within 12 h after the decrease in the growth temperature (Fig. 5). This observation suggests that the newly synthesized protein(s) that stabilizes the oxygen-evolving machinery remains stable at low temperatures without undergoing any changes, such as dissociation or proteolysis. The thermal stability of the oxygen-evolving machinery during de-acclimation was unaffected even by the presence of inhibitors of protein synthesis (data not shown). Thus it seems unlikely that continuous synthesis of proteins is required for maintenance of the thermal stability of the oxygen-evolving machinery at low temperatures.

Absence of Contribution by Hsps to Thermal Stability

Considerable attention has been paid to the roles of Hsps in terms of protection of the PSII complex against heat stress (Waters, 1995; Eriksson and Clarke, 1996; Heckathorn et al., 1998). The synthesis of Hsps is not, in general, induced at the high temperatures such as 35°C used in the present study (Gromoff et al., 1989; Vierling, 1991). To examine whether Hsps might be involved in the enhancement of the thermal stability of the oxygen-evolving machinery, we investigated the temperature-dependent

induction of the synthesis of chloroplast-localized Hsps by western analysis (Fig. 6).

Three proteins were detected immunologically as homologs of Hsp60 and the largest one was absent from the chloroplast fraction (Fig. 6A). Therefore, we postulated that the other two proteins might be homologs of Hsp60 in the chloroplast. Levels of these homologs of Hsp60 in the chloroplast did not change when cells were incubated at temperatures below 37°C for 2 h, but they increased significantly during incubation at 39°C to 41°C for 2 h. We obtained essentially the same result for the homolog of Hsp70 (data not shown). Hsp22, a small Hsp that is located exclusively in the thylakoid membranes of *C. reinhardtii* (Schuster et al., 1988), did not accumulate at all within 2 h at temperatures below 37°C. It did, however, accumulate during incubation at 39°C to 41°C for 2 h.

We also examined levels of these homologs of Hsps after longer incubation (Fig. 7). The levels of the homologs of Hsp60 in the chloroplast did not change during incubation at 35°C for 12 h even though the thermal stability of the oxygen-evolving machinery increased considerably (Fig. 3). Hsp22 did not accumulate at all under the same conditions. Since the enhancement of the thermal stability of the oxygen-evolving machinery occurred at moderate temperatures below 35°C, it seems unlikely that Hsps are involved in this phenomenon.

Our conclusion is supported by the results of an analysis of thermal stability after heat shock. Table I
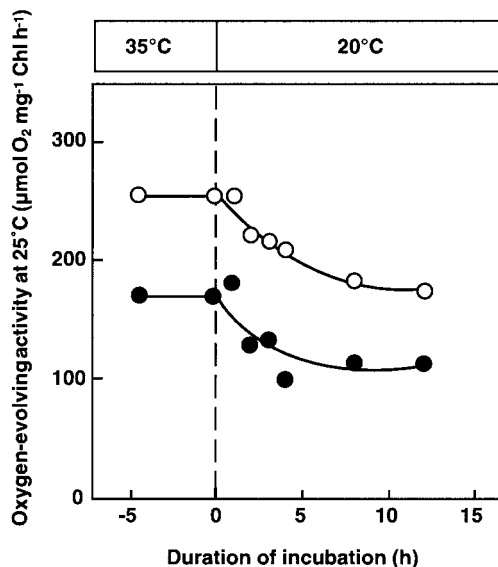

