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Double Reduction of Plastoquinone to Plastoquinol in Photosystem 1

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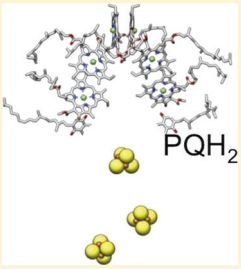
Supporting Information

ABSTRACT: In Photosystem 1 (PS1), phylloquinone (PhQ) acts as a secondary electron acceptor from chlorophyll ec_3 and also as an electron donor to the iron−sulfur cluster F_X. PS1 possesses two virtually equivalent branches of electron transfer (ET) cofactors from P_{700} to F_X, and the lifetime of the semiquinone intermediate displays biphasic kinetics, reflecting ET along the two different branches. PhQ in PS1 serves only as an intermediate in ET and is not normally fully reduced to the quinol form. This is in contrast to PS2, in which plastoquinone (PQ) is doubly reduced to plastoquinol (PQH_2) as the terminal electron acceptor. We purified PS1 particles from the *menD1* mutant of *Chlamydomonas reinhardtii* that cannot synthesize PhQ, resulting in replacement of PhQ by PQ in the quinone-binding pocket. The magnitude of the stable flash-induced P_{700}^+ signal of *menD1* PS1, but not wild-type PS1, decreased during a train of laser flashes, as it was replaced by a ∼30 ns back-reaction from the preceding radical pair ($P_{700}^+A_0^-$). We show that this process of photoinactivation is due to double reduction of PQ in the *menD1* PS1 and have characterized the process. It is accelerated at lower pH, consistent with a rate-limiting protonation step. Moreover, a point mutation (PsaA-L722T) in the PhQ_A site that accelerates ET to F_X ∼2-fold, likely by weakening the sole H-bond to PhQ_A, also accelerates the photoinactivation process. The addition of exogenous PhQ can restore activity to photoinactivated PS1 and confer resistance to further photoinactivation. This process also occurs with PS1 purified from the *menB* PhQ biosynthesis mutant of *Synechocystis* PCC 6803, demonstrating that it is a general phenomenon in both prokaryotic and eukaryotic PS1.



Oxygenic photosynthesis employs two chlorophyll-binding, multisubunit photosystems, PS1 and PS2, to absorb sunlight and convert the energy into charge-separated states, ultimately producing chemical energy in the form of NAPDH and ATP, which are used to drive carbon fixation and other endothermic reactions. Both photosystems funnel excitation energy from a relatively large network of chlorophylls (and other pigments, such as carotenoids) in the antenna toward a much smaller network in the reaction center (RC). In the RC, other cofactors (quinones and iron−sulfur clusters) are involved in achieving stable charge separation. PS1 and PS2 are members of two distinct classes of RCs. PS2 uses plastoquinone (PQ = 2,3-dimethyl-5-prenyl-1,4-benzoquinone) as its terminal electron acceptor. PQ is doubly reduced to plastoquinol (PQH_2) via a plastosemiquinone intermediate ($PQ^{\bullet-}$). PQH_2, which has a lower affinity for the Q_B site in the PS2 RC, exits the Q_B-binding pocket and is replaced by a new PQ.[1] PS1 uses phylloquinone (PhQ = 2-methyl-3-phytyl-1,4-naphthoquinone), which has a reduction potential lower than that of PQ, as an intermediate ET cofactor. In PS1, PhQ is only an intermediate in ET (termed "acceptor A_1" before its identity as PhQ was established), and the phyllosemiquinone anion rapidly passes the electron to the Fe_4S_4 cluster F_X, which in turn reduces the terminal pair of Fe_4S_4 clusters, F_A and F_B, bound by the PsaC subunit, which resembles a bacterial ferredoxin.[2] These subsequently reduce ferredoxin, a soluble one-electron carrier with a low-potential Fe_2S_2 cluster.[3]

Forward ET leading to stable charge separation (CS) through PS1 up to and including the formation of the $P_{700}^+F_{A/B}^-$ radical pair is very rapid, occurring within hundreds of nanoseconds.[4,5] ET from PhQ^- to F_X exhibits biphasic kinetics with lifetimes of ∼200 ns for PhQ_A and ∼20 ns for PhQ_B.[6] It is now widely accepted that both branches of ET cofactors are active, although most investigators agree that the A-branch is more heavily utilized than the B-branch, and this functional asymmetry seems to be stronger in cyanobacteria than in green algae.[7−9] However, it has also been suggested that the dominance of the A-branch may be overestimated, because of the occurrence of spectroscopically invisible interquinone ET from PhQ_B^- to PhQ_A.[10] Backward ET (i.e., charge recombination) from the radical pairs $P_{700}^+A_0^-$, $P_{700}^+A_1^-$, $P_{700}^+F_X^-$, and $P_{700}^+F_{(A/B)}^-$ occurs on time scales of ∼30 ns, ∼200 μs, ∼1 ms, and ∼100 ms, respectively,[4] and these decay rates can allow identification of the radical pair that is recombining.

Ke[11] has described the history of the discovery and verification of the chemical identity of A_1 in great detail. PhQ was discovered in plants in 1941, although at the time the only known biological function was in blood clotting in animals;

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thus, it is often termed "vitamin K". Not until the mid-1980s was its role in photosynthetic ET described.[12−14] Biggins and co-workers[15,16] and Itoh and co-workers[17,18] have explored the characteristics of PS1 in which quinones had been extracted with solvent and reloaded with artificial quinones. More recently, Pushkar et al.,[19,20] using a similar method, and van der Est et al.,[21] employing mutants unable to make PhQ (described below), have further illustrated more details of PS1 loaded with non-native benzoquinones, naphthoquinones, and anthroquinones in the A_1-binding site. All of these studies indicate that the A_1-binding pocket of PS1 does not stringently discriminate among a set of related quinones. Generally, quinones with one (benzoquinone), two (naphthoquinones), or three rings (anthraquinones) and with various (even multiple) substitutions are capable of occupying the A_1-binding site of PS1. In some cases, they can even participate in forward ET to generate relatively stable charge-separated states. However, these studies also agree that naphthoquinones with a long alkyl tail (and usually with a 2-methyl group, like PhQ) are best suited to successfully function as electron acceptors from A_0^- and donors to F_X. Quinone substitution at the Q_A site has also been demonstrated extensively in proteobacterial type 2 RCs.[22−26] Similar to the reports of non-native quinone recruitment to the A_1-binding pocket of PS1, these studies described dynamics of quinone binding plasticity with respect to cofactor structure and RC functionality and found that a wide range of quinones could occupy the Q_A site, some of which could functionally substitute for the native quinone, depending upon their reduction potential.

