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Photosystem II Reaction Center Damage and Repair in *Dunaliella salina* (Green Alga)[1]

Analysis under Physiological and Irradiance-Stress Conditions

Jeong Hee Kim, Jeff A. Nemson, and Anastasios Melis*

Department of Plant Biology, University of California, Berkeley, California 94720

Mechanistic aspects of the photosystem II (PSII) damage and repair cycle in chloroplasts were investigated. The D1/32-kD reaction center protein of PSII (known as the *psbA* chloroplast gene product) undergoes a frequent light-dependent damage and turnover in the thylakoid membrane. In the model organism *Dunaliella salina* (green alga), growth under a limiting intensity of illumination (100 μmol of photons m^{-2} s^{-1}; low light) entails damage, degradation, and replacement of D1 every about 7 h. Growth under irradiance-stress conditions (2000 μmol of photons m^{-2} s^{-1}; high light) entails damage to and replacement of D1 about every 20 min. Thus, the rate of damage and repair of PSII appears to be proportional to the light intensity during plant growth. Low-light-grown cells do not possess the capacity for high rates of repair. Upon transfer of low-light-grown cells to high-light conditions, accelerated damage to reaction center proteins is followed by PSII disassembly and aggregation of neighboring reaction center complexes into an insoluble dimer form. The accumulation of inactive PSII centers that still contain the D1 protein suggests that the rate of D1 degradation is the rate-limiting step in the PSII repair cycle. Under irradiance-stress conditions, chloroplasts gradually acquire a greater capacity for repair. The induction of this phenomenon occurs with a half-time of about 24 h.

Vascular plants and green algae respond to changes in the light environment in which they grow. Different light regimens influence chloroplast development as well as the composition, structure, and function of the photochemical apparatus (Anderson, 1986; Melis, 1991). Irradiance stress during plant growth is a condition manifested whenever the incident light intensity is greater than that needed to saturate photosynthesis. Such stress causes photoinhibition of photosynthesis (Kok, 1956), which is manifested as lower electron transport activity and oxygen evolution in chloroplasts (Powles, 1984) and, consequently, as lower plant growth and productivity. It is generally agreed that the target of this light-dependent damage is a functional component in the D1/32-kD reaction center protein of PSII (Kyle et al., 1984; Ohad et al., 1984; Demeter et al., 1987).

Earlier research revealed a highly unusual property for the D1/32-kD PSII reaction center protein. This protein accounts for less than 1% of the total thylakoid membrane protein content; yet, the rate of its synthesis is comparable to that of the abundant large subunit of the Rubisco in the chloroplast (Bottomley et al., 1974; Eaglesham and Ellis, 1974; Edelman and Reisfeld, 1978; Mattoo et al., 1984). Because steady-state levels of the D1/32-kD polypeptide in thylakoids are low, it was inferred that rates of degradation must be correspondingly high. The frequent turnover of the D1/32-kD protein implied a need for a frequent repair of PSII. The mechanistic details of this unique phenomenon are unclear; however, it is believed that damage to PSII is a consequence of the specialized function performed by this chloroplast complex. Indeed, the D1/32-kD reaction center protein helps stabilize four Mn atoms on the luminal side of the thylakoid membranes (Ghanotakis and Yocum, 1990), it contains a redox-active Tyr residue that serves in the electron-transport process (Barry and Babcock, 1987), and it helps bind the photochemical reaction center P680, a pheophytin, a non-heme Fe^{2+} cation, and a quinone molecule (Nanba and Satoh, 1987). The function of D1 is to facilitate a photochemical charge separation that generates a strong oxidant ($P680^+$) capable of driving the oxidation of H_2O molecules.

Investigators from this laboratory (Smith et al., 1990; Melis, 1992; Harrison et al., 1992) recently reported that chronic irradiance stress during plant growth lowers the Chl content of chloroplasts and limits the photosystem light-harvesting antenna size. Furthermore, chronic irradiance stress causes a chronic photoinhibition condition in chloroplasts and induces modifications in thylakoid membrane structure and photosystem composition. Noteworthy was the greatly elevated PSII/PSI stoichiometry ratio (approximately 12/1) in irradiance-stressed chloroplasts. Most of these PSII units contained a modified form of the D1/32-kD reaction center protein (Callahan et al., 1990; Kettunen et al., 1991; Melis, 1992) and could not perform a photochemical charge separation (Smith et al., 1990). The results suggested enhanced biosynthesis/assembly and enhanced concentration of PSII relative to other thylakoid membrane complexes under chronic photo-

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* Corresponding author; fax 1-510-642-4995.

Abbreviations: D1, the 32-kD reaction center protein of PSII encoded by the chloroplast *psbA* gene; D2, the 34-kD reaction center protein of PSII encoded by the chloroplast *psbD* gene; HL, high light; LL, low light; Q_A, primary electron-accepting plastoquinone of PSII.

inhibition. Clearly, an enhanced concentration of a labile component in the thylakoid membrane might serve to counter the adverse effect of irradiance stress.

To gain a better understanding on the mechanism of chloroplast response to chronic irradiance stress, we measured the rate of damage and degradation/replacement of the D1/32-kD reaction center protein of PSII in the model organism *Dunaliella salina* (green alga) under physiological and irradiance-stress conditions. The results provided information concerning the chloroplast strategy for repair under divergent intensities of illumination. Moreover, insight was gained regarding the rate-limiting step and the temporal sequence of events in the PSII damage and repair cycle of chloroplasts.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Dunaliella salina cultures were grown in an artificial hypersaline medium similar to that of Pick et al. (1986) containing 1.5 M NaCl, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.1 mM KH_2PO_4, 20 μM EDTA, 2 μM $FeCl_3$, 5 mM NH_4Cl, and 40 mM Tris-HCl (pH 7.5), supplemented with a mixture of micronutrients. Carbon was supplied as $NaHCO_3$ in the growth medium at an initial concentration of 25 mM. Cultures were grown in flat bottles (optical pathlength = 3 cm) at 30°C under illumination at 100 μmol of photons m^{-2} s^{-1} (LL) or at 2000 μmol of photons m^{-2} s^{-1} (HL). Care was exercised, by means of shaking and by the use of reflectors, to ensure illumination that was as uniform to the culture as possible. Cells were grown to the late natural logarithmic phase (ln A_{678} = approximately −2.0 for LL-grown cultures, ln A_{678} = approximately −3.8 for HL-grown cultures, respectively [Fig. 1]), and treatments (transfer to different irradiance conditions and/or addition of antibiotics) were performed. The ln A_{678} was measured at room temperature using the technique of Shibata (1958) as described elsewhere (Naus and Melis, 1991).

