Response of the Photosynthetic Apparatus in Dunaliella salina (Green Algae) to Irradiance Stress[1]

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ABSTRACT

The response of the photosynthetic apparatus in the green alga Dunaliella salina, to irradiance stress was investigated. Cells were grown under physiological conditions at 500 millimoles per square meter per second (control) and under irradiance-stress conditions at 1700 millimoles per square meter per second incident intensity (high light, HL). In control cells, the light-harvesting antenna of photosystem I (PSI) contained 210 chlorophyll a/b molecules. It was reduced to 105 chlorophyll a/b in HL-grown cells. In control cells, the dominant form of photosystem II (PSII) was PSII$_\alpha$ (about 63% of the total PSII) containing >250 chlorophyll a/b molecules. The smaller antenna size PSII$_\beta$ centers (about 37% of PSII) contained 135 ± 10 chlorophyll a/b molecules. In sharp contrast, the dominant form of PSII in HL-grown cells accounted for about 95% of all PSII centers and had an antenna size of only about 60 chlorophyll a molecules. This newly identified PSII unit is termed PSII$_\gamma$. The HL-grown cells showed a substantially elevated PSII/PSI stoichiometry ratio in their thylakoid membranes (PSII/PSI = 3.0/1.0) compared to that of control cells (PSII/PSI = 1.4/1.0). The steady state irradiance stress created a chronic photoinhibition condition in which D. salina thylakoids accumulate an excess of photochemically inactive PSII units. These PSII units contain both the reaction center proteins and the core chlorophyll-protein antenna complex but cannot perform a photochemical charge separation. The results are discussed in terms of regulatory mechanism(s) in the plant cell whose function is to alleviate the adverse effect of irradiance stress.

Vascular plants and green algae respond to changes in the light environment in which they grown. Long-term light-intensity variations induce changes in the composition, structure and function of the photochemical apparatus (1, 17). These changes involve both the size and composition of the Chl antenna of PSI and PSII, and the PSII/PSI stoichiometry in the thylakoid membrane.

It is known that Chl b is present only in the auxiliary Chl

a/b of LHCI[2] and LHCII, whereas the core complexes of PSI and PSII contain only Chl a. The amount of the LHC associated with each photosystem can vary, resulting in variable Chl a/Chl b ratios in the thylakoid membrane (10, 11, 14, 25). There is evidence in the literature suggesting that variations in the Chl a/Chl b ratio occur naturally as a plant responds to changes in irradiance (12, 14, 28). Variations in the Chl a/Chl b ratio imply a variable PSU size for PSI and PSII in the thylakoid membrane.

It is generally accepted that low-light intensity promotes an increase in the Chl antenna size of both PSI and PSII (larger photosynthetic unit size). Correspondingly, high-light intensities promote a smaller Chl antenna size. This response appears to be well conserved in all photosynthetic systems examined. The mechanism of the response at the molecular and membrane levels is currently unknown.

In spite of the ability to adapt to changes in light-intensity, photosynthetic organisms are damaged upon exposure to excess visible light (21). The phenomenon is known as photoinhibition and is characterized by a lower rate of growth, and a lower light-saturated rate and quantum yield of photosynthesis (22). The underlying cause of photoinhibition is damage to the photochemical reaction center of PSII (3, 21, 24). Photoinhibitory damage is thought to occur when PSII cannot dissipate all excitation energy via useful photochemistry (18). Thus, under circumstances where electron transport is restricted, such as in CO_2-depleted conditions, photoinhibition will occur even at low-light intensities (21).

The present work examines the long-term response of the green alga Dunaliella salina to steady state irradiance-stress conditions that cause chronic photoinhibition but are not sufficiently adverse to prevent cell growth. The results suggest that photosynthetic cells respond to irradiance stress by adjusting the Chl antenna size of each photosystem and also by adjusting the PSII concentration in the thylakoid membrane.

MATERIALS AND METHODS

Cell Culture

Dunaliella salina cultures were grown in an artificial hypersaline medium containing 2.0 M NaCl (20). Carbon was supplied as $NaHCO_3$ at an initial concentration of 20 mM. Cultures were grown at 30°C under a mixture of incandescent and fluorescent illumination at 500 $mmol \cdot m^{-2} \cdot s^{-1}$ (control

[2] Abbreviations: LHCI, Chl a/b light-harvesting complex of PSI; LHCII, Chl a/b light-harvesting complex of PSII; P700, photochemical reaction center of PSI, Q_A, primary quinone electron acceptor of PSII; Pheo, pheophytin; PSU, the aggregate of Chl-protein complexes that defines a photosystem; MV, methylviologen.

cells) or at 1700 mmol·m^{-2}·s^{-1} (high light:HL-grown cells). Cultures were harvested at low cell densities ($1-4 \times 10^6$ cells/ mL determined by microscopic cell count) to avoid self-shading effects or light quality gradients through the culture.

Thylakoid Membrane Isolation

Working in dim light, control cells were harvested by centrifugation at 1,500g for 3 min and washed once in 50 mM Tricine (pH 7.8) buffer containing 0.4 M sucrose, 10 mM NaCl, and 5 mM MgCl$_2$, then recentrifuged at 1,500g for 3 min. The pelleted cells were suspended in a hypotonic buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM MgCl$_2$. Cells were broken by passing once through a Yeda press at 13.7 MPa. Cells grown in HL were treated similarly except that isolation buffers contained 2% PVP and 1% BSA. Additional precautions against protease activity were taken with cells grown in HL whenever thylakoid membranes were used for SDS-PAGE analyses. These cells were washed and broken into hypotonic buffer containing 30 μg/mL each *p*-amino caproic acid, PMSF, and amino benzamidine. However, comparison of gels from HL thylakoids showed no difference in protein banding pattern or immunological response with either preparatory technique. Following the Yeda press treatment, the slurry was centrifuged at 3,000g for 5 min to remove unbroken cells and large cell fragments. The supernatant was then centrifuged at 45,000g for 10 min. The thylakoid membrane pellet was resuspended in hypotonic buffer. Chl and carotenoid concentrations were determined in 80% acetone extract using the equations of Lichtenthaler (13). Thylakoid membrane preparations were kept in the dark at 0°C until analysis.