More than 10 years ago, mutants of the cyanobacterium *Synechocystis* PCC 6803 were created in which the biosynthetic pathway of PhQ was interrupted. Disruptions of the *menA* and *menB* genes, encoding the 1,4-dihydroxy-2-naphthoate phytyltransferase and 1,4-dihydroxy-2-naphthoate synthase enzymes, respectively, both resulted in a complete lack of PhQ. Unexpectedly, the quinone-binding pocket of PS1 was found to be occupied by PQ in the absence of the native quinone. These studies revealed that PQ is not as effective an ET intermediate as the native PhQ, which is not surprising given the reduction potentials of the two quinones.[27] The *menA* and *menB* mutants could grow photoautotrophically, albeit somewhat poorly, and PS1 with PQ in the A_1 site exhibited a modest decrease (15−20%) in the level of light-driven reduction of flavodoxin.[28] It also displayed a rate of forward ET to F_X that was reduced by 2 orders of magnitude (compared to that of PhQ_A), while the rate of charge recombination from $P_{700}^+F_{(A/B)}^-$ was accelerated by ∼25-fold.[29] Taken together, these facts indicate a stabilization of the semiquinone state at the A_1 site when it is occupied by PQ, as compared to PhQ.

Recently, mutants in PhQ biosynthesis have been obtained in photosynthetic eukaryotes. Mutants in either the *MENA*[30] or the *PHYLLO* gene (which encodes a fusion of the eubacterial MenF/MenD/MenC/MenH gene products[31]) in the plant *Arabidopsis thaliana* have drastically reduced PS1 levels. A nuclear mutant of the *MEND* gene, which encodes the enzyme 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase, has been isolated in the green alga *Chlamydomonas reinhardtii*.[32] In the *menD1* mutant, the biosynthetic pathway for PhQ is interrupted, leading to a complete lack of PhQ, similar to the case for the *menA* and *menB* mutants of *Synechocystis* PCC 6803. In this mutant, there is also a drastic retardation of ET from the quinone to the FeS clusters. Moreover, it was shown that in anoxic *menD1* cells, the PQ pool would become reduced and PQH_2 would exchange with PQ in PS1, resulting in PS1 RCs that underwent charge recombination from the $P_{700}^+A_0^-$ state.

In vivo reconstitution experiments indicated that exogenous quinones added to *Synechocystis* PCC 6803 *menB* cells could exchange with PQ in the A_1-binding pocket of PS1 within 15 min.[28,33] Lefebvre-Legendre et al.[32] also found that the addition of PhQ to media could alleviate sensitivity of the *C. reinhardtii menD1* mutant to high levels of light, a common phenotype of cells with low PS1 activity.[34] Snyder et al.[35] had observed the partial restoration of the A_1^- EPR signal following removal of chemical reductants used to reduce PhQ to $PhQH_2$ in vitro, and Sieckman et al.[36] observed recovery of spin-polarized EPR signals similar to native PS1 samples following addition of PhQ to solvent-extracted PS1 particles. On the basis of these results and a multispecies PhQ exchange study,[37] it would thus seem that quinones can enter and exit the A_1 site of PS1 on a time scale of minutes to tens of minutes.

We initially set out to use the PQH_2-exchanged PS1 purified from *menD1* cells to observe charge recombination in PS1 without the use of harsh reductants, like sodium dithionite. However, along the way, we discovered that even PQ-loaded PS1 could progressively enter a back-reacting state after a train of laser flashes. We show here that these particles are capable of doubly reducing PQ to PQH_2 in the A_1-binding pocket and explore the factors that influence the process. This phenomenon is not species-specific, as we also observe it in cyanobacterial PS1 from the *menB* mutant. Thus, in some regards, PS1 can mimic a type 2 RC, as it can doubly reduce a quinone in the A_1 pocket, but by employing a distinct mechanism.

EXPERIMENTAL PROCEDURES

Sources of Material. The *menD1* mutant[32] was provided by J.-D. Rochaix (University of Geneva, Geneva, Switzerland). The PsaA-L722T mutant[10] and His_6-tagged *psaA* exon 1[38] are chloroplast loci that were described previously. The nuclear and chloroplast mutations were combined by mating.[39] PS1 particles purified from the *menB* mutant[28] were generously provided by T. W. Johnson (Susquehanna University, Selinsgrove, PA) and J. H. Golbeck (The Pennsylvania State University, State College, PA). An *Escherichia coli* BL21(DE3) strain expressing recombinant *C. reinhardtii* plastocyanin under control of the *lac* promoter and protocols for purifying it by anion exchange chromatography (DEAE-Sepharose) and gel filtration were provided by S. Kulhgert and M. Hippler (University of Münster, Münster, Germany; personal communication). Phylloquinone was purchased from Sigma-Aldrich and was dissolved in dimethyl sulfoxide to a stock concentration of 6 mM. A plastoquinone-9 standard was generously provided by B. Diner (DuPont Experimental Station). Plastoquinol was prepared by treatment of PQ-9 with KBH_4, followed by solvent extraction, lyophilization, and resuspension in methanol as described previously.[40]

Sample Preparation. Cells were grown in standard Tris-acetate-phosphate medium[39] at room temperature under fluorescent lights at ∼40 μmol of photon (photosynthetically active radiation) m^{-2} s^{-1}. His_6-tagged PS1 particles were purified via nickel affinity chromatography[38] or on linear sucrose density gradients following solubilization according to the method described in ref 41 with minor changes. Briefly, thylakoid membranes at a chlorophyll concentration of 0.8−1.0 mg/mL were solubilized on ice for 20 min with gentle stirring in the presence of 5 mM Tricine (pH 7.5, NaOH), 0.1 M sorbitol, 5 mM $CaCl_2$, 5 mM $MagCl_2$, and 0.9% *n*-dodecyl β-D-maltoside.

After centrifugation at 64000*g* to remove unsolubilized material, the supernatant was loaded onto a continuous sucrose density gradient formed by freezing and thawing of a tube containing 5 mM Tricine-NaOH (pH 8.0), 0.3 M sucrose, 0.3 M betaine, and 0.05% *n*-dodecyl *β*-D-maltoside. Following a 20-h centrifugation at ∼120000*g*, the lower PS1-containing band was isolated and concentrated for storage or prepared for assay in assay buffer [5 mM Tricine-NaOH (pH 8.0), 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.05% *n*-dodecyl *β*-D-maltoside].

