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Dynamics of Photosystem II heterogeneity during photoinhibition: depletion of PS II_β from non-appressed thylakoids during strong-irradiance exposure of *Chlamydomonas reinhardtii*

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Thylakoid membranes of *Chlamydomonas reinhardtii* were fractionated into appressed membranes and non-appressed vesicles. The latter had a Chl a/b ratio of 3.2 and contained 1.12 mmol PS II (mol Chl $a+b)^{-1}$. The PS II centers in the non-appressed regions displayed the kinetic characteristics of PS II_β centers. They were able to generate a stable charge-separation to Q_A, but they were unable to transfer electrons from Q_A^- to Q_B (Q_B-nonreducing centers). After a strong-irradiance treatment (SIT) of cells by exposure to 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 15 min, a portion of the PS II_α centers in the appressed membranes was damaged due to photoinhibition. PS II_β centers were resistant to photoinhibitory damage; however, SIT caused a lowering of the PS II content in the non-appressed fraction to 0.60 mmol PS II (mol Chl $a+b)^{-1}$. Concomitantly, there was a conversion of Q_B-nonreducing centers to a form able to reduce the plastoquinone pool (Q_B-reducing centers). Low-temperature during SIT inhibited both the depletion of PS II_β from the non-appressed membrane region and the activation of Q_B-nonreducing centers. These results are consistent with a 'PS II repair cycle' which translocates PS II units from the stroma-exposed membranes into the appressed thylakoids during activation of PS II_β from a Q_B-nonreducing to a Q_B-reducing form. Since the overall distribution of Chl-protein complexes between the stroma-exposed and appressed membrane regions was unaffected by SIT, it is suggested that damaged PS II units from the appressed membrane region are exchanged with PS II_β from the stroma-exposed membranes.

Introduction

Strong-irradiance stress of plants and algae causes the loss of Photosystem II (PS II) function, known as photoinhibition [1]. Recent investigations have focused on the molecular mechanism for the repair of irradiance-damaged PS II reaction centers. The repair is known to require removal of PS II apoproteins and incorporation of de novo synthesized proteins into the thylakoid membrane [2–5].

Photosystem II complexes are localized primarily in the appressed membrane region of the thylakoid membrane of higher plants and green algae but are also found in the stroma-exposed region [6,7]. A large antenna form, PS II_α, is localized exclusively in the appressed membrane region, while the non-appressed membranes contain the smaller antenna form, PS II_β [8,9]. Under physiological conditions, it has been shown that PS II_α displays the full photochemical activity associated with water oxidation and plastoquinone reduction [10]. On the other hand, PS II_β has a functional reaction center and forms a stable charge separation upon illumination but cannot reduce plastoquinone. Thus, PS II_β has been termed Q_B-nonreducing, in comparison with the Q_B-reducing PS II_α centers [11].

Abbreviations: Chl, chlorophyll; LHCII, light-harvesting chlorophyll a/b complex of PS II; Pheo, pheophytin; PS I, Photosystem I; PS II, Photosystem II; P-680, reaction center of PS II; P-700, reaction center of PS I; Q_A and Q_B, primary and secondary quinone electron acceptor of PS II; PS-II-Q_B-nonreducing, PS II center with impaired Q_A–Q_B interaction; SIT, strong-irradiance treatment.

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The physiological significance of PS II heterogeneity has been explained in terms of a 'PS II repair cycle' operating between appressed and non-appressed membrane regions [12]. It was postulated that, once damaged, PS II_α units dissociate into the peripheral LHCII and a PS II_β-like complex. These photochemically inert PS II_β-like centers are translocated into the stroma-exposed region. Damaged reaction centers in the stroma exposed region are then repaired, a process that includes replacement of the apoprotein(s) of the PS II reaction center. Repaired centers accumulate in the nonappressed region as a reserve pool of PS II in the form of PS II_β-Q_B-nonreducing. In a subsequent step, these centers are 'activated' to a PS II_β reducing form and become incorporated into the appressed membrane region as fully functional PS II units. It was further postulated that the size of this reserve pool is regulated at the activation step so that incorporation of new Q_B-reducing centers can be dynamically coupled to irradiance [13]. Such a cycle is consistent with the localization and movement of the PS II reaction center 'D1' polypeptide which first appears as a precursor in the stroma-exposed region [14,15]. The mature protein is then obtained after post-translational modification and intra-membrane transfer to the appressed membranes [14]. Intramembrane translocation has also been suggested to occur for certain other PS II reaction center polypeptides [16] and intact PS II complexes [17].