Photosystem Quantitation

PSII (Q$_A$) and PSI (P700) quantitation measurements were made with a laboratory-constructed, split-beam difference spectrophotometer (16). Actinic excitation of 75 mmol·m^{-2}·s^{-1} was provided in the blue region of the spectrum using a combination of Corning CS 4–96 and CS 3–73 filters. The optical pathlength of the cuvette for the measuring beam was 0.185 cm and for the actinic beam was 0.13 cm.

The concentration of P700 was determined from the amplitude of the light-induced absorbance change at 700 nm (ΔA$_{700}$) using a differential extinction coefficient of 64 mM^{-1}· cm^{-1} (7). The reaction mixture contained about 100 μM Chl, 0.02% SDS, 250 μM MV, and 2.5 mM Na-ascorbate. The concentration of Q$_A$ was determined from the amplitude of the light-induced absorbance change at 320 nm (ΔA$_{320}$) applying a differential extinction coefficient of 13 mM^{-1}·cm^{-1} (27). The reaction mixture contained approximately 100 μM Chl, 20 μM DCMU, and 2.0 mM K$_3$Fe(CN)$_6$. The absorbance difference measurements at 320 nm were corrected for the effect of particle flattening by the procedure of Pulles *et al.* (23).

The concentration of the primary electron acceptor pheophytin of PSII was determined from the light-induced absorbance change at 685 nm (ΔA$_{685}$) (3, 8). Actinic excitation of 600 μmol·m^{-2}·s^{-1} was provided in the blue region using a Corning CS 4–96 filter. The half-bandwidth of the measuring

beam was 1 nm. A differential extinction coefficient of 65 mM^{-1}·cm^{-1} was applied (3). The reaction mixture contained approximately 10 μM Chl suspended in 20 mM Tris-HCl (pH 7.8) containing 35 mM NaCl, 2 mM MgCl$_2$, 2 μM MV, 2 μM indigosulfonate, and sufficient sodium dithionite to lower the redox potential to -490 mV. The optical pathlength of the curvette was 1 cm and the absorbance-difference measurements were corrected for the effect of particle flattening (23).

Kinetic Analysis

PSII (fluorescence induction) and PSI (P700 photooxidation) kinetic measurements were performed using the above described difference spectrophotometer. Broadband actinic excitation of 40 μmol·m^{-2}·s^{-1} in the green region of the spectrum was provided by a combination of Corning CS 4–96 and CS 3–68 filters. The rate of light absorption by PSII was determined under light-limiting conditions from the kinetics of the area growth over the fluorescence curve of DCMU-treated membranes. The reaction mixture contained 50 μM Chl and 20 μM DCMU. The rate of light absorption by PSI was determined from the kinetics of the absorbance change at 700 nm. The reaction mixture contained 100 μM Chl, 20 μM DCMU, and 200 μM K$_3$Fe(CN)$_6$.

Functional antenna size estimates (N) for PSII$_\alpha$, PSII$_\beta$, and PSI were made, from the solution of the following system of equations (16):

$$\frac{Chl}{[PSI]} = \frac{N_\alpha[PSII_\alpha]}{[PSI]} + \frac{N_\beta[PSII\beta]}{[PSI]} + N_I \quad (1)$$

$$N_\alpha = cK_\alpha$$

$$N_\beta = cK_\beta \quad (2)$$

$$N_I = cK_I \quad (3)$$

$$(4)$$

where K is the experimental rate of light absorption. The proportionality constant c depends on the quantum yield of charge separation at each photosystem and on the incident light intensity. The same value of c was applied to PSII$_\alpha$, PSII$_\beta$, and PSI in control cells (16). In HL-grown cells, and because of the presence of photochemically silent PSII Chl in the thylakoid membrane, a value for c could not be determined experimentally. In this case, we used the value of c determined from the control cells. This approximation is justified because, in all kinetic measurements, the incident intensity of the actinic beam was the same (40 μmol·m^{-2}· s^{-1}), and because functional PSII and PSI units have a quantum yield of charge separation that approaches unity, independent of the size of their light harvesting Chl antenna (12, 26)

Thylakoid Membrane Polypeptide Analysis

Thylakoid membrane proteins were resolved by SDS-PAGE using the discontinuous buffer system of Laemmli (9) with a gradient resolving gel of 12.5 to 22.0% and a 5% stacking gel. The samples were solubilized in an equal volume of 500 mM Tris-HCl (pH 7.0) buffer containing 20% glycerol, 7% SDS, 2 M urea, and 10% β-mercaptoethanol and stored

Table I. *Cell Growth and Photochemical Apparatus Characteristics in Dunaliella salina*

The rate of cell growth is given as cell divisions per day. Pigment content and photosynthetic membrane component quantitation is given for control (500 μmol·m^{-2}·s^{-1}) and HL (1700 μmol·m^{-2}·s^{-1}) grown *D. salina*. Numbers in parentheses refer to number of replicate cultures. Component quantitation (mol:mol) is presented on a total Chl (a + b) basis (±SD).

Property	Control	HL-Grown
Rate of cell growth	5.2 ± 0.8 (4)	2.2 ± 0.3 (4)
Chl/cell (mol × 10^{-13})	1.8 ± 0.1 (4)	0.3 ± 0.05 (4)
Chl a/Chl b	4.6 (10)	16.5 (10)
Chl/Carotenoid	3.1 (10)	1.45 (10)
Chl/P700	620 ± 15 (10)	1090 ± 145 (5)
Chl/Q$_A$	440 ± 35 (8)	350 ± 75 (2)
Chl/Pheophytin	450 ± 25 (3)	360 ± 55 (3)
PSII/PSI	1.4 ± 0.2	3.0 ± 0.3

at -80°C. Before loading, samples were thawed and warmed to 70°C. Electrophoresis on 0.15 x 20 x 32 cm slabs was performed at a constant current of 18 mA for 20 h. The gels were loaded with equal amounts of functional PSI or PSII reaction centers, determined spectrophotometrically from the light-minus-dark difference amplitude of P700 or Q$_A$, respectively. Gels were stained with 0.1% Coomassie brilliant blue R for protein visualization.