Transient Optical Spectroscopy. Nanosecond kinetics of purified PS1 particles were obtained using a dual-laser-flash system, as described previously.[42] P_{700}^+ bleaching kinetics over the course of ≥1 h were measured using an LED-driven JTS-10 kinetic spectrophotometer (Bio-Logic). The sample consisted of PS1 particles at a total Chl concentration of 80 or 50 μg/mL for algal or cyanobacterial PS1, respectively. Sodium ascorbate (10 mM) and phenazine methosulfate (PMS, 1 μM) were added as artificial electron donors to P_{700}^+.[43] The reaction buffer was the same as described above, except that Tricine was replaced by MES (pH 5.5) or CHAPS (pH 9.0) for experiments at a lower or higher pH value. A frequency-doubled Nd:YAG laser (Continuum Electro-Optics, Inc., Santa Clara, CA) emitting 20-mJ pulses at 532 nm (6 ns duration) and 15 Hz provided actinic flashes. At specific times, the actinic flashes were stopped for 1 min, and a single laser flash was given, followed by measuring flashes (10-μs red LED passed through a 705-nm interference filter) to monitor oxidation of P_{700} by the saturating laser flash and its ensuing re-reduction. Measuring flashes commenced 300 μs after the laser flash and continued for 18 s, long enough for the bleaching signal to recover entirely. The concentrations of artificial electron donors were kept sufficiently low to reduce P_{700}^+ between measurements (for centers in which the electron had "escaped" from the RC) but not to compete with the back-reaction.[44,45] For experiments with much higher flash frequencies, a Fianium (Southampton, U.K.) fiber-based laser system (model SC-450-PP) was operated at 20 MHz, and the beam was passed through a 520-nm band-pass filter to deliver 1-nJ pulses (6 ps duration).

HPLC Analysis of Quinones. PS1 particles before or after photoinactivation were concentrated 20-fold using microfiltration (Amicon Ultracel 50-kDa cutoff membranes) and extracted with 20 volumes of acetone. Acetone-extracted pigments were evaporated to near dryness and dissolved in 5 volumes of methanol prior to injection of 200 μL onto a C-18 column (Ultrasphere, 250 mm × 4.6 mm, 5 μm). Reverse-phase HPLC was performed as described previously[46] using a flow of 1.5 mL/min of a methanol/hexane mixture (17:1, v/v). Elution was monitored with both a JASCO MD-2018Plus photodiode array detector and a Perkin-Elmer LS55 fluorescence spectrophotometer with a flow cell (excitation at 290 nm and emission at 330 nm).

RESULTS

Increase in the Extent of Back-Reaction Observed in *menD1* PS1 Particles. It was previously reported that forward ET from PQ in PS1 in living *menD1* cells under anoxic conditions would be replaced by an ∼30-ns decay because of charge recombination from the $P_{700}^+A_0^-$ state.[32] This effect was presumably due to exchange between the quinone in the A_1 site and PQ/PQH_2 in the thylakoid membrane quinone pool, which would become progressively reduced as the cells became anaerobic. To obtain superior samples for observing the back-reaction in a high quantity without prior treatment with a strong reductant like dithionite (which can be problematic for various reasons), we purified PS1 particles from *menD1* cells that had been shifted to anaerobic conditions ∼1 h before being harvested; solutions were degassed with N_2 up to the point of membrane solubilization to minimize exposure to oxygen.

After a saturating actinic laser flash, there was an unresolved instantaneous bleaching centered at 430 nm in the blue region of the spectrum, due to oxidation of P_{700} in these particles. Approximately half of this bleaching decayed with an ∼30-ns decay time, due to back-reaction from the $P_{700}^+A_0^-$ state (see Figure S1A of the Supporting Information). We also observed a small phase (∼10% of the decay) in tens of microseconds, which we tentatively attribute to decay of $^3P_{700}$ formed during charge recombination from $P_{700}^+A_0^-$.[47] The remainder decayed in tens of milliseconds, due to a combination of back-reaction from $P_{700}^+F_A/F_B^-$ and reduction by PMS and ascorbate. However, we observed that, as the experiment continued, the fraction undergoing rapid back-reaction progressively increased. After several thousand laser flashes (at 10 Hz), the fraction undergoing the ∼30-ns decay had increased from ∼50% to ∼70% at the expense of the slowest component. At that point, we recorded spectra 10 and 200 ns after the laser flash (see Figure S1B of the Supporting Information). The difference between them corresponds to the spectrum of the population decaying during that period (i.e., $P_{700}^+A_0^-$) and agrees very well with similar spectra obtained previously,[48] except that the relative amplitudes of the peaks differ slightly.

Loss of Stable P_{700} Photobleaching in *menD1* PS1 during Actinic Flashes in Vitro. We were surprised to see an increase in the fraction undergoing 30-ns charge recombination in *menD1* PS1 particles simply by exposing them to laser flashes in the presence of the weak reductants PMS and ascorbate, and in the absence of PS2 or other enzymes normally capable of doubly reducing PQ to PQH_2. Our hypothesis was that the PS1 RC itself was performing such a double reduction. To test this, our experimental protocol was to expose PS1 particles to a series of saturating 532-nm laser flashes at 15 Hz and then periodically turn off the laser and assess the ability to stably photo-oxidize P_{700} by a single laser flash. This was done using a series of weak 10-μs measuring flashes at 705 nm to monitor P_{700} before and after the flash. As the first measuring flash is given 300 μs after the actinic laser flash, this protocol would be blind to any P_{700} bleaching that decayed within 300 μs. Thus, inactivation of the quinone would lead to a loss of "stable" P_{700} photobleaching, as charge recombination from the $P_{700}^+A_0^-$ state would be complete within 1 μs. Here we operationally define stable to mean anything that lives longer than 300 μs. Back-reaction from F_A/F_B (∼100 ms) in wild-type (WT) PS1 would be visible with this procedure[49] (also see Table S1 of the Supporting Information). The PMS concentration was kept low (1 μM), so that it would not significantly compete with the back-reaction from F_A/F_B (see the inset of Figure 1).[44,45]

We used PS1 purified from aerobically grown *menD1* cells, which therefore should have PS1 loaded with PQ. In the beginning of the experiment, we saw strong flash-induced P_{700} photobleaching in the *menD1* PS1 sample, which decayed with an apparent time constant of 11.5 ms (Table S1 of the Supporting Information). This is ∼10 times faster than what we typically observe in WT PS1, an acceleration similar in magnitude to what was observed previously in PS1 from the cyanobacterial *menB* mutant.[29] After a train of 64000 laser flashes given over the course of ∼70 min at pH 8, we found that