Cells were incubated in the absence or presence of the chloroplast protein synthesis inhibitor chloramphenicol (500 μg mL^{-1}) for variable times and under a combination of different light regimens (see figure legends). Similar results were obtained whenever chloramphenicol was replaced by other chloroplast protein biosynthesis inhibitors, e.g. lincomycin.

Thylakoid Membrane Isolation

Cells were harvested by centrifugation at 3000*g* for 3 min at 4°C. Pellets were resuspended in 0.5 mL of fresh growth medium and stored frozen at −20°C until all samples were ready for processing. Samples were thawed on ice and diluted with sonication buffer containing 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.2% sodium ascorbate, 0.2% PVP, 1 mM aminocaproic acid, and 1 mM aminobenzamidine (pH 6.8). Cells were disrupted by sonication for 30 s in a Branson Sonifier (Cell Disruptor 200) operated in the pulsed mode with a 50% duty cycle and an output power setting of 5. Unbroken cells and other large cell fragments were removed by centrifugation at 3000*g* for 3 min at 4°C. The supernatant was then centrifuged at 75,000*g* for 30 min at 4°C. The thylakoid membrane pellet was resuspended in solubilization buffer containing 0.5 M Tris-HCl, 7% SDS, 20% glycerol, and 2 M urea (pH 6.8) and incubated at room temperature for 15 min. This approach (denaturation of thylakoid membrane proteins at room temperature by SDS-urea) was chosen to avoid aggregation of the hydrophobic D1/D2 polypeptides. In our experience, heat denaturation resulted in the formation of high molecular mass aggregates that appeared either as a smear in the stacking gel and upper portion of the running gel or as a high molecular mass band of protein immobilized at the stacking/running gel interface. Before electrophoresis, samples were centrifuged in a microcentrifuge for 5 min to remove unsolubilized material.

Chl concentrations were determined in acetone according to the method of Arnon (1949). β-Mercaptoethanol was added to samples to give a final concentration of 10%, and samples were diluted accordingly to yield equal Chl concentrations. Samples were stored on ice until used or otherwise stored at −80°C.

PSII Activity

Thylakoid membranes were isolated as described above except hypotonic buffer was used for the resuspension of the final pellet instead of solubilization buffer. Hypotonic buffer contained 10 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tricine/NaOH (pH 8.0). Light-induced absorbance difference measurements in the near-UV region of the spectrum (ΔA_{320}) provided an estimate of semiquinone anion formation (Q_A^-), thus a measure of functional PSII centers in thylakoids (Melis et al., 1992). The light-induced absorbance-difference measurements were corrected for the effect of particle flattening (Pulles et al., 1976).

Thylakoid Membrane Protein Analysis

Thylakoid membrane proteins were resolved by SDS-PAGE using the discontinuous buffer system of Laemmli (1970) with 15% acrylamide, 0.2% bis-acrylamide, and 4 M urea. The stacking gel contained 4.5% acrylamide and 1 M urea. The gel lanes were loaded with 4 nmol of Chl (a + b) for SDS-PAGE or with 2 nmol of Chl (a + b) for immunoblot analysis unless otherwise indicated. Electrophoresis on 0.15- × 14- × 16-cm slab gels was performed at 2°C at a constant current of 8 mA for 18 h. Gels were stained with 0.1% Coomassie brilliant blue R for protein visualization.

Immunochemical Analysis

Identification of reaction center polypeptides was accomplished with immunoblot analysis using specific polyclonal antibodies raised in this laboratory in rabbit against the PSII reaction center D1/32-kD (*psbA* gene) and D2/34-kD (*psbD* gene) proteins of spinach. The antigens (the D1 and D2 proteins) were isolated following SDS-PAGE of PSII reaction center preparations according to the method of Nanba and Satoh (1987). This approach prevented antigen contamination by other thylakoid membrane proteins and resulted in the formation of monospecific polyclonal antibodies (Melis, 1992). Our test results (not shown) suggested no cross-reactivity of the D1 antibody with the D2 protein.

Electrophoretic transfer of the SDS-PAGE resolved *D. salina* thylakoid membrane polypeptides to nitrocellulose, and the subsequent incubations with the antibodies and with alkaline phosphate-conjugated antibodies were performed as described previously (Smith et al., 1990). Cross-reaction was quantitated by scanning the nitrocellulose membranes with an LKB-Pharmacia XL laser densitometer. Results shown are the average values from three different experiments.

RESULTS

Cell Growth and Chl Content under Physiological and Irradiance-Stress Conditions

D. salina was grown under uniform illumination either at LL (100 μmol of photons m^{-2} s^{-1}) or HL (2000 μmol of photons m^{-2} s^{-1}) conditions. Cell growth was monitored by counting the cell density with a Neubauer ultraplane and, more conveniently, by the ln A_{678} of Chl. We determined that the Chl/cell ratio remained fairly constant under continuous LL or HL growth conditions (Naus and Melis, 1991). Therefore, the ln A_{678}, when plotted as a function of time after culture inoculation (Fig. 1), provides a measure of Chl accumulation and of cell growth under LL and HL conditions. It shows a logarithmically linear phase of growth and a gradually slower phase, followed by the stationary phase.