Identification of reaction center polypeptides was accomplished with Western blot analysis using polyclonal antibodies to the D1/32 kD polypeptide of PSII in pea, and antibodies to two PSI polypeptides, the 22 kD ferredoxin-binding protein (30) and the 19 kD plastocyanin-binding protein in spinach (29). Chl a/b light-harvesting complex (LHCII) polypeptides were identified by reaction with antibodies to maize LHCII. Electrophoretic transfer of the SDS-PAGE resolved polypeptides to nitrocellulose, and the subsequent incubations with the above antibodies and with alkaline-phosphate conjugated antibodies were performed as described previously (2).

RESULTS

Absorbance Spectra and Photosystem Component Quantitation

In the logarithmic phase of *Dunaliella salina* growth, we measured approximately 5.2 cell divisions per day in control cultures (Table I). However, in HL-grown cultures the rate of cell growth was lowered to 2.2 cell divisions per day and the cultures had a yellow-green color compared to the bright green color of control cells. Microscopic inspection of the cultures revealed highly active cells in both cases. A slight morphological difference existed between control cells, which were roughly spherical in shape, and the HL-grown cells which were somewhat more elongated.

Figure 1 presents the absorbance spectra of isolated *D. salina* thylakoids from control cells grown at 500 μmol·m^{-2}·s^{-1} and from HL cells grown at 1700 μmol·m^{-2}·s^{-1}. When the spectra were normalized to the 679 nm Chl a absorbance peak, the HL thylakoids exhibited an elevated Soret absorb-

ance attributed to the greater carotenoid content of these cells (Table I). The lower Chl b absorbance at 650 nm in HL thylakoids correlated with the higher Chl a/Chl b ratio in these cells when compared to control cells (Table I).

Table I presents a summary of the photochemical apparatus component quantitation in control and irradiance-stressed *D. salina*. The results show that cells respond to irradiance stress in several ways. In HL-grown cultures, the total Chl/cell and Chl/carotenoid ratios decreased while the Chl a/Chl b ratio increased (19, 25). At HL intensities the Chl/P700 ratio increased from 620:1 in the control, to 1090:1. This response of the apparent photosynthetic unit size to HL conditions was unexpected and did not conform with the 'model' response of plants to increased light-intensity (10, 28). The Chl/P700 ratio is only a statistical measure of the total Chl and PSI content in the sample and does not provide information about the distribution of Chl between the two photosystems. The underlying cause of the greater Chl/P700 in the HL-grown cells was investigated in more detail (see below).

The Chl/PSII was measured independently as Chl/Q$_A$ and Chl/Pheo. It was slightly lower in HL than control thylakoids (350 *versus* 440, Table I). The resulting PSII/PSI stoichiometry was 1.4 for control cells, but it was 3.0 for HL-grown *D. salina*. To gain better insight into the underlying changes observed, the functional Chl antenna size of each photosystem was determined.
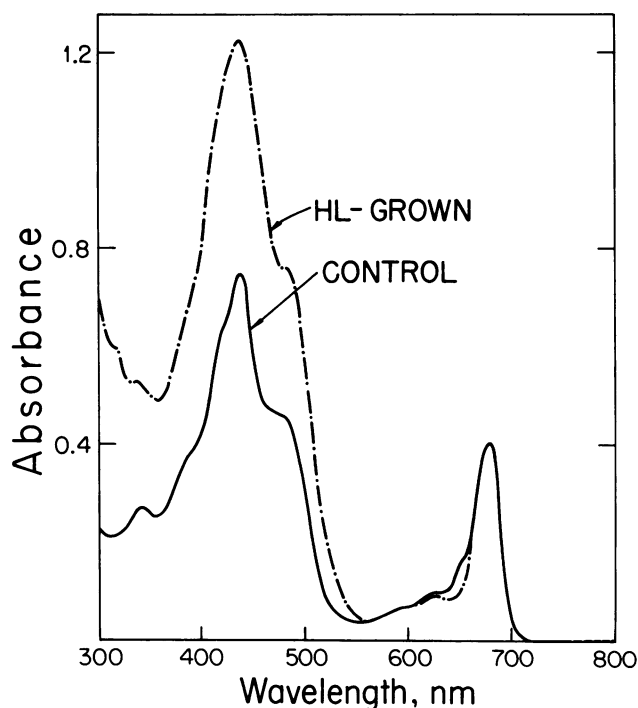

Figure 1. Absorbance spectra of isolated *D. salina* thylakoid membranes from cells grown at 500 μmol·m^{-2}·s^{-1} (control) and cells grown at 1700 μmol·m^{-2}·s^{-1} (HL-grown) conditions. The elevated absorbance of HL-grown thylakoids in the Soret region is attributed to a greater carotenoid/Chl ratio in these samples relative to the control cells.

Functional Chl Antenna Size of the Photosystems

The functional Chl antenna of each photosystem *in situ* was estimated by measuring the rate of light utilization by each reaction center (16). Figure 2 compares the P700 photooxidation kinetics in control and HL-grown *D. salina* thylakoids. The first order kinetic analysis (inset) shows that the rate of light utilization is greater in control than HL-grown cells. The slope of the straight lines in the semilogarithmic plots defined $K_I = 7.2$ photons·s^{-1} in control and $K_I = 3.7$ s^{-1} in HL grown thylakoids. The slower rate of light utilization by PSI in HL thylakoids is attributed to a smaller PSI Chl antenna size under irradiance stress.