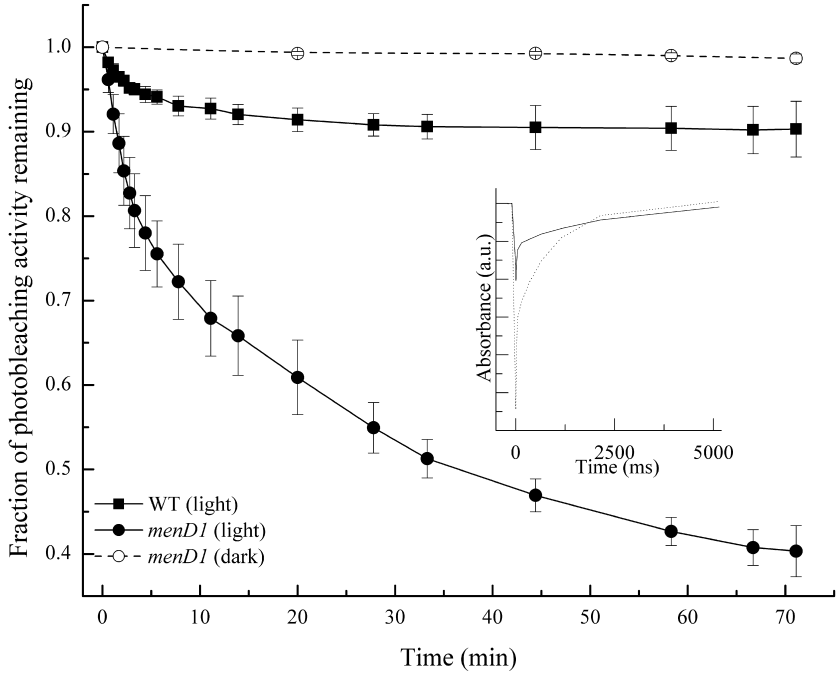

Figure 1. Photoinactivation of PS1 particles purified from WT (■) and *menD1* (●) cells. PS1 particles in assay buffer at pH 8 were flashed at 15 Hz with saturating laser flashes (532 nm, 6 ns duration) for the indicated time and then periodically assayed with a single flash after a 1-min dark recovery period. As a control, *menD1* PS1 was incubated in the dark for the same amount of time (○). Data are the average of three independent experiments ± the standard error. The inset reveals two single-flash experiments from the *menD1* data set at pH 8.0 in which the amplitude of the P_{700}^{+} bleaching signal was assayed before (···) and after 64000 accumulated flashes (—).

the sample had lost >60% of its stable P_{700} photobleaching activity (see the inset of Figure 1). As shown in Figure 1, this loss was progressive and slowed with time; the sample had lost ∼30% of its stable P_{700} photobleaching within 10 min but took >1 h to lose an additional 30% [Figure 1 (●)]. We saw only a small apparent loss of activity in WT PS1 containing PhQ_A as seen before [Figure 1 (■)]; normally, PS1 is very stable to long trains of laser flashes. This is consistent with the hypothesis that PQ_A, which is easier to reduce than PhQ_A, is being converted to PQH_2 in *menD1* PS1 during the illumination period. An alternate hypothesis is that PQ_A, which has a lower affinity for the A_1 site of PS1 than PhQ_A,[33] is simply dissociating from the site with time. We tested this by repeating the experimental protocol without the laser flashes and saw essentially no loss of activity in *menD1* PS1 in the dark [Figure 1 (○)]. Thus, the loss of the P_{700} bleaching signal, which we will here term "photoinactivation", requires both light and PQ_A in the A_1-binding pocket.

pH Dependence of Photoinactivation. If photoinactivation represented the double reduction of PQ_A, then we would expect it to occur more effectively at lower pH, because protonation is likely to be at least somewhat limiting for this process, as it is for type 2 RCs.[1,50] We tested this prediction by repeating the experiment under somewhat more basic (pH 9.0) or acidic (pH 5.5) conditions. At the lower pH, both the rate and extent of loss of stable P_{700} photobleaching were increased in *menD1* PS1 (Figure 2). We saw no effect of increasing the pH (Figure 2). The enhanced loss of activity at pH 5.5 in *menD1* PS1 was still light-dependent (see Figure S2 of the Supporting Information), arguing against denaturation of the protein induced by low pH.

Although we saw a slightly larger decrease in the activity of WT PS1 at pH 5.5, this recovered in 20 min back to the same residual level (∼91%) as at pH 8 [∼92% (Figure 2 and Table S2 of the Supporting Information)]. The level of recovery seen with WT PS1 after the laser flashes was usually of the order of 1−9% and likely reflects re-reduction of the PMS pool by ascorbate rather than any change in PS1 per se (data not shown; see Discussion). Included in this small fraction of irreversible loss seen in WT PS1 may be a portion of RCs that have accumulated $PhQH_2$. In all cases, >90% of the stable photobleaching activity survived in WT PS1 after the 64000-flash treatment and 20-min recovery (Figure 2 and Table S2 of the Supporting Information).

Direct Detection of PQ and PQH_2 by Reverse-Phase HPLC. To test directly the hypothesis that PQ_A is being converted to PQH_2 by PS1, we took particles before and after photoinactivation, concentrated them 20-fold, and then extracted them with solvent (see Experimental Procedures for details). The extracts were analyzed by reverse-phase HPLC. The peak at 35 min (Figure 3A) corresponds to PQ_A as judged by comigration with an authentic sample of PQ-9 and by its UV−visible absorption spectrum, which matched that of PQ-9 (data not shown). This peak had largely disappeared after the photoinactivation regime (Figure 3B). The small amount of PQ_A left (∼10−15%) corresponded roughly to the residual stable photobleaching activity in the photoinactivated particles. PQH_2 elutes much earlier in the profile, overlapping with the large peak of chlorophylls and major pigments in the first 15 min, which precluded the use of absorption spectroscopy to measure it. However, we were able to use the fluorescence properties of PQH_2, as established previously,[46] to measure its abundance in the extract. A sample of PQH_2 (prepared by reduction of PQ-9 with KBH_4) eluted at ∼10 min (data not shown). Initially, there was no measurable PQH_2 in the *menD1* PS1 particles (Figure 3C). However, after photoinactivation, we observed the appearance of the PQH_2 peak. Integration of the PQH_2 peak (and comparison to a standard) allowed us to