Figure 5. Changes in the thermal stability of the oxygen-evolving machinery after transfer of cells from a high temperature to a low temperature. Cells that had been grown photomixotrophically at 35°C were transferred to 20°C and incubated in the light for the indicated periods of time. Cells were incubated at 35°C (○) or 45°C (●) for 20 min in darkness and then the oxygen-evolving activity was measured as described in the legend to Figure 1. Experiments were performed three times with independent cultures and essentially the same results were obtained in each case.
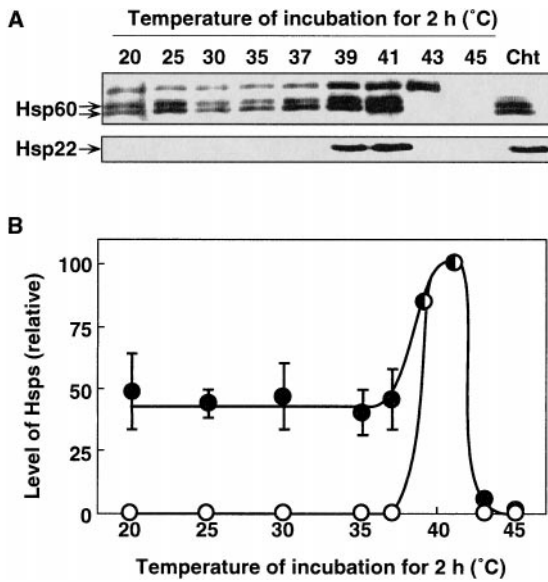
Figure 6. Levels of homologs of Hsps after incubation of cells at various temperatures. Cells that had been grown photomixotrophically at 20°C were transferred to the indicated temperatures and incubated for 2 h. Proteins were prepared from whole cells and intact chloroplasts (Cht) as described in the text. A, Levels of homologs of Hsp60 and Hsp22 were determined by western analysis with antiserum against Hsp60 from *Synechococcus vulcanus* and with antiserum against Hsp22 from *C. reinhardtii* as described in the text. Arrows indicate homologs of Hsps in the chloroplast. B, Quantitation of the results shown in A. The levels of homologs of Hsp60 shown represent the sum of the levels of the two chloroplast homologs. ●, Hsp60; ○, Hsp22. Each point represents the average of results from three independent experiments. Bars indicate SD.

Figure 7. Levels of the homologs of Hsps during acclimation to a high temperature. Cells that had been grown photomixotrophically at 20°C were transferred to 35°C for the indicated times. HS indicates the results after incubation at 41°C for 2 h. A, Analysis of the levels of homologs of Hsp60 and Hsp22 was performed as described in Figure 6A. B, Quantitation of the results shown in A. ●, Hsp60; ○, Hsp22. Each point represents the average of results from three independent experiments. Bars indicate SD.

shows the effects of heat shock on the thermal stability of the oxygen-evolving machinery. Heat shock was applied by incubating cells at 41°C for 2 h, a treatment that induced maximum levels of the homologs of Hsps. When cells that had been grown at 20°C were subjected to such heat shock, the T_{50} of the oxygen-evolving activity shifted from 42.5°C to 45.3°C. This increase might have resulted from acclimation during the increase in temperature since incubation of cells at 35°C for 2 h shifted the T_{50} to 44.3°C (data not shown). However, when cells that had been grown at 35°C were subjected to the heat shock, T_{50} did not increase any further (Table I).

The close relationship between the thermal stability of the oxygen-evolving machinery and the growth temperature also tended to rule out a contribution by Hsps, which might be expected to produce a more abrupt change in thermal stability (Fig. 2). In addition, no Hsps have yet been found in the lumen of thylakoid membranes, namely, at the site of the oxygen-evolving machinery (Waters, 1995). Thus it is unlikely that Hsps are involved in enhancement of the thermal stability of the oxygen-evolving machinery, which occurs during the acclimation of cells to high temperatures.
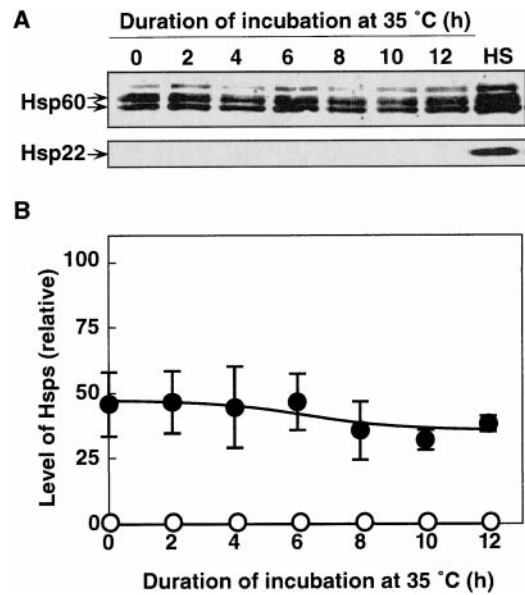
Possible Roles of Hsps