Figure 3 compares the fluorescence induction kinetics in isolated thylakoid membranes from control and HL-grown *D. salina*. The kinetics of variable fluorescence provide a measure of the rate of Q_A photoreduction under the experimental conditions employed in this work (4). There were significant differences in the fluorescence yield properties and induction kinetics of the two samples. Thylakoids from HL-grown cells had a significantly lower yield of variable fluores-
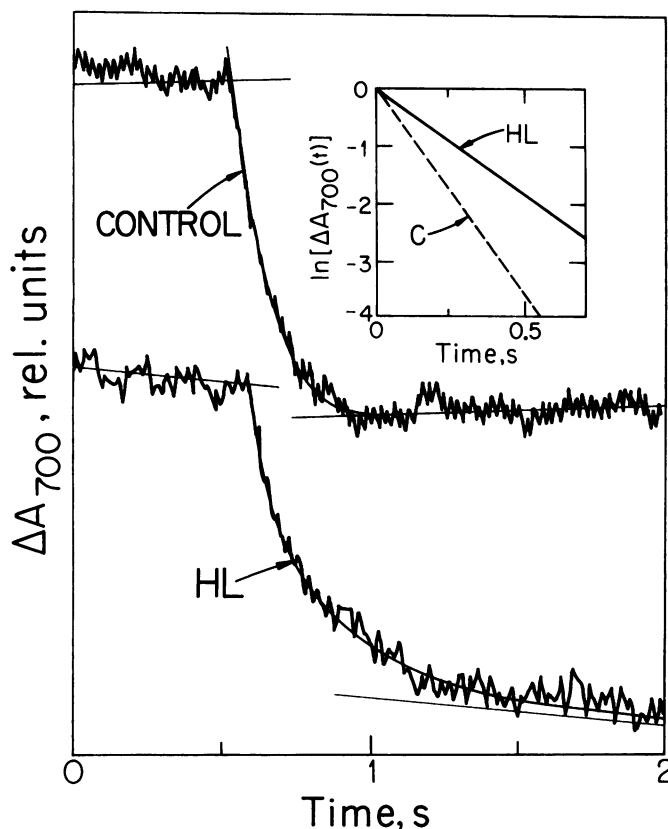

Figure 2. Photooxidation kinetics of the PSI reaction center (P700) in thylakoid membranes isolated from control (*C*) and HL-grown *D. salina*. The actinic excitation was turned on at 0.5 s and turned off at 3 s. Broadband green actinic light (Corning filters CS 4–96 and CS 3–68) was adjusted to an intensity of 40 μmol·m^{-2}·s^{-1}. Hand drawn best fit curves were used for the kinetic analysis. Inset: Semilogarithmic analysis of the $\Delta A_{700}(t)$ in control and HL thylakoids. The slopes of the straight lines defined the rate of light utilization by PSI (control, $K_I = 7.2$ photons·s^{-1}; HL, $K_I = 3.7$ photons·s^{-1}).
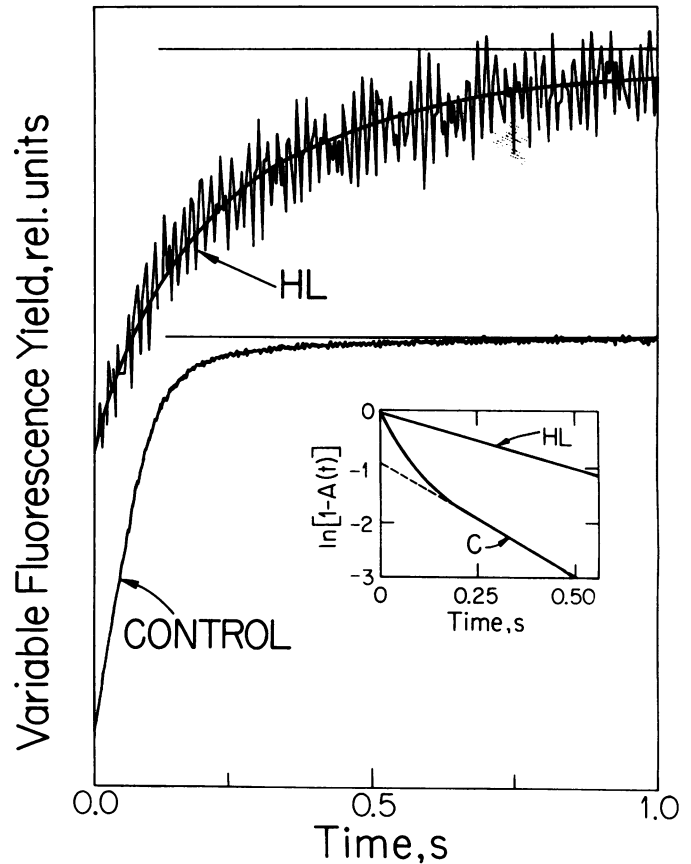

Figure 3. Fluorescence induction kinetics of isolated thylakoid membranes from control and HL-grown *D. salina* exposed to broadband green light. Thylakoid membranes were suspended in the presence of 20 μM DCMU. Different ordinate scales were used for the display of the variable fluorescence in control and HL-grown samples. Inset: Semilogarithmic analysis of the area growth over the fluorescence induction revealing a biphasic process for control and mostly monophasic exponential process for HL-thylakoids. Deconvolution of the kinetics in control thylakoids defined a rate of light utilization by PSII$_\alpha$, $K_\alpha = 19.5$ photons·s^{-1}. The rate of light utilization by PSII$_\beta$ was $K_\beta = 4.9$ photons·s^{-1}. The fluorescence induction kinetics in HL-grown cells was dominated by a component with a rate of light utilization $K_\gamma = 2.1$ photons·s^{-1} suggesting the presence of PSII units (PSII$_\gamma$) with antenna size smaller than that of PSII$_\beta$.

cence and a low F_v/F_o ratio compared to control thylakoids. This is probably a consequence of the small PSII antenna size and the presence of photochemically silent PSII Chl in HL-grown samples (see below). It is evident from the results of Figure 3 that HL-grown thylakoids exhibit slower induction kinetics, suggesting a smaller PSII antenna size than for control samples. The first order kinetic analysis of the area over fluorescence induction curve (Fig. 3, inset) provided more information about the kinetic properties of PSII in the two samples. It showed biphasic kinetics for control thylakoids and mostly monophasic exponential kinetics for HL thylakoids.

