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Membrane protein damage and repair: Selective loss of a quinone–protein function in chloroplast membranes

(photoinhibition/*Chlamydomonas*/herbicide-binding protein/photosynthesis/triazine herbicide)

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ABSTRACT **A loss of electron transport capacity in chloroplast membranes was induced by high-light intensities (photoinhibition). The primary site of inhibition was at the reducing side of photosystem II (PSII) with little damage to the oxidizing side or to the reaction center core of PSII. Addition of herbicides (atrazine or diuron) partially protected the membrane from photoinhibition; these compounds displace the bound plastoquinone (designated as Q_B), which functions as the secondary electron acceptor on the reducing side of PSII. Loss of function of the 32-kilodalton Q_B apoprotein was demonstrated by a loss of binding sites for [^{14}C]atrazine. We suggest that quinone anions, which may interact with molecular oxygen to produce an oxygen radical, selectively damage the apoprotein of the secondary acceptor of PSII, thus rendering it inactive and thereby blocking photosynthetic electron flow under conditions of high photon flux densities.**

Exposure of leaves or chloroplasts of higher plants to supraoptimal light intensities results in a loss of photosynthetic activity (1). This phenomenon is referred to as photoinhibition (reviewed in refs. 2 and 3). Photoinhibitory damage to chloroplast membranes (thylakoids) involves inactivation of photosystem II (PSII), but the molecular mechanism damage has not been established.

A thylakoid polypeptide of ≈32 kDa is synthesized at rates equal to or greater than the most abundant chloroplast proteins, yet it remains as a minor membrane constituent; this has been attributed to a rapid turnover of the polypeptide (4, 5). The rate of turnover was recently found to increase in plants grown at increasing light intensities (4). The rapidly turned-over 32-kDa polypeptide is identical to a triazine receptor protein (6) that is a structural component of the PSII core complex. Since atrazine (a commonly used triazine herbicide) is a competitive inhibitor of the binding of plastoquinone (PQ) analogues (7), the rapidly turned-over, herbicide-binding protein is identified as the apoprotein of the secondary electron transport carrier on the reducing side of PSII. The redox cofactor associated with this carrier is a bound PQ designated as Q_B; the 32-kDa polypeptide is therefore called the Q_B protein (8).

In this manuscript we demonstrate a correlation between photoinhibition (PSII inactivation) and the rate of turnover of the Q_B protein.

MATERIALS AND METHODS

Cell Growth and Photoinhibition. Cells of *Chlamydomonas reinhardtii* (var. y_{-1}) and an atrazine-resistant mutant [Ar4(+); kindly provided by L. Mets, University of Chicago] were grown on a medium containing acetate as a carbon source (9) at 25°C under continuous illumination at 350 microeinsteins per m^2/sec. The cells were then harvested and resuspended to a concentration of about 50 μg of chlorophyll (Chl) per ml in the growth medium in clear tubes (3 cm in diameter) and immersed in a temperature-regulated, transparent water bath for photoinhibition experiments. A high-intensity quartz–halogen lamp provided a photoinhibitory light intensity of 3500 microeinsteins per m^2/sec, during which the cells were stirred vigorously to ensure a uniform illumination.

Photosynthetic Membrane Preparation, Fluorescence, and Photochemical Assays. Following photoinhibition treatments, cells were washed and resuspended in a 30 mM Tricine/NaOH (pH 7.8) buffer containing 30 mM KCl and 2 mM $MgCl_2$ to a concentration of 250 μg of Chl per ml before sonic disruption. The cell homogenate was centrifuged at 2000 × *g* for 2 min to remove unbroken cells or large debris. The supernatants were used for subsequent measurements.