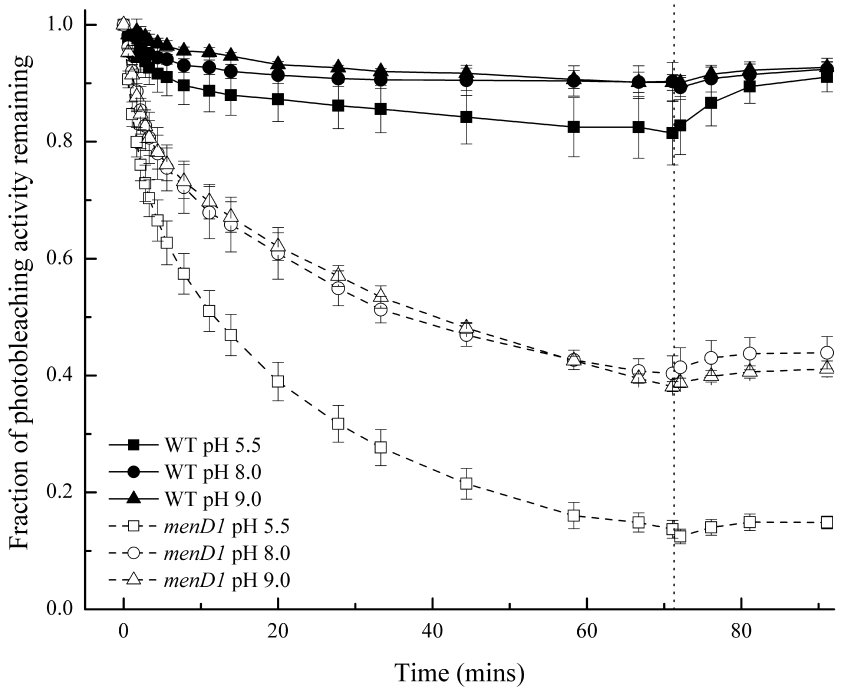

Figure 2. Photoinactivation of PS1 particles purified from WT (filled symbols) and *menD1* (empty symbols) cells. Experiments were conducted as described in the legend of Figure 1, except that the pH was 5.5 (squares), 8.0 (circles), or 9.0 (triangles). The 20-min dark recovery following each illumination period (indicated by the vertical dotted line) is also shown. Averages of three independent experiments ± the standard error.

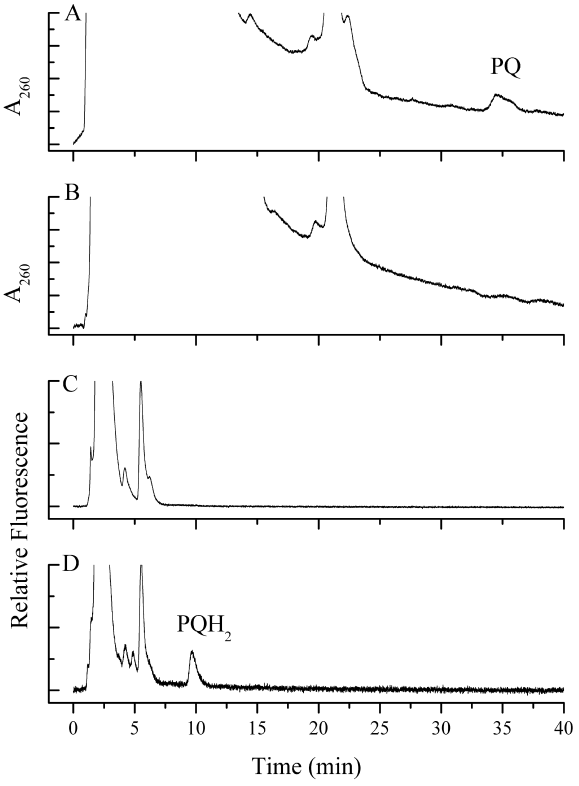

Figure 3. Pigments extracted from *menD1* PS1 particles following dark incubation (A and C) or photoinactivation (B and D) were subjected to reverse-phase HPLC. Chromatograms monitored absorbance at 260 nm (A andB) or fluorescence emission at 330 nm using excitation at 290 nm (C and D). The PQ and PQH_2 labels indicate the elution times of those species, as determined using PQ-9 and PQH_2-9 standards, respectively (not shown).

estimate that the amount of PQH_2 in the extract of the photoinactivated particles corresponded to ~75% of the amount of PQ in the extract of PS1 particles before photoinactivation, which is fairly close to the amount expected (~85%) if one assumes quantitative recovery of PQH_2 in the particles. Because the train of laser flashes causes both the disappearance of PQ and the appearance of PQH_2, we conclude that photoinactivation of *menD1* PS1 entails double reduction of the PQ in the A_1 site to PQH_2.

Rescue of Activity by Addition of Exogenous Phylloquinone. On the basis of the results from quinone exchange studies,[28,32,33,35−37] we expected that addition of an excess of the native quinone would result in the restoration of PhQ to the A_1 site of the PS1 RC. Thus, if our hypothesis (that inactivation of *menD1* PS1 is solely due to double reduction of the PQ in the A_1 site) is correct, we would predict that one should be able to reverse the inactivation by addition of PhQ, as it should be able to exchange with PQH_2 in the A_1 site.

We tested this prediction by adding a 10-fold molar excess of PhQ to *menD1* PS1 particles that had been photoinactivated at pH 5.5 until they had lost ~90% of their stable P_{700} photobleaching. Within 1 h, more than half of the lost activity had been regained, and the particles had been restored to >70% activity after 3 h [Figure 4 (▲)]. Further incubation overnight at room temperature allowed restoration to ~80−85% of the original activity. A mock treatment, in which the solvent used for PhQ (dimethyl sulfoxide) was added without quinone and all other treatments were the same, did not result in the significant rescue of photobleaching beyond the low level typically seen after cessation of the laser flashes [Figure 4 (△)]. Interestingly, after rescue with PhQ, the PS1 sample became much more resistant to subsequent photoinactivation (Figure S3 of the Supporting Information), behaving like WT PS1, consistent