LL and HL cultures reached approximately the same cell density in the stationary phase (approximately 2×10^6 cells mL^{-1}). However, they differed significantly in the Chl/cell ratio. The LL-grown cells contained about 4×10^{-13} mol of Chl/cell, whereas the HL-grown cells reached approximately 0.5×10^{-13} mol of Chl/cell (ln $A_{678} = -1$ versus ln $A_{678} = -3$). These quantitative differences are consistent with earlier findings from this laboratory (Smith et al., 1990; Harrison et al., 1992). In the following, protein levels (D1) and functional cofactors (Q_A) in LL- and HL-grown cells will be given on a Chl basis. However, the interested reader could easily convert such measurements to a per cell basis.

Figure 2 shows Chl *a*/Chl *b* ratio adjustments in the thylakoid membrane of *D. salina* occurring upon transfer of a LL culture to HL conditions and vice versa. In this experiment, cultures were grown under either LL or HL conditions to the late log phase and then switched to the converse light regimen. LL-grown cultures had an initial Chl *a*/Chl *b* ratio of approximately 5. Following transfer to HL, and after a lag period of about 24 h, we observed that the Chl *a*/Chl *b* ratio of the LL culture started to increase. This change represents acclimation of the cells to HL and underlines a decrease in the Chl antenna size of the photosystems (Smith et al., 1990).

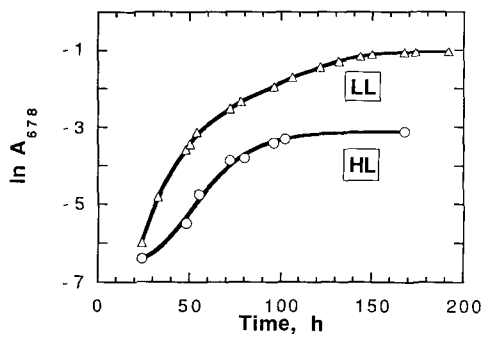

Figure 1. Growth curves of *D. salina*. The ln A_{678} of Chl is plotted as a function of time during cell growth under LL and HL conditions. Cells were grown either under physiological (LL; 100 μmol of photons m^{-2} s^{-1}, Δ) or irradiance-stress conditions (HL; 2000 μmol of photons m^{-2} s^{-1}, O).

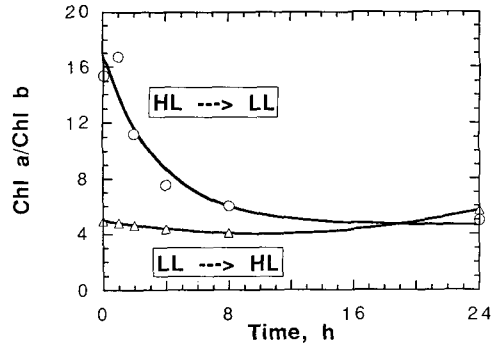

Figure 2. Adjustments in the Chl *a*/Chl *b* ratio in response to change in the level of irradiance during *D. salina* cell growth. The Chl *a*/Chl *b* ratio of *D. salina* cells is plotted as a function of time (0–24 h) following an LL → HL (Δ) or HL → LL (O) transition.

HL-grown cultures had a much greater Chl *a*/Chl *b* ratio than LL-grown cultures (17 versus 5). When HL-grown cells were switched to LL, the Chl *a*/Chl *b* ratio decreased promptly and reached a value of about 5 with a half-life of about 2 h. No lag in the HL → LL response was evident. These changes in the Chl *a*/Chl *b* ratio of *D. salina* show a response of the photosynthetic apparatus to irradiance stress and reveal different kinetics of change in the HL → LL and LL → HL direction.

PSII Reaction Center Damage and Repair under LL Conditions

To assess the rate of PSII damage and repair under the two different irradiance regimens, the amount of the D1 protein was quantified as a function of time in the absence and presence of the protein biosynthesis inhibitor, chloramphenicol. *D. salina* was grown under LL to the late log phase and cells were harvested at 0, 1, 2, 4, 8, and 24 h after addition of chloramphenicol to the growth medium. Control samples were taken at the same time intervals from a culture that was maintained under LL in the absence of chloramphenicol. Thylakoid membranes were isolated, and total protein in each sample was resolved by SDS-PAGE and visualized by Coomassie brilliant blue R staining. Figure 3 shows the LL-grown thylakoid membrane polypeptide profile of the chloramphenicol-treated samples (0–24 h). All lanes were loaded on an equal Chl (*a* + *b*) basis. It is apparent that samples from each time contain about the same amount of the major thylakoid membrane polypeptides.

Thylakoid membrane proteins were transferred to nitrocellulose, and the proteins on the membrane were probed with polyclonal antibodies raised against the D1 polypeptide of the PSII reaction center (Fig. 4). The antibody showed

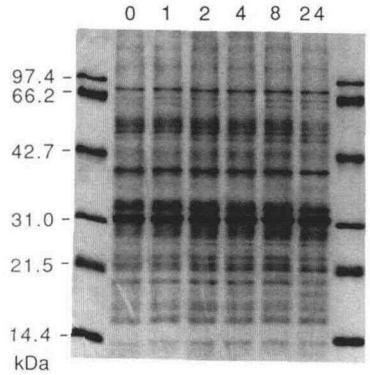

Figure 3. Profile of Coomassie-stained thylakoid membrane polypeptides from LL *D. salina* incubated in the presence of chloramphenicol. Cells were grown under LL to the late log phase, and samples were harvested at 0, 1, 2, 4, 8, and 24 h after chloramphenicol addition to the culture. Thylakoid membranes were isolated as described in "Materials and Methods." Note that samples contained about the same amount of the major thylakoid membrane proteins.

specific cross-reaction with a 32-kD protein (D1). Minor cross-reactions occurred with proteins at approximately 60 kD. The latter reflect the presence of unsolubilized dimers of D1 or D1/D2 (Nanba and Satoh, 1987; Seibert et al., 1988). The amount of the 60-kD dimers varied from preparation to preparation; however, it was always small compared to the amount of the 32-kD protein. Furthermore, the 60-kD dimers appeared to be more pronounced in LL thylakoids and negligible in HL thylakoids. In light-shift and chloramphenicol experiments, described below, we noted that the amount of the 60-kD dimers followed kinetics similar to those of the 32-kD protein, suggesting that they originated from functional PSII reaction centers (Nanba and Satoh, 1987; Seibert et al., 1988).