Chl fluorescence induction transients were monitored at room temperature (10). Photochemical reduction of dichlorophenol-indophenol (DCPIP) using either water (H_2O to DCPIP) or 1.0 μM diphenylcarbazide (DPC to DCPIP) as electron donors was measured spectrophotometrically (10). Photoreduction of the diaminodurene/ferricyanide couple using water (H_2O to DAD/FeCN) as the electron donor was measured polarographically as oxygen evolution. Photosystem I (PSI) activity was measured polarographically as oxygen uptake in a reaction using 0.5 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine reduced with 2.5 mM ascorbate as electron donor and methyl viologen (TMPD to methyl viologen) as acceptor. Photoreduction of silicomolybdate ($SiO_2 \cdot 12MoO_3$) with water (H_2O to $SiO_2 \cdot 12MoO_3$) or 1 μM DPC (DPC to $SiO_2 \cdot 12MoO_3$) as electron donors was measured spectrophotometrically according to Barr *et al.* (11).

Protein Analysis and Measurement of Herbicide Binding. Herbicide binding assays using [^{14}C]atrazine (13.4 mCi/mmol; 1 Ci = 37 GBq; kindly provided by Homer LeBaron, CIBA-Geigy) were carried out as described (10).

Azido[^{14}C]atrazine (50 mCi/mmol) was added to *Chlamydomonas* thylakoids (50 μg of Chl per ml) and incubated at 5°C under UV light for 30 min as described by Vermaas *et al.* (7). Thylakoids were then pelleted and solubilized in 0.2% lithium dodecyl sulfate ($LiDodSO_4$) as described in ref. 12 (60°C for 1 min), and electrophoresis was carried out by using the buffer system of Laemmli (13) with the inclusion of 4 M urea and on 8–12% acrylamide gradient.

RESULTS

Characterization of Photoinhibition. The effect of high photon flux densities (10× the intensity at which the cells were grown) on *Chlamydomonas* was monitored by using *in*

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Abbreviations: PSI and PSII, photosystems I and II; PQ, plastoquinone; Chl, chlorophyll(s); DCPIP, dichlorophenol-indophenol; DPC, diphenylcarbazide; DAD, diaminodurene; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron); $Pheo^-$, reduced pheophytin; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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vivo room temperature Chl fluorescence inductions in the presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea [diuron (DCMU)]. Variable fluorescence (F_v/F_0) decreased rapidly ($t_{1/2} \cong 10$ min) (Fig. 1*a*) due to two factors. First, a small but significant increase in initial fluorescence occurred in the first period of treatment; this was followed by a decline to the original F_0 level. Second, and more importantly, there was a time-dependent decrease in the maximal fluorescence (F_m). Parallel with the loss of F_v was a loss of PSII activity measured in isolated thylakoids (Fig. 1*b*).

The herbicide atrazine competitively displaces the quinone cofactor of the secondary electron acceptor of PSII and thereby prevents transfer of an electron from the primary to secondary quinone electron carriers of PSII (7, 14). Atrazine (5 μM), added during the photoinhibitory treatment, partially prevented the inactivation of PSII, as measured by both F_v and a PSII partial reaction (Fig. 1). On the other hand, dibromothymoquinone (DBMIB) inhibits the oxidation of the plastohydroquinone pool (15) and enhanced the rate of loss of both the F_v and PSII activity that occurred during incubation under photoinhibitory conditions (Fig. 1).

Electron transport in an atrazine-resistant mutant of *Chlamydomonas* [Ar4(+)] was inhibited by only 10–15% in the presence of 10 μM atrazine (data not shown). This mutant exhibited a greater degree of photoinhibitory damage than the atrazine-sensitive strain in the presence of the herbicide (Fig. 1*a*), indicating that the ability of atrazine to protect from photoinhibition damage is dependent upon its ability to bind to the Q_B protein (7).