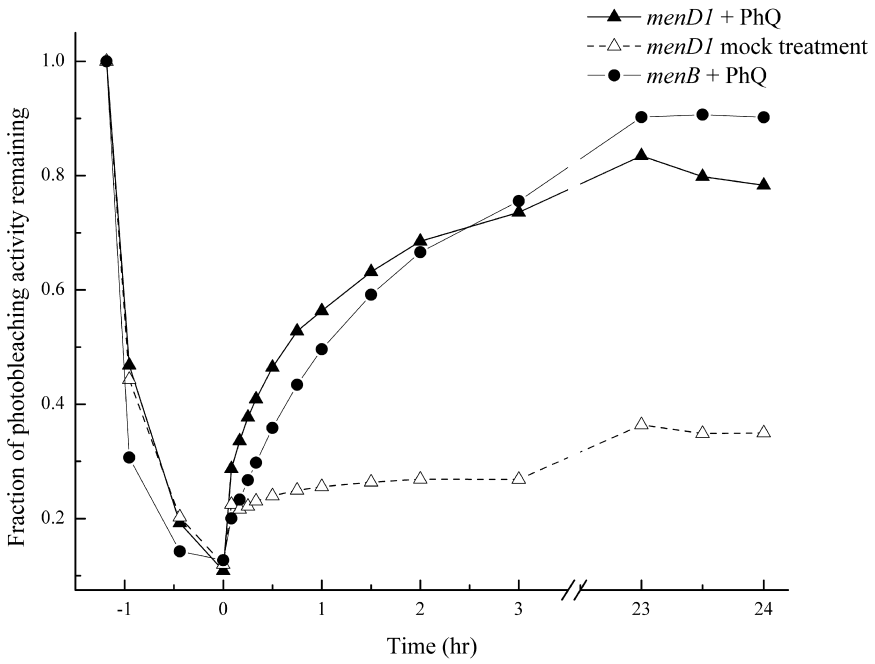

Figure 4. Reactivation of photoinactivated PS1 by addition of exogenous PhQ. PS1 particles purified from the *C. reinhardtii menD1* mutant (▲) or the *Synechocystis* PCC 6803 *menB* mutant (●) were subjected to photoinactivation at pH 5.5 for 70 min as before. At time zero, phylloquinone was added to a final concentration of 6 μM (10-fold excess over PS1) and the solution was gently mixed and maintained at room temperature in the dark. Photobleaching activity was periodically monitored with single saturating flashes as before. Data for the mock mechanical treatment of photoinactivated *C. reinhardtii menD1* PS1 using solvent alone are shown (△).

with the idea that PQ/PQH_2 had been replaced by PhQ. This experiment demonstrates that double reduction of the quinone in PS1 is responsible for the vast majority of the photoinactivation seen with *menD1* PS1.

Species Dependence of PQH_2 Accumulation. The *Synechocystis menA* and *menB* mutants were first described in 2000 and have been extensively characterized.[28,29,33,51−53] Because of the lack of PhQ biosynthesis, PS1 in these cells contains PQ. However, the phenomenon we describe above for *C. reinhardtii menD1* PS1 had not been previously reported. Our initial hypothesis was that eukaryotic PS1 might somehow be different from cyanobacterial PS1 in a way that would allow it to perform the double reduction of PQ. J. H. Golbeck and T. W. Johnson generously provided samples of PS1 purified from the *Synechocystis menB* mutant and WT cells, permitting us to test this hypothesis.

We found, however, that *Synechocystis* PCC 6803 *menB* PS1 was susceptible to photoinactivation and its rate of inactivation was even higher than that seen with *C. reinhardtii menD1* PS1 (compare Figure 5 with Figure 2). Moreover, the inactivation displayed a similar pH dependence, becoming faster at pH 5.5 [Figure 5 (□)]. PS1 purified from WT *Synechocystis* PCC 6803 cells was resistant to photoinactivation, demonstrating the requirement for PQ in the A_1 pocket. Photoinactivated *menB* PS1 could also be rescued by addition of exogenous PhQ [Figure 4 (●)]. Thus, we have no evidence of the specificity of this process for algal, or even eukaryotic, PS1; it appears to be a general phenomenon of PS1 loaded with PQ.

Effect of a Mutation in the PhQ_A-Binding Pocket. Leu722 of PsaA donates an H-bond from its amide N to one of the oxygens of PhQ_A,[54] which would be expected to stabilize the phyllosemiquinone anion. Recently, mutation of this residue to Trp in *Synechocystis* PCC 6803 PS1 was shown to result in acceleration of forward ET from PhQ_A,[42] which was interpreted as being due to an increased driving force resulting from a weakening of the H-bond. The mutation also caused the disappearance of the phyllosemiquinone transient EPR signal (A_1^-) after multiple flashes at low temperatures in the presence of dithionite.[55] This result was explained as being due to an increased likelihood of protonation of the semiquinone, which would normally be blocked by the H-bond to Leu722 of PsaA, thereby facilitating double reduction of PhQ to $PhQH_2$ in the altered site. We decided to test if a similar mutation would accelerate the photoinactivation rate, because this process also represents double reduction of a quinone. Unfortunately, the PsaA-L722W mutation in *C. reinhardtii* destabilizes PS1,[10] precluding its use here. The PsaA-L722T mutation, however, has a very similar effect on forward ET from PhQ_A, accelerating it ~2-fold,[10] without any effect upon protein stability. We therefore combined the nuclear *menD1* mutation with the chloroplast *psaA-L722T* mutation to create double mutants harboring PS1 with a mutated PhQ_A site and containing PQ instead of PhQ.

We first examined the rate of charge recombination in PsaA-L722T PS1, loaded with either PhQ or PQ, as these have not been previously reported. The major phase of P_{700}^+ decay in PS1 purified from the *psaA-L722T* mutant had a decay time of ~200−250 ms, indicating a rate of $P_{700}^+F_{(A/B)}^-$ charge recombination at least twice as slow as that in WT PS1 from *C. reinhardtii* (Table S1 of the Supporting Information). This is consistent with a weakened H-bond to PhQ_A in this mutant, increasing the energy of the intermediate $P_{700}^+PhQ_A^-$ state, making it more difficult to be used during the back-reaction. PS1 purified from the *menD1*[*psaA-L722T*] double mutant exhibited a P_{700}^+ decay time of ~14.7 ms, indicating that the PsaA-L722T mutation slowed charge recombination by

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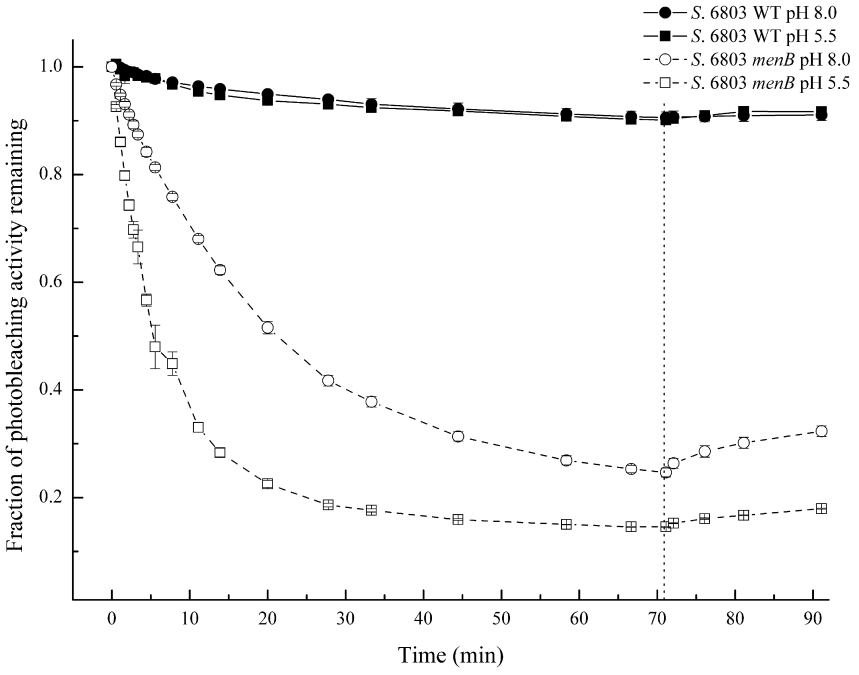