Additional antibody cross-reaction with a 160-kD protein complex was also detected (see below for detailed discussion

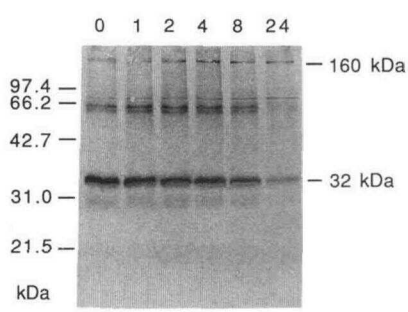

Figure 4. Immunoblot analysis of thylakoid membrane proteins from LL-grown *D. salina* incubated in the presence of chloramphenicol. Thylakoid membrane proteins [4 nmol of Chl (*a* + *b*)] were resolved by SDS-PAGE and subsequently transferred to nitrocellulose. Levels of the 32-kD protein were estimated from the cross-reaction with specific polyclonal antibodies. The level of the 32-kD protein gradually decreased as a function of time (0–24 h) under LL in the presence of chloramphenicol.

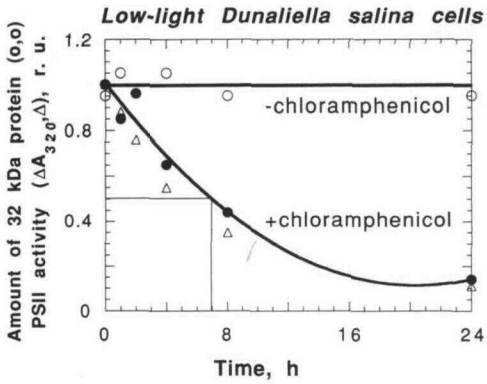

Figure 5. Quantitation of the cross-reaction between polyclonal antibodies and the 32-kD protein as determined by laser densitometry. The level of the 32-kD protein is plotted as a function of time in control (−chloramphenicol, ○) or chloramphenicol-treated (+chloramphenicol, ●) *D. salina* grown under LL conditions. The level of the 32-kD protein decreased with a half-time of about 7 h in the presence of chloramphenicol under LL, whereas the level of this protein stayed constant in the absence of chloramphenicol. The amount of photoreducible Q_A (ΔA_{320}, △) declined in the presence of chloramphenicol with kinetics similar to those for the 32-kD protein. In LL-grown cells, one relative unit (r.u.) of ΔA_{320} corresponds to a Q_A/Chl ratio of 2.2:1 (mmol:mol ratio).

of the 160-kD complex). The level of the 32-kD protein in thylakoids, as shown in Figure 4 (0-h lane), is the steady-state amount resulting from the continuous damage, degradation, and de novo biosynthesis of D1. Chloramphenicol blocks the biosynthesis of organelle proteins; therefore, the intensity of the cross-reaction with the 32-kD protein declined as a function of time after addition of this antibiotic.

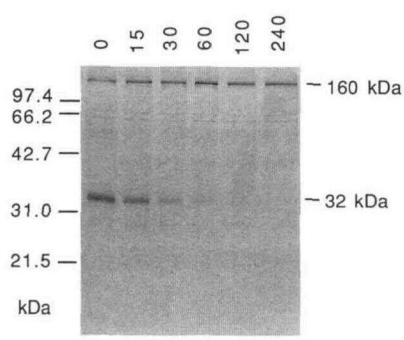

Figure 6. Immunoblot analysis of thylakoid membrane proteins from HL-grown *D. salina* incubated in the presence of chloramphenicol. Cells were grown under HL to the late log phase and samples were harvested at 0, 15, 30, 60, 120, and 240 min after chloramphenicol addition to the culture. Thylakoid membrane proteins were resolved by SDS-PAGE and subsequently transferred to nitrocellulose. Levels of the 32-kD protein and of the 160-kD protein complex were estimated from the cross-reaction with specific D1 polyclonal antibodies. The level of the 32-kD protein decreased rapidly as a function of time (0–120 min) under HL in the presence of chloramphenicol. The level of the 160-kD protein complex increased transiently (0 ≤ time ≤ 120 min) and then declined (time > 120 min).