Schuster et al. (1988) reported that pre-incubation of *C. reinhardtii* cells at 40°C for 2 h prevented the photoinhibition of the PSII complex under heat. These authors suggested that Hsp22, which had accumulated in the grana of thylakoid membranes during the pre-incubation, protected either the PSII complex itself or the synthesis de novo of the D1 protein. We assume that the effect of the pre-incubation may be largely due to the acclimation of cells to high temperature, as shown in this study (Table I). However, it is also possible that a particular Hsp such as Hsp22 participates in the repair of the PSII complex

Table I. *Effects of heat shock on the thermal stability of the oxygen-evolving machinery*

Cells that had been grown at 20°C and 35°C were incubated at 41°C for 2 h in light at 70 μE m^{-2} s^{-1}. After incubation, the temperature for 50% inactivation (T_{50}) of the evolution of oxygen was determined as described in the legend to Figure 1. Values are means $\pm$ SD of results from three independent experiments.

Growth Temperature	T_{50} before and after Heat Shock (41°C, 2 h)	
	Before	After
	°C	
20	42.5 ± 0.2	45.3 ± 0.1
35	46.0 ± 0.1	46.5 ± 0.3

from the photo-induced inactivation when cells are exposed to light and heat stress together.

Eriksson and Clarke (1996) showed that inactivation of the gene for ClpB (Hsp100) in *Synechococcus* sp. decreased the ability to enhance the thermal stability of the oxygen-evolving machinery after heat shock. ClpB is constitutively expressed at moderate temperatures and might function as a molecular chaperone in *Synechococcus* sp. (Porankiewicz and Clarke, 1997). We cannot exclude the possibility that in *C. reinhardtii* such a Hsp participates in proper folding and translocation of newly synthesized proteins that are necessary for enhancing the thermal stability of the oxygen-evolving machinery.

Possible Mechanisms for the Acclimation of the Photosynthetic Machinery to High Temperature

The activities of the photosynthetic machinery are regulated by proteins encoded by the nuclear and the chloroplast genome. However, little is known about the contribution of these genomes to acclimation of the photosynthetic machinery to high temperature. Our results demonstrate that the cooperation of proteins that are synthesized de novo from both genomes is necessary for enhancement of the thermal stability of the oxygen-evolving machinery. We have three working models that might explain the cooperation of proteins encoded by the nuclear and the chloroplast genome in the acclimation to high temperature, as follows.

High temperature induces the synthesis of some nucleus-encoded protein(s) that regulates the synthesis of some chloroplast-encoded protein(s) that stabilizes the oxygen-evolving machinery. Transcriptional, post-transcriptional, and translational controls of the expression of chloroplast genes are largely dependent on nucleus-encoded proteins (Schmidt et al., 1985; Goldschmidt-Clermont, 1998). For example, mutation of the F35 gene in the nuclear genome of C. *reinhardtii* revealed that some nucleus-encoded proteins are required for translation of the *psbA* gene for the D1 protein of the PSII complex, which is encoded by the chloroplast genome (Yohn et al., 1996). In our first model, the synthesis of some nucleus-encoded protein(s) is induced after an increase in growth temperature. This protein(s) is translocated into the chloroplast and stimulates the synthesis of some chloroplast-encoded protein(s). Subsequently, the newly synthesized chloroplast-encoded protein(s) is translocated to the lumen of thylakoid membranes and stabilizes the oxygen-evolving machinery.