Figure 5. Photoinactivation of PS1 particles purified from WT (filled symbols) and *menB* (empty symbols) *Synechocystis* PCC 6803 cells at pH 8 (circles) and pH 5.5 (squares). A 20-min dark recovery followed each illumination period (indicated by the vertical dotted line). Averages of three independent experiments ± the standard error.

25−30% when PQ was in the A_1 site. This effect indicates that there is likely an H-bond to PQ in the context of the WT PsaA polypeptide, and that it is weakened by the PsaA-L722T mutation. The fact that the effect of the mutation is not quantitatively identical in the two quinone contexts is not entirely surprising, as we do not know where on the Marcus curve the relevant reactions lie.

Interestingly, we saw no effect of the PsaA-L722T mutation on the photoinactivation of PS1 particles containing PhQ (Figure 6A). Thus, we see no evidence of accelerated double reduction of PhQ in the PsaA-L722T mutant. In contrast, the photoinactivation of PS1 particles containing PQ was accelerated by the PsaA-L722T mutation (Figure 6B). Although the effect of the PsaA-L722T mutation was seen at both pH 5.5 and 8, the acceleration of photoinactivation was somewhat more pronounced at the higher pH. Thus, although the chemical identity of the quinone is the most important factor in the efficient photoaccumulation of a doubly reduced quinone, the environment of the quinone can also play a role.

Effect of Flash Frequency and P_{700}^+ Re-Reduction Rate on Photoinactivation. As a last test of the hypothesis that photoinactivation represents reduction of PQ to PQH_2, we attempted to vary two key parameters: the frequency of CS events and the rate of donation to P_{700}^+. We reasoned that double reduction of a quinone requires two electrons and therefore two charge separation events in the PS1 RC. Moreover, when the second CS event occurs, the electron must still be on the acceptor side (i.e., on the quinone or Fe−S cofactors) to have a chance to doubly reduce a quinone. Thus, we would expect photoinactivation to be sensitive to the actinic flash frequency, as the longer the delay between flashes, the longer the system has to perform charge recombination, thereby resetting the system. In the *menD1* PS1 particles, this decay time is ∼11−12 ms. Thus, when operating at 15 Hz (the upper limit of our laser), we would expect that only ∼0.3% of the PS1 RCS that had undergone CS would still have an electron on the acceptor side before the next flash occurs. This places a severe limitation on the double-reduction process.

A PS1 RC must be "reset" by reduction of P_{700}^+ before it can perform a second CS, making it even worse.[56] We intentionally kept the concentration of PMS low to prevent it from serving as an electron acceptor,[44,45] but the consequence of this would be a very slow re-reduction of P_{700}^+ [∼0.5−1 s by our measurements of the slow rate of P_{700} bleaching decay (data not shown)], corresponding to RCs in which the electron had "escaped" from the FeS clusters (perhaps to PMS or some other exogenous acceptor). Thus, we would expect that use of a faster donor to P_{700}^+ would increase the rate of photoinactivation. Simply increasing the concentration of PMS is problematic, as it can also reoxidize the FeS clusters. The in vivo donor to P_{700}^+ is plastocyanin (Pc) and is a better choice. Prebound Pc has been reported to reduce P_{700}^+ in 6 μs, while the second-order rate of reduction can be in the range of 50−60 μs.[57] When we replaced PMS with recombinant Pc to a concentration equivalent to a 10-fold molar excess relative to PS1, we observed that photoinactivation of *menD1* PS1 became markedly faster (Table S3 of the Supporting Information).

We were unable to increase the frequency of our laser, so we made use of an ultrafast laser operating at 20 MHz; a 520-nm interference filter was used to keep the actinic light wavelength similar. Under these conditions, each pulse was 1 nJ and 6 ps, thus providing only 20 mW of power, which is much lower than the 300 mW of power provided by our Nd:YAG laser operated at 15 Hz (with 20 mJ/pulse). The situation was also made worse by the fact that the setup could illuminate only ∼30% of the sample volume (as it was gently stirred). Despite these drawbacks, the nonsaturating flashes at 20 MHz resulted in more photoinactivation than the saturating flashes at 15 Hz (Table S3 of the Supporting Information). When the use of Pc as a reductant was combined with the high-frequency excitation, we observed the highest rate of photoinactivation [∼69% loss of stable photobleaching in 10 min (Table S3 of the