Localization of Photoinhibition Damage to the Reducing Side of PSII. Partial reactions of the photosynthetic electron transport chain were utilized to define the primary cause of

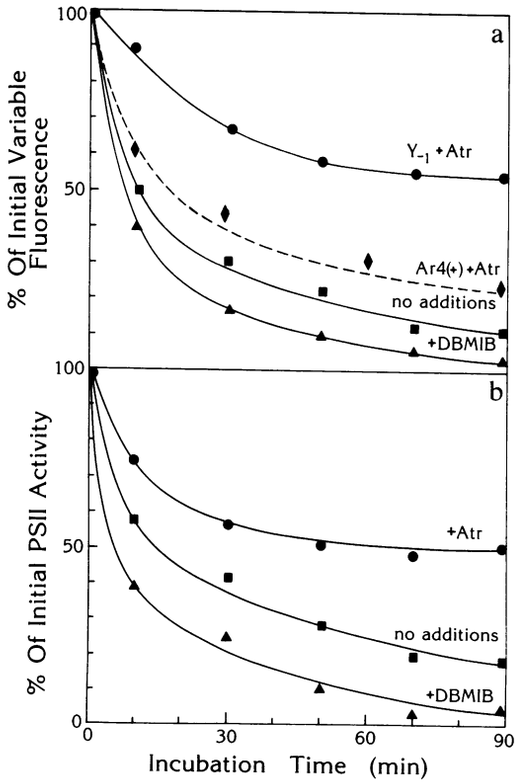

FIG. 1. Effect of addition of certain inhibitors on the loss of fluorescence (*a*) and PSII activity (*b*) from *Chlamydomonas* as a function of time under photoinhibitory conditions in the presence of 5 μM atrazine (Atr; ●) or 1 μM DBMIB (▲) or in the absence of either inhibitor (■). Initial variable fluorescence (F_v/F_0) = 2.0; initial PSII activity (H_2O to DPIP) = 150 μequiv per mg of Chl per hr. The loss of F_v is shown for the atrazine-resistant strain Ar4(+) in the presence of 10 μM atrazine (◆).

Table 1. Effect of a 90-min high-light incubation of *Chlamydomonas* cells (photoinhibition) on photosynthetic electron transport partial reactions

Partial reaction	Reaction rate, μequiv per mg of Chl per hr		Ratio
	Control	Photoinhibited	
1. H_2O to DCPIP	240	48	0.20
2. DPC to DCPIP	250	62	0.25
3. H_2O to DAD	376	72	0.19
4. TMPD to methyl viologen	300	316	1.05
5. H_2O to SiO_2·12MoO_3	168	135	0.80
6. DPC to SiO_2·12MoO_3	240	228	0.95

Reactions 1 and 2 require both PSII and PSI; reaction 3 requires PSII only; reaction 4 requires PSI only; reactions 5 and 6 are PSII reactions that do not require the participation of the Q_B protein (11). Reactions 1, 2, 5, and 6 were carried out in 100 mM potassium phosphate buffer (pH 6.8) and reactions 3 and 4 were carried out in 25 mM Tricine (pH 7.8). DCPIP = 30 μM; DPC = 12.5 mM; DAD = 1.2 mM (oxidized with 2mM FeCN); TMPD = 0.5 mM (reduced with 2.5 mM ascorbate); methyl viologen = 0.1 mM; SiO_2·12MoO_3 = 100 μg/ml. All reactions were uncoupled with 0.1 μM gramicidin and also contained 0.1 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. Ratio = photoinhibited/control ratio.

PSII inactivation that occurred during a 90-min exposure of *Chlamydomonas* to high light. The interpretation of the results in Table 1 confirms previous reports that the principal effect of photoinhibition is near PSII with little or no effect on PSI (1–3). Furthermore, by using assays specific to the PSII reaction center and not involving the secondary quinone electron acceptor Q_B (SiO_2·12MoO_3 reduction), it was apparent that little damage had occurred to either the water-splitting enzymes or the primary reaction center.

The rate of PSII turnover can be estimated through the use of Chl fluorescence induction transients; the time required to increase fluorescence to a maximum in a sample of isolated thylakoids is inversely proportional to the number of PSII turnovers that occurs and thereby causes the reduction of the electron acceptor pool. Following 90 min of photoinhibition, the longer half-rise time for F_v from either isolated membranes or whole cells (Table 2) indicates a decreased rate of reduction of the PQ pool in the photoinhibited sample. The rate of reduction of the primary stable acceptor of PSII can be estimated from the fluorescence rise time of samples in the presence of 5 μM DCMU. This rate increased following photodamage, indicating that photoinhibition results in an all-or-none loss of F_v from photoinhibited PSII reaction centers and that the antennae pigments of the inactive centers now serve the remaining functional PSII traps. The addition of hydroxylamine or dithionite (or both) did not restore the room temperature F_v component (data not shown).