In the second model, high temperature independently induces the synthesis of both nucleus-encoded and chloroplast-encoded proteins that are necessary to stabilize the oxygen-evolving machinery. Nucleus-encoded proteins associate with chloroplast-encoded proteins to form some functionally active protein complexes, such as the PSII complex and Rubisco (Erickson, 1998). Some chloroplast-encoded proteins are activated by their interaction with nucleus-encoded proteins, such as Cyt *f* of the Cyt b_6/f complex (Goldschmidt-Clermont, 1998). In our second model, after an increase in growth temperature, synthesis of the relevant nucleus-encoded and chloroplast-encoded proteins is induced by separate and independent mechanisms. These proteins are then translocated to the lumen and act cooperatively to stabilize the oxygen-evolving machinery. In this case if any one of the proteins is absent, no enhancement of the thermal stability of the oxygen-evolving machinery can be achieved. Inhibition of the synthesis of either nucleus-encoded or chloroplast-encoded proteins prevents the enhancement (Fig. 3).

In our third model, high temperature induces the synthesis of some chloroplast-encoded protein(s) that regulate the synthesis of some nucleus-encoded protein(s) that stabilizes the oxygen-evolving machinery. Kropat et al. (1997) reported that a precursor to Chl, the synthesis of which is regulated by chloroplast-encoded protein(s), might be involved in the induction of a nucleus-encoded protein in *C. reinhardtii*. In our third model, the synthesis of some chloroplast-encoded protein(s) is induced after an increase in growth temperature. This protein transmits a signal to the cytosol that stimulates the synthesis of some nucleus-encoded protein(s). The newly synthesized nucleus-encoded protein(s) is in turn translocated to the chloroplast where it becomes associated with the oxygen-evolving machinery, with resultant enhancement of the thermal stability of the oxygen-evolving machinery.

It is not clear whether the protein(s) that is newly synthesized at high temperatures can stabilize the oxygen-evolving machinery directly. It is possible that the newly synthesized protein(s) activates the synthesis and/or transport of compatible solutes that stabilize the oxygen-evolving machinery against heat-induced inactivation. However, in our previous studies with cyanobacteria we demonstrated that inactivation of the *psbU* gene for PsbU, an extrinsic protein of the PSII complex, resulted in the loss of the capacity for enhancement of the thermal stability of the oxygen-evolving machinery upon acclimation to high temperatures (Nishiyama et al., 1999). This finding suggests a mechanism for stabilization of the oxygen-evolving machinery; some as-yet-unidentified protein factor(s) stabilizes the oxygen-evolving machinery by strengthening the binding of the extrinsic proteins to the core of the PSII complex. The physiological aspects of the photosynthetic acclimation to high temperature are very similar in cyanobacteria (Nishiyama et al., 1993) and *C. reinhardtii* (this study). Therefore, it seems likely that some newly synthesized protein(s) might act directly to stabilize the oxygen-evolving machinery.

MATERIALS AND METHODS

Strains and Culture Conditions

Two strains of *Chlamydomonas reinhardtii*, CC-2986 (mt$^+$, arg7-8, and nit1) and CC-3403 (mt$^-$, arg7, nit1, and cw15), were obtained from the *Chlamydomonas* Genetics Center, Duke University, (Durham, NC). Cells were grown photomixotrophically in Tris [tris(hydroxymethyl)amino-methane]-acetate-phosphate medium or photoautotrophically in high-salt medium (Harris, 1989) at designated temperatures with aeration by sterile air that contained 1% (v/v) CO_2 under illumination at 70 μmol m^{-2} s^{-1}. Both media were supplemented with 50 μg Arg mL^{-1}.

Assays of Thermal Stability

In the present study we distinguished terms of high temperature and heat. We defined high temperature as moderately high temperatures that do not affect the growth of cells, such as 35°C, and defined heat as very high temperatures that inhibit the growth of cells, such as 41°C.

All assays of thermal stability were performed using strain CC-2986. To characterize profiles of heat-induced inactivation of the oxygen-evolving machinery, we prepared 1.5-mL aliquots of a suspension of cells in 15-mL tubes at the early-stationary phase of growth at a cell density of 30 to 50 μg Chl mL^{-1}. Each aliquot was incubated at the designated temperature for 20 min in darkness and then promptly cooled to 25°C. We examined changes in the thermal stability of the oxygen-evolving machinery during acclimation of cells to high temperature as follows. Cells were grown photomixotrophically at 20°C in 70-mL glass tubes. Then the suspensions of cells at early-stationary phase were transferred to 35°C and incubated in light at 70 μmol m^{-2} s^{-1} or in darkness. Aliquots of 1.5 mL of each suspension were removed at designated times and were incubated at 35°C or 45°C for 20 min in darkness and then oxygen-evolving activity was measured at 25°C.