In the chlorophyte microalga *Chlamydomonas reinhardtii*, a large demand for reaction center repair is created when PS II_α centers are damaged due to photoinhibition [13]. Such strong-irradiance stress is also associated with a sharp reduction in the pool size of the Q_B-nonreducing centers, which are apparently converted to the Q_B-reducing form [13]. These 'activated' PS II_β centers counteract the impact of photoinhibition by contributing to whole chain electron transport (oxygen evolution). In the present report, we have analysed the distribution of reaction center complexes in appressed and non-appressed membrane regions of *C. reinhardtii* following exposure to strong irradiance. Both mechanical and detergent fractionation methods were employed in the isolation of membrane vesicles enriched in appressed and non-appressed regions. We show that a selective depletion of PS II_β from the non-appressed membrane region occurs during strong-irradiance exposure of *C. reinhardtii*. The results are consistent with the operation of a repair cycle that translocates PS II complexes between non-appressed and appressed membrane regions during photoinhibition.

Materials and Methods

Chlamydomonas reinhardtii was grown as previously described [18] using a growth irradiance of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Intact cells were exposed to 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 15 min as described [13]. Strong irradiance treatments at 0 °C were performed as described in Ref. 19. In order to provide sufficient material for fractionation, approximately 10 liters of culture were used in each treatment. Cells were harvested in a refrigerated continuous centrifuge (Sharples, Model T-1). The cells were protected from light exposure during harvesting.

Cells were resuspended in 50 mM Tricine/NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% bovine serum albumin (BSA) and 0.2% ascorbate and sonicated for 90s in a 50% duty cycle pulse mode. All operations were performed under dim light at 4 °C. The homogenate was centrifuged for 5 min at $3000 \times g$ in order to remove unbroken cells and large cell fragments. Thylakoid membrane vesicles were precipitated by differential centrifugations at $10000 \times g$ for 30 min ('10k' fraction), $40000 \times g$ for 30 min ('40k' fraction), and $140000 \times g$ for 60 min ('140k' fraction). The 10k and 40k fractions were washed once in hypotonic buffer to ensure complete removal of BSA and ascorbate. Control (unfractionated) membranes were obtained by direct centrifugation of the crude membrane suspension at $140000 \times g$ for 60 min.

For detergent fractionation, sonication power was lowered to produce relatively larger membrane fragments. Unbroken cells were sedimented upon centrifugation at $1000 \times g$ for 5 min. The membranes in the supernatant were sedimented at $10000 \times g$ for 30 min. The pellet was resuspended in hypotonic buffer to a final concentration of 300 μM Chl $(a+b)$ and 0.5% (w/v) digitonin. The concentration of $MgCl_2$ was increased to 10 mM during the incubation to ensure complete stacking of the membranes. The suspension was stirred on ice for 15 min in the dark and then the incubation was stopped by a 10-fold dilution with hypotonic buffer. The fractionated membranes were successively centrifuged to obtain 10k, 40k and 140k fractions as described above except that the 10k and 40k fractions were not washed.

The P-700 and Q_A content of thylakoid membrane suspensions was determined from measurements of the light-induced absorbance changes at 700 nm and 320 nm, respectively, as described in Ref. 18. The amplitude of the absorbance change at 320 nm was corrected for the effects of particle flattening and for any contribution of P-700 as described [18]. The photoreduction of the primary electron acceptor of PS II, Pheo, was measured using the absorbance change at 685 nm as previously described [13]. Since the ability of PS II to produce a stable charge separation between P-680 and Q_A is affected by detergent treatment [20], only the photoreduction of Pheo was used to measure the PS II content of membranes after digitonin treatment. A total of four or five preparations were assayed for each thylakoid membrane component determination. In order

to control for variations between cultures, all results were normalized to the corresponding quantitation in control thylakoids isolated from cells maintained at physiological growth irradiance. The results presented are given relative to the average control values over all determinations. Using this approach the standard error as a percentage of the mean was about $\pm 6\%$ for PS II content and $\pm 4\%$ for PS I content.

The kinetics of PS II photochemistry were determined from measurements of the fluorescence induction curve of thylakoid membranes in the presence of DCMU as previously described [13]. The analysis concentrated on the α/β phases of the area increase over the fluorescence induction curve. The fluorescence induction curves can also have a very slow rising phase ('γ' phase) with kinetics at least 5-times slower than the β phase [21]. The γ phase has a negligible contribution to the total area over short light exposures (i.e., 2 s under our conditions). The fluorescence induction of membranes in the presence of 2.5 mM FeCN was measured in order to determine the F_0 to F_{pl} variable component as previously described [10].

Total chlorophyll content (*a* and *b*) was measured in 80% acetone extracts using the equations of Ref. 22. The ratio of Chl *a* to Chl *b* was determined using a modification of the Arnon equations as previously described [23].