Table 2. Fluorescence half-rise times measured with isolated *Chlamydomonas* thylakoids in the presence or absence of 0.01 mM DCMU (diuron)

Cells	Fluorescence half-rise time, msec	
	With DCMU (P_{680} to Q)	Without DCMU (P_{680} to PQ pool)
Control	47	891
Photoinhibition	28	2030
+ Atrazine	41	1370

Values are shown for nonphotoinhibited cells (control) and cells subjected to a photoinhibitory treatment for 90 min in the presence or absence of 5 μM atrazine.

EPR has been used to demonstrate that an electron carrier that serves as a donor to the PSII reaction center can be detected via characteristic light-induced electron spin signals (16). These are designated EPR signal II_f (measured in Tris-washed membranes), signal II_{vf} (untreated membranes), or signal II_s (measured in the dark) (defined in ref. 16). No significant differences in signal II_f or II_{vf} relative to signal II_s were detected when comparing thylakoids obtained from control or photoinhibited *Chlamydomonas* cells (Fig. 2). We interpret these EPR data to indicate that the functional integrity of the donor side of PSII is not initially affected by the high-light treatment.

Localization of Damage to the Q_B Protein. Since atrazine has been shown to be a competitive inhibitor of quinone binding at the Q_B protein (7), we have used this herbicide to probe the functional integrity of this binding site. A change from 650 to 2000 Chl per binding site reflects a loss of over two-thirds of the herbicide-binding sites (as a consequence of photoinhibition) (Fig. 3). This number correlates well with the PSII activity lost during photoinhibition when DPC was used as an electron donor (DPC to DCPIP; Table 1). No change was observed in the binding affinity of the remaining sites following photoinhibition.

Chloroplast thylakoids were isolated from cells exposed to 0, 30, or 90 min of high-light exposure, tagged with azido-[^{14}C]atrazine and subjected to polyacrylamide gel electrophoresis. Autoradiograms of the samples (Fig. 4) revealed that the herbicide was specifically associated with a diffuse, poorly staining polypeptide of ≈32 kDa in control membranes. The same polypeptide was labeled to lesser extents in the samples from cells exposed to high light for increasing time periods. These data confirm the measurements of Fig. 3 and further demonstrate that the loss of herbicide-binding sites correlates with the loss of the Q_B protein function.

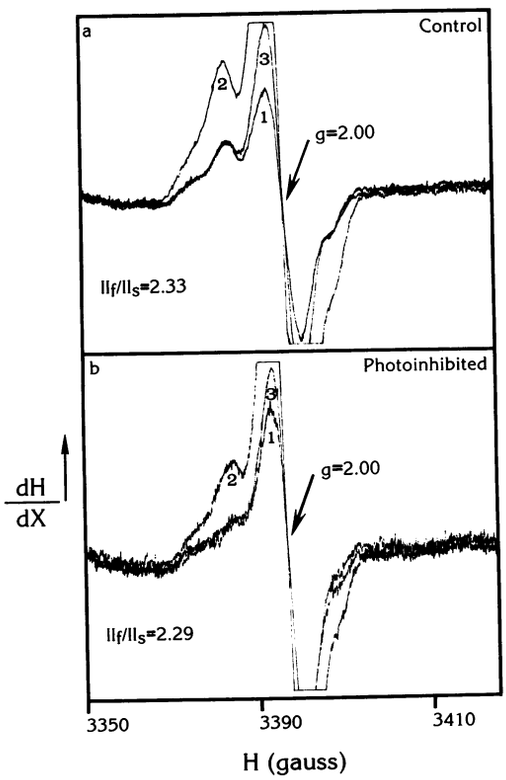

FIG. 2. EPR spectra of Tris-washed thylakoids prepared from photoinhibited cells (*b*) and noninhibited cells (*a*). Spectra are shown for the samples prior to illumination (peak 1), during illumination (peak 2), and following illumination (peak 3). The Tris washing procedure was identical to that given in ref. 16.