The biphasic fluorescence kinetics in *D. salina* (and in other grana-containing chloroplasts) underline the existence of two

Table II. *Photosystem Ratio and Antenna Size Estimates (N) in Control and HL Grown Dunaliella salina Thylakoids.*

The proportion of different forms of PSII, (PSII$_\alpha$, PSII$_\beta$, and PSII$_\gamma$) in the thylakoid membrane of control and HL-grown cells is given as percent of the photochemically competent PSII reaction centers (± SD). The number of functional Chl ($a + b$) antenna molecules associated with PSI, PSII$_\alpha$, PSII$_\beta$, and PSII$_\gamma$ is given by N$_I$, N$_\alpha$, N$_\beta$, and N$_\gamma$, respectively. Estimates were based on the rates of light utilization reported in the text.

Property	Control	HL-Grown
% PSII$_\alpha$	63 ± 6 (5)	0 ± 6 (5)
% PSII$_\beta$	37 ± 6 (5)	5 ± 5 (3)
% PSII$_\gamma$	0 ± 5 (5)	95 ± 5 (3)
N$_I$	210 ± 5 (6)	105 ± 10 (3)
N$_\alpha$	560 ± 20 (6)	—
N$_\beta$	140 ± 7 (9)	130 ± 10 (6)
N$_\gamma$	—	60 ± 5 (6)

populations of PSII units with distinct Chl antenna size. In control *D. salina*, the slow linear phase (Fig. 3, inset) reflects the photochemical activity of the smaller antenna PSII$_\beta$ units. The intercept of the straight line with the ordinate at zero time (dashed line in Fig. 3, inset) revealed that in control cells PSII$_\beta$ accounted for about 37% of the total PSII present. The rate of light utilization by PSII$_\beta$ (K_β) was measured from the slope of this straight line ($K_\beta = 4.9$ s^{-1}). In control *D. salina*, the fast kinetic phase (Fig. 3, inset) reflects the photochemical activity of PSIIα centers that have a larger Chl antenna size. The corresponding parameters for PSIIα in control cells were [PSII$_\alpha$] = 63% of the total PSII, and $K_\alpha = 19.5$ s^{-1} (Table II).

Analysis of the fluorescence induction in HL thylakoids (Fig. 3, inset) showed that the kinetics were mostly monophasic. The fast and sigmoidal PSII$_\alpha$ component was entirely missing. Moreover, the PSII$_\beta$ component constituted only up to 5% of the total PSII centers. The majority of PSII centers (about 95%) reacted with even slower exponential kinetics that corresponded to PSII centers with an antenna size smaller than PSII$_\beta$. These centers will be termed PSII$_\gamma$. The slope of the straight line in HL-grown thylakoids (Fig. 3, inset) defined $K_\gamma = 2.1$ photons·s^{-1} and $K_\beta = 4.6$ s^{-1}.

Based on the measured rates of light utilization (K) (Figs. 2 and 3) and on the Chl/PS ratios (Table I), the functional Chl antenna size servicing each reaction center was estimated (see "Materials and Methods"). The size of the Chl antenna of PSI was 210 Chl ($a + b$) molecules in control cells. The size of the Chl antenna of PSII$_\alpha$ and PSII$_\beta$ was 560 and 140 Chl ($a + b$) molecules, respectively, in these thylakoids (Table II). In HL-grown cells, the functional Chl antenna size of PSI was estimated to be 105 Chl molecules (Table II). The dominant form of PSII (PSII$_\gamma$, see "Discussion") contained about 60 Chl molecules. The small number of PSII$_\beta$ reaction centers which were present in the thylakoid membrane of HL-grown cells (about 5% of the total PSII) contained 130 Chl ($a + b$) molecules. Interestingly, PSII$_\alpha$ centers were totally absent from the thylakoid membrane of HL-grown cells (Table II). Thus, in HL-grown cells, and in spite of the significantly greater Chl/P700 (Table I), the functional Chl antenna size of both PSI and PSII is considerably smaller than that of

control cells. The results suggested that a sizable fraction of Chl in the thylakoid membrane of HL-grown cells was photochemically inert. This phenomenon was investigated in more detail.

Thylakoid Membrane Polypeptide Quantitation

Quantitation measurements of P700, Q$_A$, and Pheo (Table I) provide information on the concentration of photochemically competent photosystems in the thylakoid membrane. However, these light-induced spectrophotometric measurements cannot sense photochemically silent units. The presence of photochemically silent PSI and/or PSII complexes with their associated Chl in the thylakoid membrane would explain the unexpectedly high Chl/P700, Chl/Q$_A$, and Chl/Pheo ratios measured in HL-grown cells (Table I).

To address the question of the total concentration of PSI and PSII in the thylakoid membrane, SDS-PAGE analysis of the thylakoid membrane polypeptides and immunoblot quantitation of PSII and PSI integral protein components was undertaken. The levels of the D1/32 kD reaction center polypeptide and of the Chl a/b LHCII of PSII, and the levels the 22 kD ferredoxin-binding protein and the 19 kD plastocyanin-binding protein of PSI, were compared in HL-grown and control cells.

Figure 4 compares the thylakoid membrane polypeptide profile of HL and control *D. salina*. Lanes 1 and 2 were loaded on an equal functional PSII reaction center (Q$_A$) basis, as determined from the photochemical charge-separation reaction (results of Table I). Lane 1 (HL) was loaded with 16
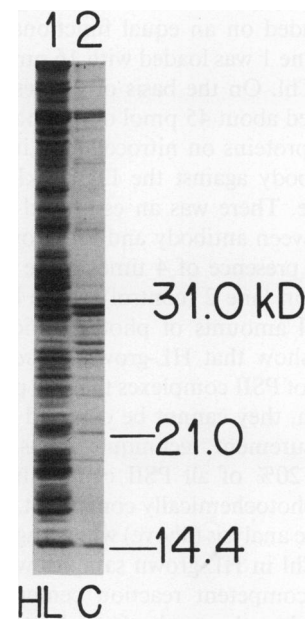