Results

Localization of photosystem complexes

Although thylakoids in *C. reinhardtii* are not organized into distinct grana stacks and stroma-exposed lamellae, they do contain regions in which bands of 3–5 thylakoids are tightly appressed as well as regions that are stroma exposed [24]. The appressed regions will be relatively resistant to mechanical breakage compared to the non-appressed regions. Therefore, the heavy 10k pellet should be enriched in the appressed membranes, and the light 140k pellet should be enriched in stroma-exposed membranes. Table I shows the properties of membrane fractions obtained by progressively stronger centrifugation of a sonicated cell homogenate. More than half of the material is recovered in the 10k fraction, whereas only about 14% appears in the 140k fraction. The Chl *a/b* ratio of 2.60 in the 10k fraction was slightly lower than the ratio of 2.74 in the starting material. The 140k fraction did have a significantly higher Chl *a/b* ratio of 3.10. This suggests that the 140k fraction is relatively depleted of LHCII complexes, which bind most of the Chl *b*. This was confirmed by direct measurements of PS I and PS II content of the membrane fractions using P-700, Q_A and Pheo quantitations. The overall stoichiometry of these complexes in the thylakoid membranes of *C. reinhardtii* is about 1.8/1.0, similar to what has been previously reported for medium light grown cultures [18]. In comparison, PS II was depleted to 0.72 PS II per PS I in the 140k fraction and enriched to 2.23 PS II per PS I in the 10k fraction. The low chlorophyll yield resulted in recovery of only about 7% of total PS II in the 140k fraction compared to the approx. 50% of PS II which are PS II_β centers (see below) and resident in the stroma-exposed lamellae [9]. The 40k fraction also showed depletion of Chl *b* (Chl $a/b = 2.85$) and a lower PS II/PS I ratio of 1.28 with respect to the starting homogenate. Overall, the 40k fraction had properties that were intermediate between the 10k and 140k fraction (Table I).

TABLE I

Content of chlorophylls a and b, P-700 and Q_A in thylakoids and in membrane fractions from C. reinhardtii

Membrane fractions were obtained by sonication of thylakoids followed by successive centrifugations at $10000 \times g$ ('10k'), $40000 \times g$ ('40k'), and $140000 \times g$ ('140k') as described in the 'Materials and Methods' section. Distribution of pigments is given for 1 μmol of starting Chl *a* and *b*.

	Thylakoids	10k	40k	140k	Total of fractions	% Recovery
Chl $a+b$ (μmol)	1.00	0.57	0.20	0.14	0.91	91
Chl a/b	2.74	2.60	2.85	3.10		
P-700 (nmol)	1.27	0.69	0.28	0.23	1.20	94
QA (nmol)	2.29	1.55	0.36	0.16	2.07	90
Chl/P-700	787	816	688	600		
Chl/QA	437	365	536	832		
QA/P-700	1.80	2.23	1.28	0.72		

The method of detergent fractionation was also used to prepare membrane vesicles that are selectively enriched in appressed and non-appressed regions [15,25]. The digitonin fractionation technique was applied to membrane vesicles obtained by mild sonication of *C. reinhardtii* thylakoids, followed by centrifugation at $10000 \times g$ for 30 min. The starting membrane vesicles had a Chl composition similar to that of the starting homogenate used in the previously described mechanical fractionation (Table II). The 10k fraction obtained after digitonin incubation had a PS II/PS I ratio of 2.5, slightly higher than that obtained by mechanical fractionation. Very little ($\leq 5\%$) of the Chl in the original suspension appeared in the 40k fraction after treatment with digitonin. Therefore, the 40k centrifugation was routinely omitted in the digitonin treatment, and the supernatant remaining from the 10k pellet was directly centrifuged at $140000 \times g$ for 60 min. The 140k fraction had a Chl *a/b* ratio of 3.28, similar to that obtained in the light vesicles after mechanical fractionation. This is consistent with the reported depletion of LHCII polypeptides from the light fraction of digitonin solubilized membranes [15,25].

One significant difference with the results of mechanical fractionation was that, after treatment with dig-

TABLE II

Content of chlorophylls a and b, P-700 and photoreducible Pheo in thylakoids and in membrane fractions from C. reinhardtii

Membrane fractions were obtained by digitonin solubilization of thylakoids followed by successive centrifugations at 10000 × *g* ('10k') and 140000 × *g* ('140k') as described in the 'Material and Methods' section. Distribution of pigments is given for 1 μmol of starting Chl *a* and *b*.

	Thyla-koids	10k	140k	Total of fractions	% Re-covery
Chl *a* + *b* (μmol)	1.00	0.45	0.16	0.61	61
Chl *a*/*b*	2.74	2.61	3.28		
P-700 (nmol)	1.27	0.47	0.25	0.72	58
Pheo (nmol)	2.29	1.20	0.18	1.38	60
Chl/P-700	787	939	620		
Chl/Pheo	437	374	894		
Pheo/P-700	1.82	2.51	0.69		

itonin, centrifugation at 140000 × *g* for 60 min was no longer sufficient to precipitate all of the chlorophyll-containing proteins. For this reason the per cent recovery of material was considerably less after digitonin treatment, averaging about 60% of total chlorophyll (Table II).