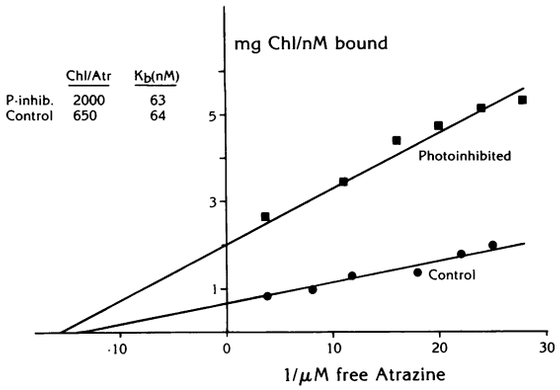

FIG. 3. [^{14}C]Atrazine (Atr) binding to thylakoids isolated from photoinhibited cells (■) and nondamaged cells (●). The ordinate and abscissa intercept values (computer calculated for best fit of data) corresponding to the binding constant (k_b) and the number of Chl per herbicide site (Chl/Atr) are shown (upper left).

DISCUSSION

Loss of Q_B Function in Photoinhibited Membranes. In previous studies of photoinhibition in higher plants it was not possible to identify the primary biochemical lesion in PSII. This may be related partially to the thickness of higher plant leaves and the fact that light levels are gradually attenuated across the leaf. The surface exposed chloroplasts probably begin to show secondary effects of photoinhibition while chloroplasts at lower cell levels were only at an early stage of inactivation. This experimental problem is overcome in the present study through the use of the unicellular alga *Chlamydomonas* in which there is a single chloroplast per cell. Short times of high-light exposure of these cells (in rapidly stirred suspensions to avoid self-shading) were found to result in a highly specific loss of electron transport activity of the bound PQ serving on the reducing side of PSII (Q_B). [It was noted that longer exposure times (>90 min) resulted in a more general secondary loss of PSII water oxidizing and reaction center capacity (data not presented).] The specific early loss of Q_B function is supported by several lines of evi-

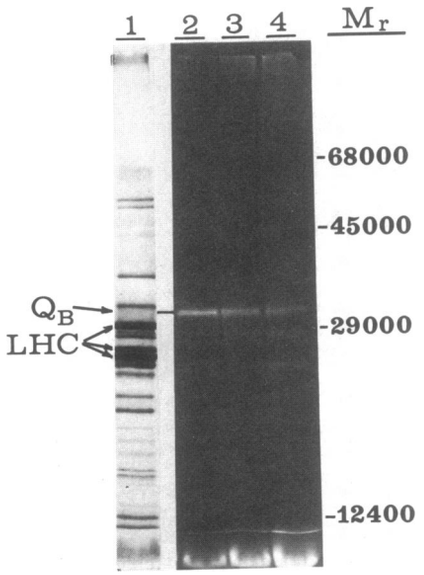

FIG. 4. Polyacrylamide gel electrophoresis of *Chlamydomonas* thylakoids in the presence of $LiDodSO_4$ and 4 M urea. Lane 1, Coomassie blue-stained gel; lanes 2–4, autoradiograms of azido[^{14}C]-atrazine-tagged thylakoids isolated from cells prior to photoinhibition (lane 2) or following 30 min (lane 3) or 90 min of incubation in high light (lane 4). LHC, light harvesting chlorophyll–protein complex.

dence. (*i*) Partial reactions of PSII (SiO_2·8$12MoO_3$ reduction) that do not require Q_B function (11) are not inhibited at photoinhibition times when PSII-mediated reduction of DAD or other electron acceptors after the PQ pool is drastically reduced (Table 1). (*ii*) PSII reaction center activity, as assayed by the amplitude of photoinduced EPR signal II, was unaffected when linear electron flow was blocked (Fig. 2). (*iii*) Herbicide-binding sites, known to be determined by the Q_B protein (6–8), were lost in concert with inactivation of PSII (Figs. 3 and 4). It seems likely that the loss of Q_B protein function may also be a primary site of PSII inactivation in higher plant leaves suffering photoinhibition but that this effect is masked by the presence of heterogeneous chloroplasts in the leaves (displaying various levels of primary and secondary damage when isolated).