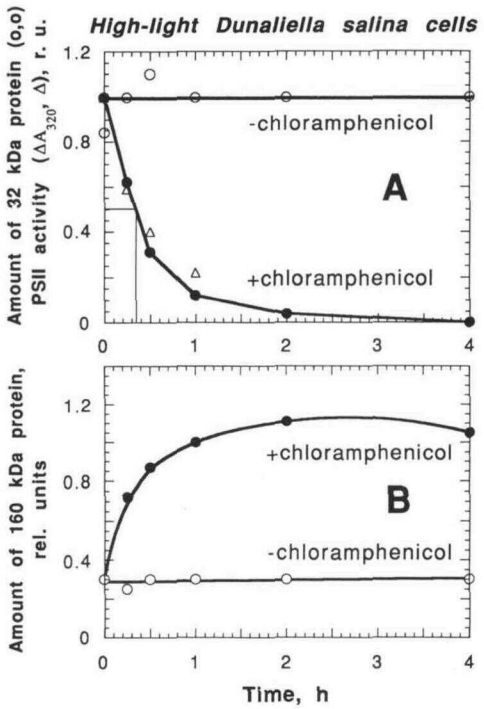

Figure 7. Quantitation of the cross-reaction between polyclonal antibodies and PSII reaction center protein as determined by laser densitometry. The level of the 32-kD protein (A) and the level of the 160-kD protein complex (B) are plotted as a function of time (0–4 h) in control (−chloramphenicol, ○) or chloramphenicol-treated (+chloramphenicol, ●) *D. salina* grown under HL conditions. A, The level of the 32-kD protein decreased with a half-time of about 20 min in the presence of chloramphenicol under HL (●). The amount of photoreducible Q_A (ΔA_{320}, Δ) decreased with similar kinetics. B, In the presence of chloramphenicol, the level of the 160-kD protein complex increased transiently ($0 \leq$ time ≤ 2 h) and then declined slowly for time ≥ 2 h (+chloramphenicol, ●). Levels of the 32-kD protein and the 160-kD complex remained constant as a function of time in the absence of chloramphenicol. In HL-grown cells, one relative unit (r.u.) of ΔA_{320} corresponds to a Q_A/Chl ratio of 3:1 (mmol:mol ratio).

Concomitantly with the decline in the density of the 32-kD protein, we noted a transient (0–4 h) increase in the density of the 160-kD complex (Fig. 4).

Laser densitometric analysis of such immunoblots showed that, upon addition of chloramphenicol to LL-grown *D. salina* cells in vivo, the amount of the 32-kD protein in the thylakoid membrane decreased exponentially with a half-time of about 7 h (Fig. 5, solid circles). Light-induced ΔA_{320} showed identical kinetics in the decline of semiquinone anion formation (Fig. 5, triangles), indicating loss of PSII primary charge separation activity under these conditions. These results provide a measure of the rate of PSII damage and D1 degradation under LL conditions. Because in the absence of chloramphenicol levels of the 32-kD protein (and of the semiquinone anion formation) remained constant (Fig. 5, open circles), it is concluded that the rate of PSII damage and repair under LL conditions occurs with a half-time of about 7 h. This estimate is consistent with pulse-chase measurements of the rate of D1 turnover under LL plant growth conditions (Mattoo and Edelman, 1987).

PSII Reaction Center Damage and D1 Degradation under Irradiance-Stress Conditions

To study the structural configuration of PSII and rate of damage and repair under chronic irradiance-stress conditions, *D. salina* cells were grown under HL to the late log phase and then chloramphenicol was added to the culture. Samples were harvested at 0, 15, 30, 60, 120, and 240 min after the addition of chloramphenicol. For the control experiment, samples were harvested at the same time intervals, but the culture was maintained under HL in the absence of chloramphenicol. Thylakoid membranes were isolated from each of the samples. SDS-PAGE, followed by immunoblot analysis and cross-reaction with anti-D1 antibody, was used to estimate the D1 protein content in thylakoids under chronic irradiance stress (Fig. 6). In the zero time sample (Fig. 6, 0-min lane), the antibody showed cross-reaction with the 32-kD protein and with the 160-kD protein complex. The relative intensity of the cross-reaction with the 160-kD aggregate was much stronger than that in LL-grown *D. salina* (compare with Fig. 4). It was determined that the 160-kD protein complex is also recognized by polyclonal antibodies raised against the D2 protein of PSII (not shown). Therefore, it is likely that the 160-kD protein complex originates from the aggregation or cross-linking of damaged PSII reaction centers, which probably occur as unsolubilized D1/D2/Cyt *b*-559 dimers (Schuster et al., 1989; Prasil et al., 1992). This observation would indicate accumulation of damaged PSII reaction centers in HL-grown *D. salina* thylakoid membranes that are photochemically inert, and, as such, they cannot be detected by the Q_A or pheophytin photoreduction assays (Smith et al., 1990; Melis, 1992; also, see below). In the presence of chlor-

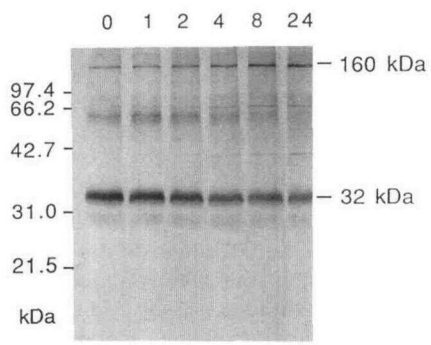

Figure 8. Adjustment of *D. salina* thylakoids to irradiance stress following an LL → HL transition in vivo. Cells were grown under LL to the late log phase and then transferred to HL conditions. Samples were harvested at 0, 1, 2, 4, 8, and 24 h after transfer of the culture to HL. Thylakoid membrane proteins were resolved by SDS-PAGE and subsequently transferred to nitrocellulose. Levels of the 32-kD protein and of the 160-kD protein complex were estimated from the cross-reaction with specific D1 polyclonal antibodies. The level of the 32-kD protein decreased transiently as a function of time (0–4 h) under HL and then gradually recovered (time > 4 h). The level of the 160-kD protein complex increased continuously during the 24-h period following the LL → HL transition.