Figure 4. Coomassie stained profile of polypeptides from *D. salina* thylakoid membranes. Lanes were loaded on an equal functional PSII reaction center (Q$_A$) basis, as determined photochemically. Lane 1 contained HL thylakoids (16 nmol Chl loaded), and lane 2 contained control thylakoids (20 nmol Chl loaded). Note the higher concentration of proteins in the HL *versus* control thylakoids.
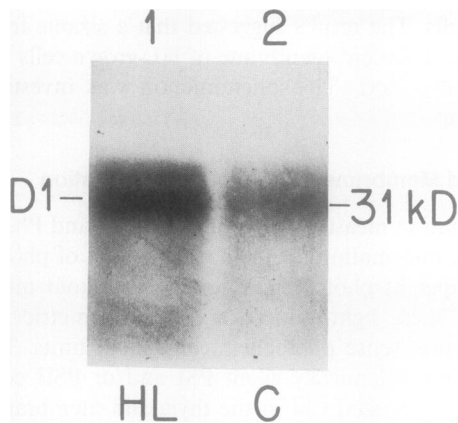
Figure 5. Western blot analysis of *D. salina* thylakoids. Lane 1 (thylakoids from HL-grown cells) was loaded with 16 nmol Chl (45 pmol of photochemically active Q_A) and lane 2 (control thylakoids) was loaded with 20 nmol Chl (45 pmol Q_A). Both lanes were probed with antibody against the D1/32 kD reaction center polypeptide of PSII. The much greater cross-reactivity of this antibody with proteins in the HL-grown cells suggested the presence of photochemically silent PSII units in these samples.

nmol Chl and lane 2 (control) with 20 nmol Chl. It is apparent from the results of Figure 4 that HL-thylakoid samples contained more total protein than control thylakoids. Because of the great abundance of protein in the HL-thylakoid sample, the PSII and PSI reaction center polypeptides could not be quantitated easily from the intensity of the Coomassie stain.

Figure 5 shows a Western blot analysis of the D1/32 kD reaction center polypeptide of PSII. Lane 1 (HL) and lane 2 (control) were loaded on an equal functional PSII reaction center basis, *i.e.* lane 1 was loaded with 16 nmol Chl and lane 2 with 20 nmol Chl. On the basis of the results in Table I, each lane contained about 45 pmol of photochemically competent PSII. The proteins on nitrocellulose in Figure 5 were probed with antibody against the D1/32 kD PSII reaction center polypeptide. There was an estimated 4 times greater cross-reaction between antibody and HL-grown sample (Fig. 5), suggesting the presence of 4 times more PSII protein in lane 1 (HL) than in lane 2 (control). Since both lanes were loaded with equal amounts of photochemically competent PSII, the results show that HL-grown *D. salina* thylakoids contain an excess of PSII complexes that are photochemically silent and, as such, they cannot be detected by the Q_A photoreduction measurement technique. Thus, in HL-grown cells, only about 20% of all PSII centers in the thylakoid membrane were photochemically competent. This is consistent with the kinetic analysis (above) which suggested that only a fraction of all Chl in HL-grown samples was coupled to a photochemically competent reaction center. This unusual phenomenon could be the result of steady state photoinhibition of PSII occurring under these high light-intensities (see below).

Figure 6 compares the relative cross-reactivity of proteins associated with the Chl *a/b* light-harvesting complex of PSII (LHCII) in HL-grown (lane 1) and control cells (lane 2). In this experiment, lane 1 was loaded with 16 nmol Chl and lane

2 with 20 nmol Chl, *i.e.* the two lanes contained equal amounts of photochemically competent PSII, as in the approach of Figure 4. The results of Figure 6 support the earlier observation (6, 25) that, under physiological conditions, *D. salina* thylakoids contain large quantities of at least five different polypeptides associated with the LHCII. These polypeptides constitute the auxiliary light-harvesting antenna of $PSII_\alpha$ and $PSII_\beta$ in control cells (Table II). High-light grown cells (lane 1) show the same qualitative pattern of proteins associated with the LHCII; however, the relative amounts are considerably less than those of the control cells (lane 2). The small quantity of LHCII polypeptides in HL-grown cells is probably associated with the small number of $PSII_\beta$ centers present in these cells (Table II).

The Western blot analysis was also performed on PSI proteins. Figure 7 compares the relative cross-reactivity of two integral PSI proteins in HL-grown cells (lane 1) and control cells (lane 2). The two lanes in this experiment were loaded on the basis of equal functional PSI reaction center (P700) content. Lane 1 was loaded with 33 nmol Chl and lane 2 with 20 nmol Chl. On the basis of the results in Table I, each lane contained approximately 30 pmol of P700. Proteins on nitrocellulose were probed with two different antibodies, one raised against the 19 kD plastocyanin-binding protein of PSI (PCbp) and the other against the 22 kD ferredoxin-binding protein of PSI (FDbp). The results of Figure 7 show approximately equal cross-reactivity for the 19 kD PCbp in HL and control cells. These results suggest that equal amounts of total PSI (30 pmol) were loaded in lanes 1 and 2, in agreement with the spectrophotometric measurements. Thus, photoinhibition did not affect the function of PSI in HL-grown *D. salina.* Interestingly, the antibody against the 22 kD FDbp cross-reacted strongly with a protein migrating at about 16 kD and with a second protein migrating at about 28 kD in the HL-grown sample only. The specificity of this reaction with different proteins from the HL-grown cells may indicate that HL-grown cells possess significant amounts
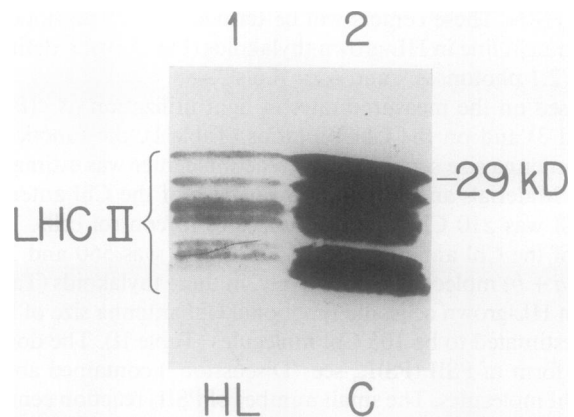