Explanations for Fluorescence Quenching in Photoinhibited Membranes. An inhibition of electron transport on the reducing side of PSII, such as that caused by addition of diuron or a similar herbicide, causes an increase in Chl fluorescence to a maximal invariant level. The fact that we propose that electron transfer properties of Q_B are lost in parallel to a reduction of the F_m levels (Fig. 1) in photoinhibited *Chlamydomonas* may therefore appear contradictory. The following ideas are presented to demonstrate that this is not necessarily the case.

Several factors determine the fluorescence yield of chloroplasts, including the rate constants for photochemistry (k_P), fluorescence (k_F), and nonradiative deactivation (k_H) of the PSII excited state Chl. It is possible that a rapid, efficient cyclic electron transfer around PSII could result when the Q_B protein function is lost. In this way futile electron cycling would generate a permanently open trap for excitons, resulting in a fluorescence quenching. Two arguments can be used to negate this idea. First, the half-rise time for fluorescence inductions in diuron-poisoned membranes was markedly faster in photoinhibited membranes (Table 2). This means that the pigments associated with inactive PSII centers were serving the remaining active centers of photoinhibited membranes; this could not occur if inactive centers were "open traps." The second argument is more intuitive; it seems unlikely that secondary damage to inactivate PSII centers would ensue if the traps were "protected" via an electron cycle. We did observe a loss of PSII reaction center activity in *Chlamydomonas* after 90 min of exposure to high light—i.e., as a secondary effect of photoinhibition.

There is now increasing evidence that F_v from PSII arises via luminescence at the PSII trap following charge recombination between reduced pheophytin ($Pheo^-$, primary electron acceptor for PSII) and the oxidized reaction center (P_{680}^+) (17, 18). If PSII particles or chloroplasts are illuminated in the presence of a low-potential electron donor, such as dithionite, a time-dependent quenching of fluorescence occurs (17, 19). This corresponds to the trapping of PSII in the state [D·P_{680}·$Pheo^-Q^-$], in which D and Q are electron donors and acceptors, respectively. When thylakoids were isolated from photoinhibited *Chlamydomonas*, dark-adapted, and analyzed via fluorescence transients, there was no fluorescence increase followed by a decay as when $Pheo^-$ accumulates in control membranes (data not presented). We interpret this to mean either (*i*) $Pheo^-$ accumulation does not occur in photoinhibited membranes (and therefore this mechanism of quenching is not relevant), (*ii*) the accumulation of the $Pheo^-$ state is irreversible (see discussion in ref. 19), or (*iii*) a component of the membrane becomes a very efficient electron donor to P_{680}^+. In the latter case, $Pheo^-$ could accumulate immediately upon onset of sample illumination. Since the $Pheo^-$ P_{680}^+ back reaction is estimated to be 2–4 nsec (20), this would require an extremely efficient alternate electron donor for P_{680}^+ to be working in photoinhibited membranes. Cytochrome b_{559} is known to be an electron donor to the PSII trap and is in a membrane domain where it is influenced by the Q_B protein (21). However, it seems very speculative to suggest that the alteration of Q_B protein function observed in photoinhibited cells could so alter cytochrome b_{559} as to make it a membrane component that can reduce P_{680}^+ within 2–4 nsec. In short, rapid $Pheo^-$ accumulation in photoinhibited membranes is not likely and does not explain the quenching.