While the resolved difference in photosystem content between the two membrane regions is not as great as that reported for higher plant chloroplasts, the evidence clearly shows that PS II and PS I are localized in separate membrane regions in the green alga *C. reinhardtii*. Furthermore, the corresponding membrane fractions have similar composition when obtained either by mechanical or detergent methods.

Fluorescence induction kinetics

A bimodal distribution of the functional antenna size of PS II has been reported for thylakoid membranes from higher plants [26] and some species of green algae [11,18]. The relative content of the larger antenna PS II_α and smaller antenna PS II_β can be determined by analysis of the biphasic kinetics of the fluorescence induction curve in the presence of DCMU [26]. We undertook to test the hypothesis that the non-appressed membranes of *C. reinhardtii* contain PS II_β while the appressed membranes contain PS II_α, as has been reported for higher plant chloroplasts [8]. The Chl *a* fluorescence induction kinetics were determined in preparations obtained by mechanical fractionation. The fluorescence induction curve of the starting thylakoid suspension of *C. reinhardtii* displays sigmoidal kinetics and a significant amount of slow β component (Fig. 1A, trace 'Thy'). The 10k fluorescence kinetics (Fig. 1A, trace '10k') are faster than those of the starting thylakoids, suggesting a specific depletion of the slow β phase. In contrast, the non-appressed membrane fraction (Fig. 1A, trace '140k') shows a much smaller variable yield and the kinetics are markedly slower than either in thylakoids or the 10k fraction.

The fluorescence kinetics shown in the upper panel of Fig. 1 were analysed using semi-logarithmic plots of the area increase over the fluorescence induction curve. By taking the intercept of the slow phase with the ordinate at zero time, we determine that the proportion of PS II_β in the 10k fraction (Fig. 1B, '10k') was only 32% compared to 50% in the original thylakoids (Fig. 1B, 'Thy'). Only a single exponential component could be resolved from the 140k fraction (Fig. 1B, '140k') which had a slope similar to the slow phase of the control and 10k fractions. These observations show that the PS II units in light 140k fraction are almost entirely PS II_β centers. In contrast, the PS II_α centers were the dominant form in the heavy 10k fraction.

A second type of heterogeneity associated with PS II is the so-called reducing side heterogeneity. Through several approaches, it has been shown that the secondary quinone acceptor, Q_B, is not reduced by PS II_β centers [10,27], which have thus been termed Q_B-nonre-

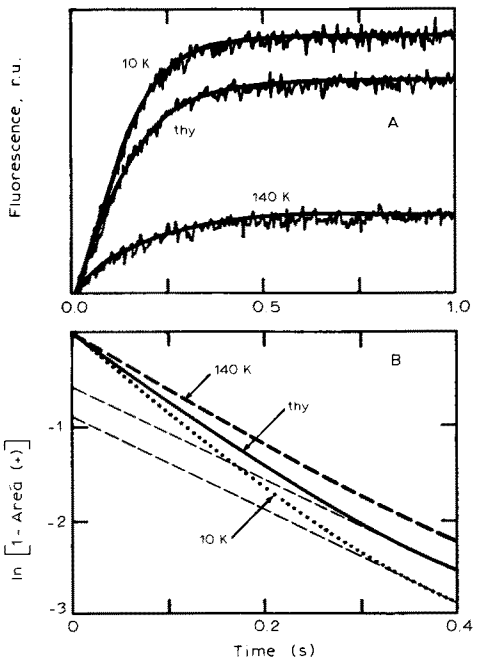

Fig. 1. Fluorescence induction curves displayed for different thylakoid membrane fractions obtained after sonication of *C. reinhardtii*. (A) Variable Chl *a* fluorescence of control thylakoids (curve 'Thy', Chl *a*/Chl *b* = 2.8), membranes precipitated at 10000 × *g*, which are enriched in appressed thylakoids (curve '10k', Chl *a*/Chl *b* = 2.6), and membranes requiring more than 40000 × *g* to be sedimented, which are enriched in non-appressed thylakoids (curve '140k', Chl *a*/Chl *b* = 3.3). (B) First-order kinetic analysis of fluorescence traces shown in (A). The relative PS II_β concentration determined by extrapolation of the slow phase slope to the zero time origin was 50% for total thylakoids (———), 32% for the appressed membrane fraction (·····), and 100% for the non-appressed membrane fraction (- - - - -). The reaction mixtures contained 20 μM Chl (*a* + *b*)/20 μM DCMU and an actinic intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of green light was used.