Changes in the thermal stability of the oxygen-evolving machinery during de-acclimation were also examined. Cells were grown photomixotrophically at 35°C until they reached early-stationary phase, when they were transferred to 20°C. After various periods of time, thermal stability was examined as described above.

Measurement of the Oxygen-Evolving Activity

The photosynthetic evolution of oxygen was measured with a Clark-type oxygen electrode. The PSII-mediated transport of electrons from water to 1.4-benzoquinone (BQ) was measured at 25°C in culture medium that had been supplemented with 2 mM BQ as the electron acceptor. Yellow actinic light at 2 mmol m^{-2} s^{-1} was provided by an incandescent lamp in conjunction with heat-absorbing (HA50; Hoya, Tokyo) and yellow (V-Y46; Toshiba, Tokyo) optical filters. Concentrations of Chl were determined as described by Arnon et al. (1974).

Immunoblotting Analysis of Hsps

Levels of homologs of Hsp60, Hsp70, and Hsp22 in cells were determined by western analysis. CC-2986 cells were harvested by centrifugation at 5,000g for 5 min and washed with 20 mM Tris HCl (pH 8.0) that contained 1 mM EDTA. Subsequent procedures were performed at 0°C to 4°C. Pelleted cells were suspended in 20 mM Tris HCl (pH 8.0) that contained 1 mM EDTA, 0.4 M Suc, and 0.5 mM phenyl-methylsulfonyl fluoride. The suspension was homogenized for 2 min with an equal volume of glass beads (diameter of 0.1 mm) in a mixer (Bead-beater, Biospec Products, Bartlesville, OK). The homogenate was centrifuged at 5,000g for 5 min to remove unbroken cells and the supernatant was collected as total cell proteins. Protein concentrations were determined as described by Bradford (1976) with bovine serum albumin as the standard. Aliquots equivalent to 2.5 μg of protein were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel for analysis of homologs of Hsp60 and Hsp70 and on a 15% (w/v) polyacrylamide gel for analysis of Hsp22. After electrophoresis, proteins were blotted onto a nitrocellulose membrane (Protran Nitrocellulose, Schleicher and Schuell, Dassel, Germany) and allowed to react with antisera that had been raised in rabbits against Hsp70 from pumpkin chloroplasts (Tsugeki and Nishimura, 1993), Hsp60 of *Synechococcus vulcanus* (StressGen Biotechnologies, Victoria, Canada), and Hsp22 of *C. reinhardtii* (Grimm et al., 1989). Immunoreactive proteins were detected with peroxidase-linked secondary antibodies and enhanced chemiluminescence western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). Levels of immunoreactive proteins were determined with a luminescent image analyzer (Fujifilm, Tokyo).

Isolation of Intact Chloroplasts

Intact chloroplasts were prepared from strain CC-3403, a mutant that is deficient in cell wall synthesis, as described by Mendiola-Morgenthaler et al. (1985) with minor modification. After heat shock at 41°C for 2 h, cells were harvested by centrifugation at 3,000g for 5 min. Subsequent procedures were performed at 0°C to 4°C. Pelleted cells were suspended in isolation buffer, which contained 35 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH (pH 7.8), 250 mM sorbitol, 1 mM MnCl$_2$, 5 mM MgCl$_2$, and 2 mM EDTA-KOH (pH 7.8). The suspension was passed through a French pressure cell (SLM Instruments, Urbana, IL) at 7.4 MPa and the resultant homogenate was centrifuged at 3,000g for 5 min at 4°C. The pellet was suspended in isolation buffer and then placed on top of layers of 40% (v/v) and 60% (v/v) Percoll in the same buffer. After centrifugation at 8,000g for 20 min, intact chloroplasts were recovered as a band between the layers of 40% and 60% (v/v) Percoll and they were washed twice with isolation buffer.

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