Quenching can also result in a fluorescing sample from either a decrease in k_F or an increase in k_H, or both. We possess no means of measuring these terms for photoinhibited membranes. These values are influenced by the environment of the emitting chromophore, and, as will be discussed immediately below, the Q_B protein is physically altered or removed (or both) from photoinhibited membranes. This would result in a change in the PSII core complex, of which the 32-kDa Q_B protein is a constituent. Therefore, we are left with the most likely explanation for a fluorescence quenching in photoinhibited membranes being that the microenvironment near the PSII trap is structurally altered, thus changing the k_F or k_H of the excited state PSII Chl that are responsible for F_v.

Mechanism of Q_B Protein Inactivation. Photoinhibition was found to proceed more rapidly in *Chlamydomonas* cells in which the oxidation of the PQ pool was prevented (DBMIB; Fig. 1). An inhibitor of PQ pool reduction (atrazine) partially prevented photoinhibition (Fig. 1). We conclude that the extent of reduction of the PQ pool is the factor that determines the rate of loss of Q_B protein function. An understanding of the reactions of PSII quinone proteins leads to an explanation of why this occurs.

Two PQ molecules designated Q_A and Q_B receive electrons sequentially from the PSII reaction center. Q_A is a one-electron acceptor that is reduced only to the semiquinone anion (Q_A) by an electron transfer from P_{680} (14, 20). Q_B is a two-electron-accepting PQ species that is reduced in two sequential reactions by Q_A; the first electron transfer results in formation of a very stable quinone anion Q_B^-. The anionic nature of the semiquinone and its stability imply that Q_B is formed in an aprotic, hydrophobic environment (20) at a unique quinone-binding site within the Q_B protein (7, 8). Reduction of Q_B^- to Q_B^{2-} is followed by dissociation of the quinone from its binding site; upon protonation it enters the PQ pool and shuttles electrons to PSI. An oxidized quinone from the PQ pool then binds to the vacated quinone-binding site and electron transfers continue.

The Q_B-binding site must involve amino acids that participate in stabilizing the Q_B anion. In mutant chloroplasts in which a single serine residue of the Q_B protein was replaced by a glycine, the Q_A^-·$Q_B \rightleftarrows Q_A$·Q_B^- equilibrium constant was shifted to the left; we hypothesized that the serine may function in hydrogen bonding to stabilize the anion Q_B^- (8). Although it is biochemically useful to stabilize the Q_B^- anion to allow time for the arrival of the second electron from P_{680}, it is definitely not of use for the chloroplast to stabilize the doubly reduced quinone within the Q_B protein (since the reduced electron carrier must diffuse away from PSII to deliver electrons to PSI).

When the PQ pool of the photosynthetic electron transport chain becomes fully reduced, the binding site of the Q_B protein cannot be filled by an oxidized quinone. It is under these conditions that we have observed the rapid onset of photoinhibition. We hypothesize that this occurs because (*i*) a fully reduced quinone can rebind to the Q_B site (possibly deprotonating in the process) and (*ii*) the presence of the relatively strong reductant (the unstabilized Q_B^{2-}) results in a reaction that directly and covalently modifies the Q_B protein, rendering it nonfunctional. We speculate that this inactivation may involve oxygen since O_2 has been shown to be required for certain photoinhibitory processes (3). Diffusion of oxygen

(which is produced in the PSII-mediated electron transport and is thus in a high local concentration) to the Q_B-binding site, and reaction of O_2 with Q_B^{2-}, would result in production of an oxygen radical within the Q_B protein.

We will demonstrate elsewhere that the 32-kDa Q_B protein disappears from photoinhibited membranes (consistent with its proposed chemical modification via Q_B^{2-} or an oxygen radical generated by reaction with the quinone) and that recovery from photoinhibition occurs when there is *de novo* chloroplast-directed synthesis of "replacement" Q_B protein (unpublished data). It therefore appears that photoinhibition is a deleterious process that occurs as a natural consequence of the function of the Q_B protein in electron transfer whenever excess light is absorbed causing over-reduction of the PQ pool. Nature has developed an acclimation strategy to compensate for this damage, which simply entails an efficient system for turnover (removal and replacement) of the damaged electron carrier.

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