Figure 6. Western blot analysis of *D. salina* thylakoids. Both lanes were probed with antibody against the Chl *a/b* light-harvesting complex of PSII (LHCII). The significantly lower cross-reactivity of this antibody with proteins from the HL-grown cells suggested a greatly reduced PSII auxiliary antenna size relative to that of control cells. For more details on experimental conditions, see legend of Figure 5.
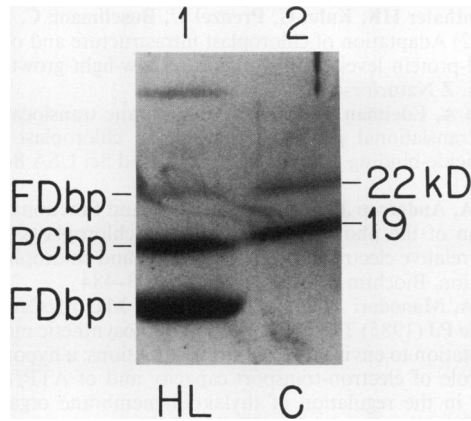
Figure 7. Western blot analysis of *D. salina* thylakoids. Lane 1 (thylakoids from HL-grown cells) was loaded with 33 nmol Chl (30 pmol P700) and lane 2 (control cells) was loaded with 20 nmol Chl (about 30 pmol P700). Both lanes were probed with antibodies against the 19 kD plastocyanin-binding protein of PSI (PCbp) and against the 22 kD ferredoxin-binding protein of PSI (FDbp), respectively. The equal cross-reactivity of the PCbp antibody with proteins from HL and control cells suggested equal total PSI reaction center content in lanes 1 and 2.

of modified ferredoxin-binding proteins which have common antigenic determinants with the 22 kD protein and which are expressed only under HL conditions. Alternatively, the 16 kD protein might be a partial degradation product of the 22 kD polypeptide, occurring upon lysis of the cells. This phenomenon was not investigated further in this work.

DISCUSSION

The response of the photosynthetic apparatus in *Dunaliella salina* to irradiance stress involved distinct but interrelated phenomena. The functional Chl antenna size of PSI was reduced significantly in response to irradiance stress during cell growth. In control cells (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$), the light-harvesting antenna of PSI contained 210 Chl ($a + b$) molecules. The proteins associated with this light-harvesting antenna include the Chl a-binding core-complex of PSI which binds about 100 Chl a molecules, and the auxiliary light-harvesting antenna of PSI (LHCI) which binds the remaining Chl a and Chl b molecules. In HL-grown cells (1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$), the light-harvesting antenna of PSI contained only about 105 Chl a molecules, suggesting the selective absence of LHCI from these samples. In agreement, previous studies have shown that 95 Chl a molecules constitute the minimum PSI unit size that will assemble in the thylakoid membrane of green plants (5). It is concluded that excessive irradiance during cell growth elicits a down-regulation of the Chl antenna size of PSI in *D. salina*.

The light-harvesting antenna size of PSII showed a similar response to irradiance stress. In control cells, the dominant form of PSII was PSII$_\alpha$ (Chl antenna size > 250 Chl [$a + b$] molecules). The smaller antenna size PSII$_\beta$ contained only about 140 ± 10 Chl ($a + b$) molecules. It is known that PSII$_\beta$ centers contain, in addition to the Chl a-binding proteins of the PSII-core, an accessory Chl a/b light-harvesting antenna

(LHCII-inner) (10, 16). The functional antenna size of PSII$_\alpha$ is considerably larger than that of PSII$_\beta$ because of the presence of the LHCII-peripheral (16). In HL-grown cells, PSII$_\alpha$ was absent and only a few PSII$_\beta$ centers were present. The dominant form of PSII in these samples contained about 60 Chl a molecules, suggesting the sole presence of a Chl a-binding PSII-core complex. This PSII antenna size is similar to that found in the Chl b-less chlorina f2 mutant of barley (5) which also lacked the LHCII-inner and LHCII-peripheral complexes from the thylakoid membrane. Thus, it appears that a stable form of PSII with 50 to 60 Chl a molecules can exist in the thylakoid membrane of vascular plants and green algae. We propose to denote this PSII unit as PSII$_\gamma$.

These results suggest that regulation of the Chl antenna size of PSI and PSII during irradiance stress involves modulation of the levels of LHCI and LHCII present in the thylakoid membrane. The molecular mechanism for this regulation is presently unknown.

A rationale for the down-regulation of Chl biosynthesis at higher light intensities is that the rate of photosynthesis is limited by the dark reactions of carbon fixation and/or by carbon dioxide availability to the cell. Under these conditions, the biosynthesis/assembly of a large Chl antenna size for PSI and PSII would be both unnecessary and metabolically expensive. A nonregulated biosynthesis/assembly of LHC may even be counterproductive for the plant since a large antenna size under high-intensity illumination would result in increased nonphotochemical dissipation of excitation at the reaction centers, leading to further photoinhibition and/or other photodamage (21). Therefore, a light-intensity dependent attenuation of biosynthesis/assembly of the LHC allows the plant to conserve significant amounts of metabolic energy for other cellular functions, and to minimize adverse effects due to the nonphotochemical dissipation of excitation.

The substantially different PSII/PSI stoichiometry ratio and the large fraction of photochemically silent PSII complexes in HL-grown cells are probably interrelated phenomena. It may be argued that, under HL-conditions, the rate of photosynthesis is limited by carbon fixation and/or carbon dioxide availability to the cells. This irradiance-stress condition results in photoinhibition of the PSII reaction center and in accumulation of damaged PSII units in the thylakoid membrane. The damaged photochemical reaction centers contain the D1/32 kD protein and a core Chl a antenna but are unable to perform charge separation reactions and must be in need of repair (3, 15, 24).

This hypothesis, describing the interplay between irradiance stress, rate of photochemistry, and carbon dioxide fixation in *D. salina*, received support from preliminary experiments in which HL-grown cell cultures were transferred to control irradiance conditions or were supplemented with gaseous CO_2 by bubbling with a gas mixture containing 3% CO_2 in air. In both instances, the Chl antenna size of the photosystems and the PSII/PSI ratio approached that of control cells (half-time of change approximately 20 h). Simultaneously, the photoinhibitory state in the thylakoid membrane was overcome. Thus, the results presented here suggest the operation of regulatory mechanism(s) designed to protect the cell against irradiance stress. The response involved adjustment and op-

timization of function in the photosynthetic apparatus via control of the Chl antenna size and of the PSII/PSI stoichiometry ratio in the thylakoid membrane.