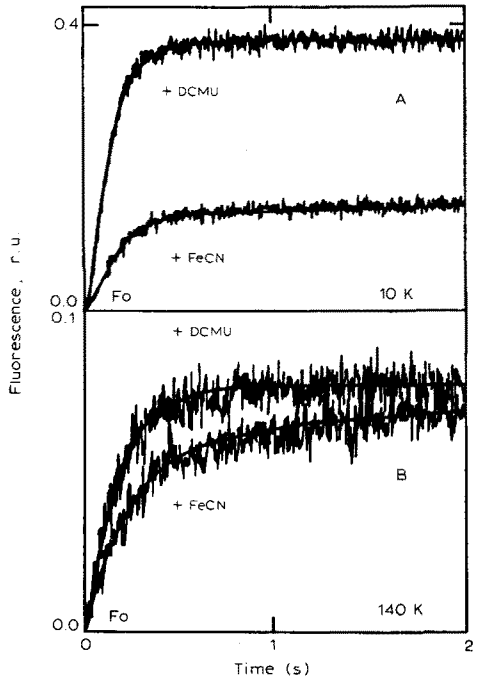

Fig. 2. Comparison of the total variable fluorescence yield in the presence of 20 μM DCMU (F_0 to F_m, '+DCMU') and the fluorescence yield in the presence of 2.5 mM FeCN (F_0 to F_{pl}, '+FeCN'). (A) Appressed membrane fraction (10k) and (B) non-appressed fraction (140k), measurement conditions as in Fig. 1.

ducing centers (cf. Ref. 28). In isolated thylakoids, the relative content of the Q_B-nonreducing centers can be determined by comparing the variable fluorescence yield in the presence of ferricyaninde with that in the presence of DCMU [10]. In the presence of ferricyanide, weak illumination will reduce Q_A in the Q_B-nonreducing centers only and the fluorescence yield will increase from a non-variable yield F_0 to an intermediate yield F_{pl}. In the presence of DCMU, all PS II centers will become reduced and the fluorescence yield will increase from F_0 to F_{max}. The F_0 to F_{pl} increase in the 10k fraction (Fig. 2A, '+FeCN') is small (33%) compared to the total variable fluorescence yield (F_v) obtained in the presence of DCMU (Fig. 2A, '+DCMU'). The slow kinetics of the F_0 to F_{pl} yield in 10k membranes suggest that this fluorescence is emitted by the residual portion of PS II_β centers present in this membrane fraction (cf. Fig. 1). In the 140k fraction, the F_0 to F_{pl} increase (Fig. 2B, '+FeCN') accounted for almost all of the variable fluorescence (Fig. 2B, '+DCMU'). In fact, the F_0 to F_{pl} amplitude in several 140k vesicle preparations was consistently 80% or more of the F_v. Taken together, the results on the α, β and PS II reducing side heterogeneity suggest that the Q_B-reducing PS II_α centers are selectively isolated in the heavy 10k fraction and the Q_B-nonreducing PS II_β centers are contained in the light 140k fraction.

The effect of strong-irradiance on the PS II content of non-appressed membranes

We have previously suggested that photoinhibition damage to PS II_α can be partially counteracted by a conversion of PS II_β, Q_B-nonreducing centers to a Q_B-reducing state [13]. The above results on fractionation of *C. reinhardtii* thylakoids indicate that these PS II_β probably originate from the non-appressed membrane region; however, previous studies indicate Q_B-reducing centers are localized exclusively in the appressed membrane regions [29]. This implied that PS II complexes may be depleted from the non-appressed membrane region in parallel with the activation of electron transport from Q_A to Q_B.

We sought to test this hypothesis by comparing the PS II content of membrane fractions isolated from cells before and after exposure to strong irradiance. Intact cells were exposed to a short (15 min) strong-irradiance treatment (SIT) of 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, which is sufficient to induce activation of Q_B-nonreducing centers with only moderate photoinhibition of Q_B-reducing

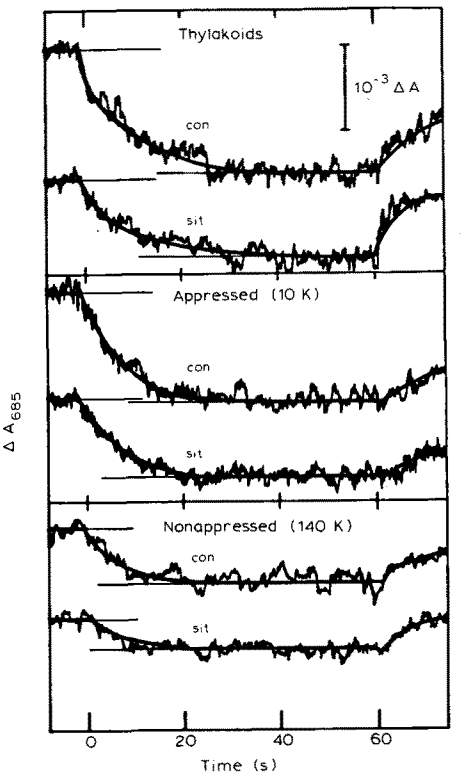

Fig. 3. Light-induced absorbance change at 685 nm (ΔA_{685}) attributed to reduction of the PS II primary electron acceptor Pheo for *C. reinhardtii* membrane fractions obtained by digitonin treatment of membranes isolated from cells maintained under normal growth irradiance (CON) or after a strong irradiance treatment (SIT) of 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 15 min. Measurement of the absorbance change was carried out as described in the 'Materials and Methods' section. Reaction mixture contained 10 μM ($a+b$) and the cuvette had 1.0 cm pathlength. Actinic light (800 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was turned 'ON' at time = 0 and 'OFF' at time = 60 s. Note the large decrease in the amplitude of the ΔA_{685} in the non-appressed thylakoids after SIT.