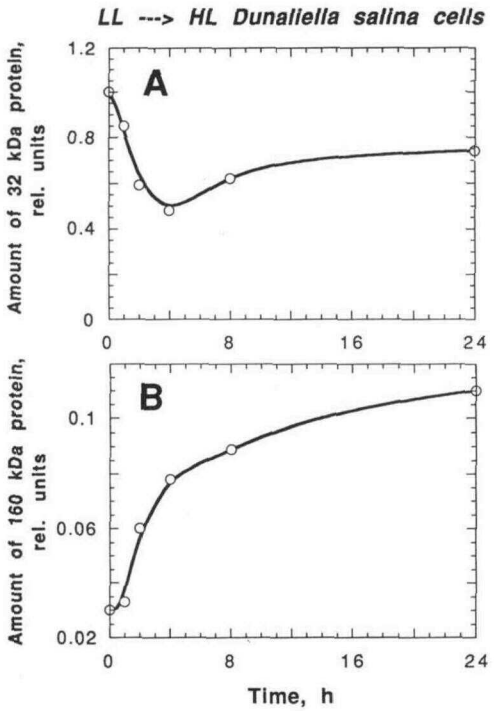

Figure 9. Quantitation of the cross-reaction between polyclonal antibodies and PSII reaction center protein as determined by laser densitometry. The level of the 32-kD protein (A) and the level of the 160-kD protein complex (B) in *D. salina* thylakoids are plotted as a function of time (0–24 h) following an LL → HL transition in vivo. A, The level the 32-kD protein decreased transiently as a function of time (0–4 h) following the transition to HL and then gradually recovered (time > 4 h). B, The amount of the 160-kD protein complex increased biphasically (fast phase from 0–4 h, slower phase from 4–24 h) to about 4-fold the initial value following the LL → HL transition.

amphenicol, the level of the 32-kD protein decreased rapidly as a function of time, whereas the level of the 160-kD protein complex increased transiently ($0 < \text{time} \leq 120$ min) and then declined for time > 120 min (Fig. 6).

Laser densitometry of such immunoblots revealed that, upon addition of chloramphenicol to irradiance-stressed *D. salina*, levels of the 32-kD protein in thylakoids declined exponentially with a half-time of only about 20 min (Fig. 7A, solid circles). Light-induced ΔA_{320} showed similar kinetics in the decline of semiquinone anion formation (Fig. 7A, triangles). Thus, decreasing levels of the 32-kD protein occur in tandem with the loss of PSII primary charge separation activity. In the absence of chloramphenicol, levels of the 32-kD protein (and of the semiquinone anion formation) remained constant (Fig. 7, open circles). It is suggested that the rate of PSII damage and the rate of repair under irradiance stress (including functional damage, D1 degradation, and de novo biosynthesis/assembly) must occur with a half-time of about 20 min, i.e. it is about 20-fold faster than that under LL conditions (compare with Fig. 5).

The steady-state level of the 160-kD aggregate remained constant in the absence of chloramphenicol, and, as mentioned earlier, it was considerably more abundant than that of LL-grown cells (Fig. 7B, open circles). It is interesting that, upon addition of chloramphenicol, the level of the 160-kD complex increased (up to approximately 4-fold of the initial level) throughout the ensuing 120 min, concomitantly with the loss of the 32-kD protein (Fig. 7B, solid circles). Subsequently, the level of the 160-kD complex declined gradually as a function of time in the presence of chloramphenicol (half-time of about 7 h, results not shown).

The results show that, under irradiance-stress conditions and in the absence of protein biosynthesis, PSII photochemical activity correlates with the amount of the D1/32-kD protein and does not correlate with the amount of the 160-kD protein complex in thylakoids.

Dynamics of Chloroplast Acclimation following a LL → HL Transition

To investigate the dynamics of chloroplast acclimation to irradiance stress, *D. salina* was grown under LL to the late log phase and then transferred to HL conditions. Samples were harvested at 0, 1, 2, 4, 8, and 24 h after the LL → HL transition. Thylakoid membranes were isolated from the samples at each time, and SDS-PAGE and immunoblot analysis were undertaken.

The antibody showed cross-reaction with both the 32-kD protein and with the 160-kD protein complex (Fig. 8). Laser densitometric quantitation of the amount of D1 protein in thylakoid membranes, following a LL → HL transition (Fig. 9A), showed that the amount of the 32-kD protein declined transiently to about 50% during the first 4 h after transfer to HL. For times greater than 4 h, the amount of the 32-kD protein began to increase and reached about 75% of the initial level after about 24 h. These results suggest that LL-grown *D. salina* cells do not possess the capacity to carry out enhanced rates of repair immediately upon exposure to HL conditions. In consequence, the rate of D1 damage exceeds the rate of repair, resulting in a net loss of functional D1 (loss of the 32-kD protein). With time in HL, however, cells appear to acquire a greater capacity for repair, resulting in a recovery

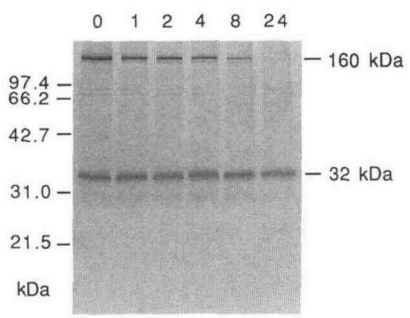

Figure 10. Recovery of *D. salina* thylakoids from irradiance stress following an HL → LL transition in vivo. Changes in the concentration of the 32-kD protein and of the 160-kD protein complex following an HL → LL transition are shown. Under these conditions, the level of the 32-kD protein decreased slightly during the 24-h period. The level of the 160-kD protein complex decreased rapidly as a function of time following the transition to LL.

of the 32-kD form of the D1 protein. The half-time of this induction phenomenon was estimated to be about 24 h.

The level of the 160-kD aggregate increased significantly upon transition to HL. The increase was biphasic with a fast phase occurring in the 0- to 4-h period and a slower phase occurring during the subsequent 24-h period (Fig. 9B). It is of interest to observe that the fast phase in the accumulation of the 160-kD complex coincided with the transient loss of the 32-kD protein from the thylakoid membrane (Fig. 9A), whereas the subsequent slow phase coincided with the recovery of the 32-kD form in thylakoids. This observation corroborates earlier results concerning the appearance of the 160-kD aggregate. It suggests that, whenever the rate of damage exceeds the chloroplast capacity for D1 degradation, the 160-kD aggregate accumulates in the thylakoid membrane.