LITERATURE CITED

1. **Anderson JM** (1986) Photoregulation of the composition, function and structure of thylakoid membranes. Annu Rev Plant Physiol **37**: 93–136
2. **Darr SC, Somerville SC, Arntzen CJ** (1986) Monoclonal antibodies to the light-harvesting chlorophyll a/b protein complex of photosystem II. J Cell Biol **103**: 733–740
3. **Demeter S, Neale PJ, Melis A** (1987) Photoinhibition: impairment of the primary charge separation between P680 and pheophytin in photosystem II of chloroplasts. FEBS Lett **214**: 370–374
4. **Duysens LNM, Sweers HE** (1963) Mechanism of two photochemical reactions in algae as studied by means of fluorescence. *In* Studies on Microalgae and Photosynthetic Bacteria. Special Issue of Plant Cell Physiology University of Tokyo Press, Tokyo, pp 353–372
5. **Glick RE, Melis A** (1988) Minimum photosynthetic unit size in System I and System II of barley chloroplasts. Biochim Biophys Acta **934**: 151–155
6. **Guenther JE, Nemson JA, Melis A** (1988) Photosystem stoichiometry and chlorophyll antenna size in *Dunaliella salina* (green algae). Biochim Biophys Acta **934**: 108–117
7. **Hiyama T, Ke B** (1972) Difference spectra and extinction coefficients of P700. Biochim Biophys Acta **267**: 160–171
8. **Klimov V, Klevanik A, Shuvalov V, Krasnovsky A** (1977) Reduction of pheophytin in the primary light reaction of photosystem II. FEBS Lett **82**:183–186
9. **Laemmli U** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680–685
10. **Larsson UK, Anderson JM, Andersson B** (1987) Variations in the relative content of the peripheral and inner light-harvesting chlorophyll ab-protein complex (LHC II) subpopulations during thylakoid light adaptation and development. Biochim Biophys Acta **894**: 69–75
11. **Leong T-A, Anderson JM** (1984) Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. I. Study on the distribution of chlorophyll-protein complexes. Photosynth Res **5**: 105–115
12. **Ley AC, Mauzerall DC** (1982) Absolute absorption cross sections for photosystem II and the minimum quantum requirement for photosynthesis in *Chlorella vulgaris*. Biochim Biophys Acta **680**: 95–106
13. **Lichtenthaler HK** (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol **148**: 350–382
14. **Lichtenthaler HK, Kuhn G, Prenzel U, Buschmann C, Meier D** (1982) Adaptation of chloroplast ultrastructure and of chlorophyll-protein levels to high-light and low-light growth conditions. Z Naturforsch **73**: 464–474
15. **Mattoo A, Edelman M** 1987 Intermembrane translocation and posttranslational palmitoylation of the chloroplast 32 kDa herbicide-binding protein. Proc Natl Acad Sci USA **84**: 1497–1501
16. **Melis A, Anderson JM** (1983) Structural and functional organization of the photosystems in spinach chloroplasts: antenna size, relative electron-transport capacity, and chlorophyll composition. Biochim Biophys Acta **724**: 473–484
17. **Melis A, Manodori A, Glick RE, Ghirardi ML, McCauley SW, Neale PJ** (1985) The mechanism of photosynthetic membrane adaptation to environmental stress conditions: a hypothesis on the role of electron-transport capacity and of ATP/NADPH pool in the regulation of thylakoid membrane organization and function. Physiol Veg **23**: 757–765
18. **Osmond CB** (1981) Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. Biochim Biophys Acta **639**: 77–98
19. **Pick U, Gounaris K, Barber J** (1987) Dynamics of photosystem II and its light harvesting system in response to light changes in the halotolerant alga *Dunaliella salina*. Plant Physiol **85**: 194–198
20. **Pick U, Karni L, Avron M** (1986) Determination of ion content and ion fluxes in the halotolerant alga *Dunaliella salina*. Plant Physiol **81**: 92–96
21. **Powles SB** (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol **35**: 15–44
22. **Powles SB, Osmond CB, Thorne SW** (1979) Photoinhibition of intact attached leaves from C₃ plants illuminated in the absence of both carbon dioxide and of photorespiration. Plant Physiol **64**: 982–988
23. **Pulles MPJ, Van Gorkom H, Verschoor G** (1986) Primary reactions of photosystem II at low pH. 2. Light-induced changes of absorbance and electron spin resonance in spinach chloroplasts. Biochim Biophys Acta **440**: 98–104
24. **Schuster G, Timberg R, Ohad I** (1988) Turnover of Photosystem II thylakoid proteins during photoinhibition of *Chlamydomonas reinhardtii*. FEBS Lett **177**: 403–410
25. **Sukenik A, Bennett J, Falkowski P** 1988 Changes in the abundance of individual apoproteins of light-harvesting chlorophyll a/b-protein complexes of photosystem I and II with growth irradiance in the marine chlorophyte *Dunaliella tertiolecta*. Biochim Biophys Acta **932**: 206–215
26. **Thielen APGM, Van Gorkom HJ** (1981) Quantum efficiency and antenna size of photosystem II_α, II_β and I in tobacco chloroplasts. Biochim Biophys Acta **635**: 111–120
27. **Van Gorkom HJ** (1974) Identification of the reduced primary electron acceptor of photosystem II as a bound semiquinone anion. Biochim Biophys Acta **347**: 439–442
28. **Wilhelm C, Wild A** (1984) The variability of the photosynthetic unit in *Chlorella*. J Plant Physiol **115**: 125–135
29. **Wynn RM, Malkin R** (1988) Interaction of plastocyanin with photosystem I: A chemical cross-linking study of the polypeptide that binds plastocyanin. Biochemistry **27**: 5863–5869
30. **Zilber AL, Malkin R** (1988) Ferredoxin cross-links to a 22 kD subunit of photosystem I. Plant Physiol **88**: 810–814