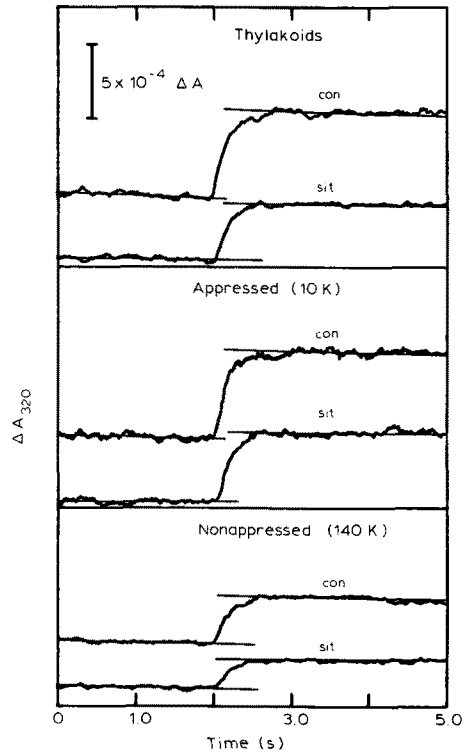

Fig. 4. Light-induced absorbance change at 320 nm (ΔA_{320}) due to reduction of the primary quinone acceptor, Q_A, of *C. reinhardtii* thylakoid fractions obtained after sonication of cells maintained under normal growth irradiance (CON) or after a strong-irradiance treatment (SIT) of 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 15 min. Measurement of the absorbance change was carried out as described in the 'Materials and Methods' section. Reaction mixture contained 100 μM Chl $a + b$ and the cuvette had a 0.1768 cm pathlength. Actinic light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) came 'ON' at time = 2 s. Note the large decrease in the amplitude of the ΔA_{320} in the non-appressed thylakoids after SIT.

centers. Following strong irradiance treatments, we found a similar decrease in the amplitude of the absorbance change of both Pheo (ΔA_{685}) and Q_A (ΔA_{320}) photoreduction, suggesting that about 30% of the initial content of photochemically active PS II were lost to photoinhibition (Fig. 3, upper panel, Fig. 4, upper panel). We also observed a lowering in the F_0 to F_{pl} yield of thylakoids in the presence of ferricyanide (data not shown, cf. Ref. 13). Fig. 3 (middle panel) shows a lowering of the amplitude of ΔA_{685} in the appressed (10k) membrane fraction after SIT, suggesting loss of the PS II primary charge separation between P-680 and Pheo due to photoinhibition in this membrane fraction. The 10k membranes contain the large antenna PS II_α which is preferentially damaged during SIT of *C. reinhardtii* [13]. Significantly, the amplitude of pheophytin photoreduction in the non-appressed (140k) fraction also decreased after exposure to strong irradiance (Fig. 3, lower panel).

Similar results were obtained when the appressed and non-appressed membrane regions were derived by mechanical fractionation and the number of photochemically active PS IIs was measured by photoreduction of Q_A (ΔA_{320}). Fig. 4 (middle panel) shows that a lower Q_A amplitude is exhibited by the 10k fraction after SIT. This suggests that the damage to the PS II_α in the appressed membrane region has equal effect on the PS II charge separation to Pheo and Q_A. Furthermore, the amplitude of Q_A photoreduction in the 140k fraction was lowered upon exposure to strong irradiance, in agreement with the relative decrease observed in the photoreduction of pheophytin (compare lower panels, Figs. 3 and 4). The latter results suggest that a depletion of photochemically active PS II has occurred in the non-appressed regions of *C. reinhardtii* thylakoids.

One possible explanation for the depletion of PS II in the 140k fraction is that PS II_β centers in stroma-exposed membranes are being damaged by strong irradiance. However, studies of photoinhibition with isolated thylakoids have consistently shown that PS II_β is resistant to photoinhibition damage, unlike PS II_α [19,30]. Moreover, using identical irradiance conditions as in this study, we have shown that total PS II_β content in thylakoids of strong-irradiance treated *C. reinhardtii* remained constant, even though PS II_α was damaged (Fig. 4 in Ref. 13). Thus, the significantly lower signal amplitudes for Pheo (Fig. 3) and Q_A (Fig. 4) photoreduction in non-appressed membranes of the 140k fraction are not likely to reflect damage of PS II_β during SIT. Rather, we suggest that a lower PS II_β content in the non-appressed membrane fraction might reflect translocation of PS II_β from the stroma-exposed vesicles to the appressed membranes regions.