Chloroplast Recovery from Irradiance Stress following a HL → LL Transition

To investigate the recovery of irradiance-stressed cells, HL-grown *D. salina* cells were transferred to LL conditions, and the level of the D1 protein was measured by immunochemical analysis. In this experiment, cells were grown to the late log phase under HL and then transferred to LL. Samples were harvested at 0, 1, 2, 4, 8, and 24 h after the HL → LL transition. Thylakoid membranes were isolated from each time point, and SDS-PAGE and immunoblot analysis were undertaken.

The antibody showed strong cross-reaction with the 32-kD protein and with the 160-kD protein complex (Fig. 10, 0 h), similar to the results in Figure 6. On a per Chl basis, the level of the 32-kD protein decreased slightly to a lower steady-state level as a function of time in LL. However, the level of the 160-kD protein complex decreased more rapidly (Fig. 10). Figure 11 shows the laser densitometric analysis of immunoblot results similar to those of Figure 10. When *D. salina* cells were switched from HL to LL, the level of the 32-kD protein was gradually lowered to about 75% of the initial value during a 24-h period (Fig. 11A). The level of the 160-kD protein complex decreased exponentially with a half-time of about 6.5 h and was virtually absent 24 h after the transition to LL (Fig. 11B). This observation further corroborates our results above (Fig. 4), which showed a negligibly low steady-state level of the 160-kD complex under physiological conditions.

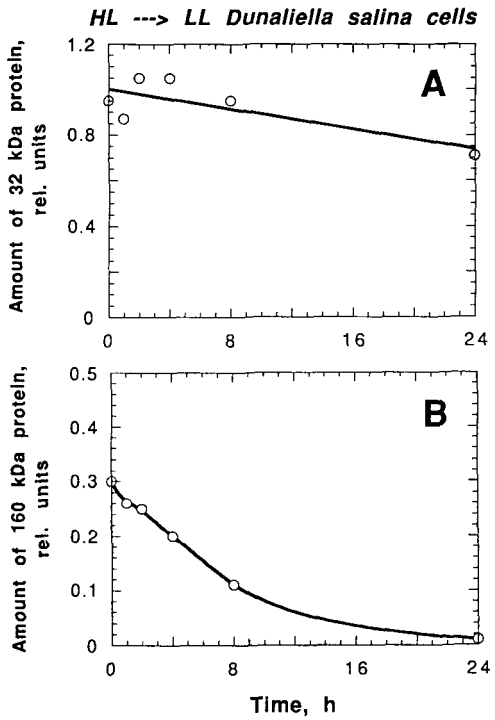

Figure 11. Quantitation of the cross-reaction between polyclonal antibodies and PSII reaction center protein as determined by laser densitometry. The level of the 32-kD protein (A) and the level of the 160-kD protein complex (B) in *D. salina* thylakoids are plotted as a function of time (0–24 h) following an HL → LL transition in vivo. A, On a Chl basis, the level of the 32-kD protein decreased to approximately 75% of its initial level during the 24-h period following the transfer of cells to LL. B, The level of the 160-kD protein complex decreased exponentially with a half-life of approximately 6.5 h following the HL → LL transition.

DISCUSSION

The Chl *a*-Chl *b* light-harvesting complexes of PSI and PSII contain both Chl *a* and Chl *b* molecules. However, the core complexes of PSI and PSII contain only Chl *a* (Green, 1988; Bassi et al., 1990; Peter and Thornber, 1991). Thus, changes in the Chl *a*/Chl *b* ratio, occurring upon plant acclimation to irradiance, imply changes in the size of the light-harvesting complex for each of the photosystems (Ley and Mauzerall, 1982; Lichtenthaler et al., 1982; Leong and Anderson, 1984; Lichtenthaler and Meier, 1984; Larsson et al., 1987; Sukenik et al., 1988). Results from this study show that LL-grown *D. salina* had a Chl *a*/Chl *b* ratio of about 5, whereas HL-grown cells had a ratio of about 17. These results are consistent with earlier study findings (Smith et al., 1990; Harrison et al., 1992) and suggest that chloroplasts from HL-grown cells have a smaller Chl *a*-*b* light-harvesting antenna than LL-grown cells. The Chl *a*/Chl *b* ratio of thylakoid membranes is dynamically adjusted in response to irradiance. Upon transition of HL-grown cells to LL conditions (HL → LL), the Chl *a*/Chl *b* ratio decreased exponentially from 17 to about 5 with a half-time of about 2.2 h (Fig. 2). A change in the light regimen in the opposite direction (LL → HL) did not produce prompt changes in the Chl *a*/Chl *b* ratio. This ratio started to increase only after a lag period of about 24 h (Fig. 2).

Irradiance conditions affect further changes in the photosystem composition and gene expression in chloroplasts. One dynamic response of the photosynthetic apparatus involves the capacity of chloroplasts to carry out the repair of damaged D1/32-kD reaction center proteins. Under LL conditions (100 μmol of photons m^{-2} s^{-1}), the rate of damage to PSII is slow and the turnover of the D1/32-kD protein occurs with a half-time of 7 h (Fig. 5). Under irradiance-stress conditions (2000 μmol of photons m^{-2} s^{-1}), the rate of damage is enhanced (half-time of approximately 20 min), approximately in direct

proportion to the light intensity (Fig. 7). At the same time, the rate of biosynthesis of the D1/32-kD protein is enhanced to match the rate of damage.

A novel aspect of the results in this work is the detection of the 160-kD protein complex that appeared in vivo both under irradiance-stress conditions and upon inhibition of chloroplast protein biosynthesis by chloramphenicol. The 160-kD protein complex may originate as a PSII reaction center dimer (D1/D2/Cyt *b*-559/psbI)$_2$, the molecular mass of which is approximately 160 kD. This complex clearly represents photochemically inert (damaged) PSII, evidenced by the fact that primary charge separation activity at PSII is proportional to the amount of the 32-kD protein in thylakoids and totally independent of the amount of the 160-kD complex (Figs. 5 and 7). Aggregates of D1 and D2 are known to form in SDS-gels, especially in heat-denatured samples. However, our solubilization conditions were chosen to avoid the fortuitous aggregation of these proteins (see "Materials and Methods"). We propose that this complex accumulates whenever the rate of reaction center damage and PSII disassembly exceeds the rate of D1 degradation/replacement in thylakoids. Figure 12 shows a temporal sequence of events beginning with the light-dependent damage to PSII reaction center (RC; step 1), followed by the disassembly (Hundal et al., 1990) of damaged PSII complexes (step 2).

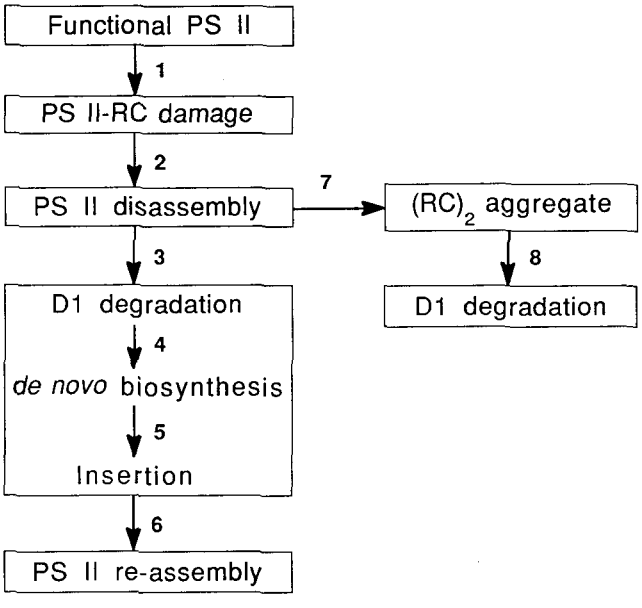

Figure 12. Schematic diagram of a temporal sequence of events in the PSII damage, disassembly, and D1 degradation. The rate of damage to PSII (step 1) is directly proportional to the incident light intensity. The rate of PSII disassembly (step 2) is not limiting under a broad range of incident intensities and/or in the presence of chloramphenicol. Direct D1 degradation (step 3) and de novo biosynthesis (step 4) become rate limiting under irradiance stress and in the presence of chloramphenicol, respectively. Under these conditions, reaction center (RC) dimers are formed from the disassembled PSII units (step 7). The results suggest some form of coordination and/or coupling between the processes of D1 degradation (step 3), de novo biosynthesis (step 4), and possibly the insertion of the nascent D1 polypeptide in thylakoids (step 5).

Under LL plant-growth conditions, damage to the reaction center is the rate-limiting step (step 1 in Fig. 12); therefore, rates of D1 degradation (step 3), de novo biosynthesis (step 4), and insertion (step 5) are not limiting. As a consequence, step 7 ([RC]$_2$ formation) is not competitive and no accumulation of the 160-kD complex is observed (Fig. 4). Under irradiance-stress conditions (Figs. 6, 8, and 10), PSII damage and disassembly (steps 1 and 2) are faster than D1 degradation/replacement (steps 3–5), thereby resulting in a steady-state accumulation of (RC)$_2$ aggregates in thylakoids (step 7 in Fig. 12). D1 in the (RC)$_2$ complex is degraded slowly with a half-time of about 6.5 h (Fig. 11B).

Upon addition of chloramphenicol to *D. salina* cultures, there is loss of the 32-kD protein and a transient accumulation of the 160-kD protein complex in thylakoids. This phenomenon is observed either under LL (Fig. 4) or irradiance-stress conditions (Fig. 6), as well as after a change in the light regimen (not shown). The transient accumulation of the 160-kD complex in the presence of chloramphenicol signifies that, in the absence of de novo D1 biosynthesis, the prompt D1 degradation (step 3) is also inhibited. Under such conditions, reaction step 7 is preferred (Fig. 12). We suggest that prompt D1 degradation (step 3) is tightly coupled to the de novo biosynthesis/insertion of this protein in thylakoids (steps 4 and 5). This interpretation is sufficient to explain why reaction step 7 is favored over step 3 when either the rate of D1 degradation or the rate of D1 biosynthesis is limiting.

Overall, the phenomenology associated with the formation of the 160-kD complex under irradiance stress suggested an emergency response of the photosynthetic apparatus to the adverse environmental condition. The 160-kD complex formation may serve as a stop-gap measure under conditions of accelerated PSII damage and disassembly when the PSII repair cycle does not have the capacity to carry out the necessary repairs. The formation of damaged reaction center dimers is not an unlikely scenario given that PSII complexes exist as dimers in the thylakoid membrane (Giddings et al., 1983; Peter and Thornber, 1991; R. Bassi, personal communication). One may argue that, upon disassembly of PSII, and in the absence of sufficient capacity for degradation/de novo biosynthesis, neighboring PSII-reaction center proteins aggregate either by hydrophobic interaction or by cross-linking of the constituent proteins (Prasil et al., 1992).

Upon transfer of LL-grown *D. salina* cells to irradiance-stress conditions, we noted that 4 h elapsed before the onset of a detectable chloroplast response and adjustment to the adverse effect (Fig. 9). This result suggested that LL chloroplasts do not possess the capacity for a rate of repair as high as that needed under irradiance-stress conditions. However, they begin to acquire this capacity after a lag of about 4 h, and they complete the response during the subsequent 24- to 36-h period following cell exposure to HL. The analysis suggested that irradiance stress induces the cellular capacity for enhanced rates of PSII repair. The mechanism for the induction of this phenomenon is unknown.

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