We tested for processes other than translocation that may result in the apparent depletion of PS II_β from the stroma-exposed regions by exposing cells to strong-irradiance under conditions that would inhibit protein trafficking in the membrane. In this respect, we note that low temperature during SIT would lower the rate of enzymatic processes and would induce phase transitions in the lipid bilayer that decrease the fluidity of the thylakoid membrane [31]. We hypothesized that under such conditions PS II_β would not be translocated from the stroma-exposed membranes. This hypothesis was tested using strong-irradiance treatment of cell suspensions maintained at 0 °C in an ice-water bath (SIT-0 °C). Fig. 5 (upper and middle panel) shows that significant photoinhibition occurred during SIT-0 °C and photochemically active PS II was lowered both in thylakoids and in the appressed membrane fraction. At the same time, there was no drop in the F_0 to F_{pl} yield of thylakoids in the presence of ferricyanide (data not shown). Significantly, no change occurred in the PS II_β content of the non-appressed fraction (Fig. 5, lower panel). The latter is in sharp contrast to the results obtained in room temperature incubation (Figs. 3 and 4). This result is consistent with the interpretation that

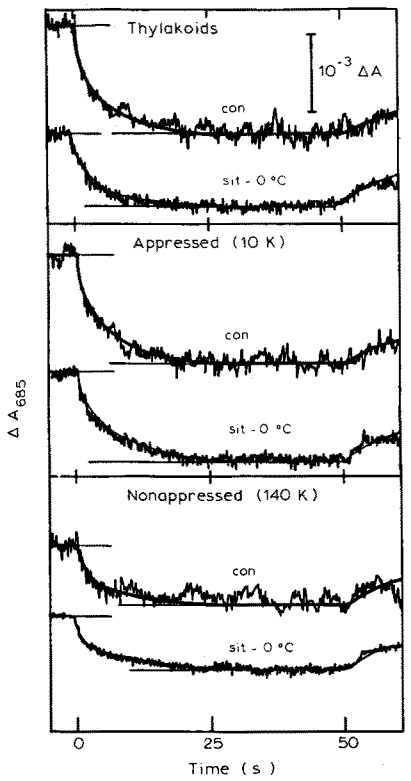

Fig. 5. Pheo photoreduction in thylakoid fractions as in Fig. 3 obtained after sonication as in Fig. 4, except strong irradiance treatments administered to cells maintained at ice temperatures (SIT-0 °C). Note that no change occurred in the amplitude of the non-appressed fraction, despite a large loss in amplitude in total thylakoids due to photoinhibition.

process(es) which result in depletion of PS II_β from the stroma-exposed membranes are inhibited at low temperature.

A summary of the properties of membrane fractions from strong-irradiance treated cells is given in Table III. Since fraction characteristics were independent of fractionation method (Tables I and II), results from digitonin and mechanical fractionations were combined. The average Chl/PS II ratio in the 140k fraction is twice as high in the SIT-treated cells when compared to control or SIT-0 °C samples. Stated as numbers of PS II per unit Chl, the PS II content of the 140k fraction fell from a mean (± S.E.) of 1.12 ± 0.08 mmol PS II [mol Chl $a+b]^{-1}$ in the control ($n=5$) to 0.60 ± 0.04 mmol PS II [mol Chl $a+b]^{-1}$ after SIT ($n=4$). However, when SIT treatment was conducted at 0 °C there was no significant decrease in the concentration of photochemically active PS II in the non-appressed (140k) membrane fraction (PS II = 1.30 ± 0.06 mmol [mol Chl $a+b]^{-1}$ $n=3$). In either case (room temperature or 0 °C) there was little difference in the Chl distribution and relative content of Chl *a* and *b* between the corresponding membrane fractions of control and SIT-treated material. This suggested that depletion of PS II from the non-appressed membranes during SIT is not simply the result of any changes in the bulk properties of thylakoid membranes exposed to strong irradiance. Similarly, strong irradiance under physiological temperatures resulted in only a slight decrease in the P-700 content of the unfractionated membranes, and the P-700 content of the 140k fraction was unaffected. Strong-irradiance treatment on ice led to a more significant loss of P-700 activity, the cause of which is unknown (Table III).

TABLE III

PS I (P-700) and PS II (Q_A or Pheo) in thylakoids and in membrane fractions from C. reinhardtii comparing control, strong-irradiance treated (SIT) and strong-irradiance treated at 0 °C (SIT-0 °C)

Membrane fractions were obtained by sonication or digitonin solubilization of thylakoids followed by successive centrifugatios at 10000 × *g* ('10k'), 40000 × *g* ('40k'), and 140000 × *g* ('140k') as described in the 'Material and Methods' section. Units: mmol of complex per mol Chl (*a* + *b*).

		Thylakoids	10k	40k	140k
PS II	control	2.29	2.75	1.86	1.12
(Q or Pheo)	SIT	1.58	1.64	1.26	0.60
	SIT 0 °C	1.68	1.89	1.27	1.30
P-700	control	1.27	1.15	1.45	1.63
	SIT	1.16	1.00	1.39	1.66
	SIT 0 °C	1.00	0.93	1.55	1.30

Discussion

The results of this study show that non-appressed membranes of *C. reinhardtii* contain PS II units which occur in the configuration of PS II_β. Furthermore, the PS II_β of the non-appressed membranes in *C. reinhardtii* are unable to transfer electrons from Q_A to Q_B (Fig. 2), a property similar to that of PS II_β in the stroma-exposed membranes of higher plant chloroplasts [10]. The results presented here also show that the lateral heterogeneity of PS II is dynamic. Strong-irradiance exposure leads to significant depletion of PS II_β from the non-appressed membrane region and this phenomenon occurs in concert with an 'activation' of Q_B-nonreducing centers to a Q_B-reducing form. Furthermore, our results indicated that Q_B-nonreducing centers can only be activated under physiological conditions [33]. In consequence, there was no evidence of depletion of PS II_β from the non-appressed membranes and the numbers of Q_B-nonreducing centers remained constant when strong-irradiance treatment occurred at 0 °C.

Despite the depletion of the PS II_β from non-appressed membranes under physiological conditions, it is notable that no major changes occurred in the bulk Chl properties of the membrane fractions obtained by the fractionation techniques used here. This suggests that the overall number of Chl-protein complexes occurring in the appressed and non-appressed membranes re-

mains relatively constant. One possible explanation for this observation is that damaged PS II units from the grana are exchanged with 'newly activated' PS II_β centers from the stroma-exposed membranes. Since damaged PS II units are photochemically inert, they can not be detected by measurements of light-induced absorbance change. However, they contribute to the Chl content of the stroma exposed membranes. Thus, under physiological conditions, the non-appressed region may constitute a 'holding area' for reserve PS II centers. However, under irradiance stress, the stroma-exposed region may be converted to a holding area for a pool of damaged PS II centers undergoing protein turnover and renewal.

The characteristics of the photochemically inert PS II centers will depend on the primary site of damage during photoinhibition. In this regard, it is important to note that photoinhibition of intact cells of *C. reinhardtii* resulted in the same proportion of lowering of the Q_A and Pheo absorbance change amplitudes (Figs. 3 and 4). This suggests that photoinhibition caused a loss of the $P\text{-}680^+$ $Pheo^-$ primary charge separation, consistent with previous results [13,32]. We emphasize that such results are obtained when intact cells are exposed to strong-irradiance under physiological conditions. The primary-charge separation may not be the only target of damage under in vitro experimental conditions, for example in detergent isolated PS II particles exposed to strong irradiance at room temperature [34–36].

The results strengthen the basic concept of PS II_β being normally resident in the non-appressed membranes and occurring in the Q_B-nonreducing form [10]. However, they also suggest a dynamic relationship with PS II_α in the appressed membrane regions such that, under irradiance stress conditions, PS II_β are activated to a Q_B-reducing form and translocated from the non-appressed to the appressed membrane regions. Further support that such a physiological mechanism accounts for the depletion of PS II_β from the non-appressed membranes during activation is the fact that low temperature inhibited both activation and changes in the PS II content of the 140k fraction. The activation of PS II from the non-appressed membrane region apparently requires operation of processes, for example enzymatic activity and/or movement of the complex in the lipid bilayer [31], which occur only at physiological temperatures. In summary, our results indicate that the PS II_β may be integrated into the appressed-membrane region in the process of being activated into the Q_B-reducing state.

While in theory the activation should then result in an overall increase in the PS II_β content of the appressed-membrane fraction, the 10k fractions prepared by the methods used here are not sufficiently free of non-appressed components (i.e., Tables I and II) in order to resolve such a shift. Further experiments are underway to examine incorporation of PS II_β into the appressed membrane region as well as other possible mechanisms that may account for the depletion of PS II_β from the stroma-exposed membranes.

The localization of different PS II complexes into appressed and non-appressed membrane regions is a characteristic of all higher plant chloroplasts [8,9]. The present work shows that such differential localization also occurs in *C. reinhardtii*, confirming previous evidence based on immunocytochemistry [7] and polypeptide composition [15,25]. Thus, it appears that localization of different PS II centers in separate membrane regions may be typical of all Chl *b* containing eukaryotic algae and higher plants.

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