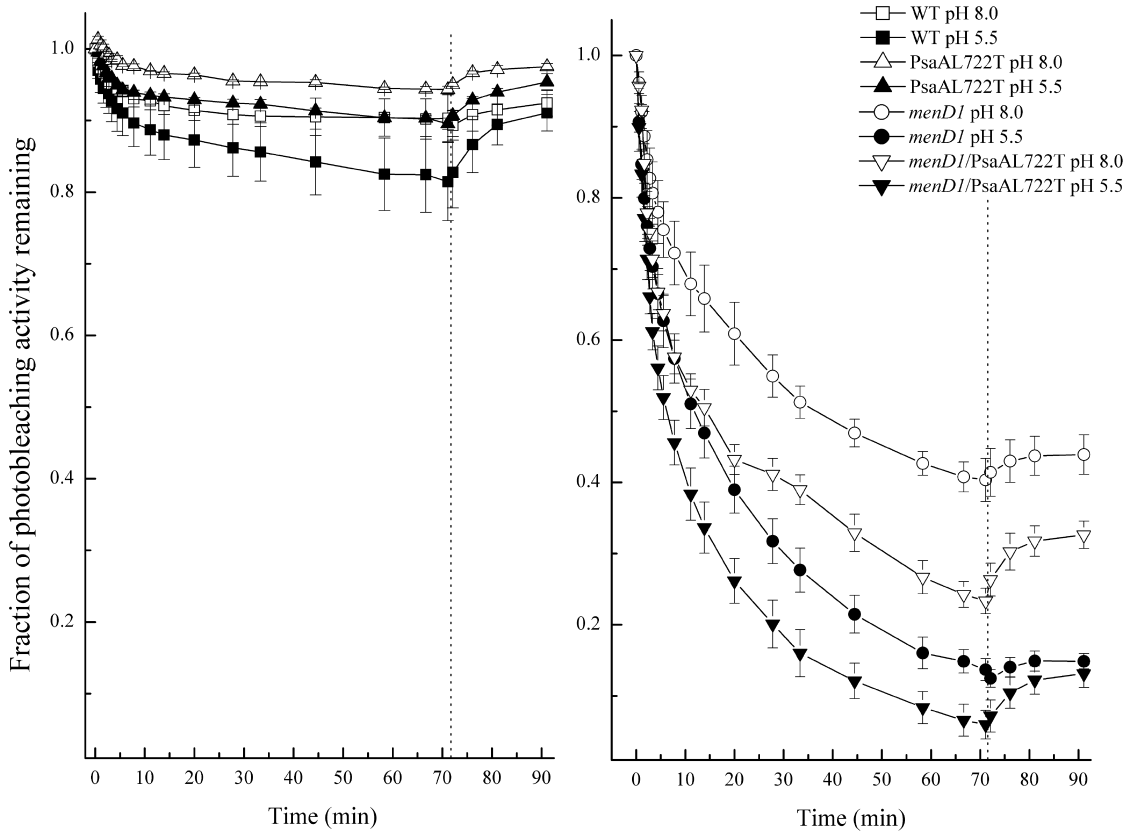

Figure 6. Photoinactivation of PS1 particles purified from *C. reinhardtii* cells (WT and PsaA-L722T backgrounds) harboring either PhQ (A) or PQ (B) in the A_1-binding pocket assayed at pH 8 (empty symbols) or pH 5.5 (filled symbols). A 20-min dark recovery followed each illumination period (indicated by the vertical dotted line). Averages of three independent experiments ± the standard error.

Supporting Information)]. Using the formula of Cho et al.[58] and the absorption spectrum of *menD1* PS1, we were able to calculate the probability of a PS1 RC absorbing a photon on a given flash under these conditions to be 7.8×10^{-6}. At a frequency of 20 MHz, the probability of a PS1 RC being excited a second time within 1 ms of the previous hit is thus 14%; this probability increases to 50% within 4.5 ms, to 83% within 11.5 ms, and to 96% within 20 ms. Thus, the interflash delay is significantly shortened under this regime versus that with 15-Hz saturating flashes (66.7 ms).

■ DISCUSSION

In this paper, we have described a phenomenon of photoinactivation that occurs after a train of many laser flashes in PS1 possessing PQ in its quinone-binding pocket instead of native PhQ. We conclude that this photoinactivation process represents accumulation of PQH_2 in the quinone-binding pocket of PS1 in the absence of strong reductants. In other words, PS1 is capable of double reducing its embedded quinones to quinols. There are several lines of evidence supporting this conclusion. Light and the presence of PQ (rather than PhQ) in the A_1-binding pocket are required for the accumulation of a quinol. We also found that PQH_2 photoaccumulates more effectively at lower pH values in *menD1* PS1 particles, suggesting that protonation is somehow limiting double reduction of PQ in the A_1-binding pocket. The effect of the PsaA-L722T mutation also supports this conclusion (discussed more below). The rise of a major 30-ns decay component with the spectral characteristics of $P_{700}{}^+A_0{}^-$ in photoinactivated particles shows that the damage must have been after A_0 and before F_X. Most importantly, addition of PhQ to photoinactivated *menD1* PS1 RCs not only restored their activity but also made them resistant to further photoinactivation. This proves that the damage was at the level of the quinone and that no defect in any other cofactor in the photoinactivated *menD1* PS1 particles was responsible for the loss of activity. Moreover, the fact that the photoinactivated state was stable for many hours (see Figure 4, mock treatment) argues against a simple single reduction of PQ, as the semiquinone would not be expected to be that stable at room temperature.[59] Finally, the direct observation of conversion of PQ to PQH_2 by HPLC (Figure 3) leaves little room for doubt.

We have also demonstrated that there is little species dependence in the ability of PQ-containing PS1 to photoaccumulate PQH_2. The pH dependence of the process was about the same in both prokaryotic and eukaryotic PS1, and both types of PS1 could be reactivated afterward by addition of PhQ. Photoinactivation of cyanobacterial *menB* PS1 occurred somewhat more quickly than with eukaryotic *menD1* PS1. This difference could be at the level of the donor side, as addition of a faster donor to eukaryotic *menD1* PS1 could accelerate its photoinactivation rate (discussed more below); it is possible that cyanobacterial $P_{700}{}^+$ is reduced more quickly by PMS and ascorbate under these conditions. Regardless, there is little difference in the final extent to which PQH_2 photoaccumulates in PS1 from these two species. Thus, the double reduction of PQ in the A_1 site of PS1 would appear to be a general phenomenon, which is not surprising when one considers how similar the rates of ET are in PS1 from different sources.[60]

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Rates of $P_{700}^+F_{(A/B)}^-$ Charge Recombination in PS1: Stability of the Semiquinone State. The rate of decay of P_{700}^+ after a saturating flash provides a measure of the energy of the $P_{700}^+Q^-$ state, which is believed to be an intermediate in the back-reaction. We had earlier found that conversion of either Trp697 of PsaA or Trp677 of PsaB to Phe in *C. reinhardtii* PS1 resulted in a slowing of forward ET of the associated quinone, PhQ_A or PhQ_B, respectively;[61] either mutation also accelerated the charge recombination reaction by a factor of 5−7.[62] Thus, stabilization of the semiquinone state results in slower forward ET from A_1 to F_X and faster charge recombination of $P_{700}^+F_{(A/B)}^-$. In the case of the *men* mutants, where PQ replaces PhQ_t the effect on both is even larger. Here we measured decay times of 11.5 ms for *C. reinhardtii menD1* PS1 and 3.4 ms for *Synechocystis* PCC 6803 *menB* PS1 (Table S1 of the Supporting Information), the latter of which agrees very well with the previously published value of 3.2 ms,[29] giving us confidence in the accuracy of our measurements. Why the effect of PQ upon the back-reaction rate is smaller in algal PS1 than in cyanobacterial PS1, despite the similar effect on forward ET, is unclear at present. We also found that the PsaA-L722T mutant PS1, which exhibited a faster rate of forward ET from PhQ_A to F_X,[10] underwent charge recombination at a slower rate (Table S1 of the Supporting Information). We also saw the effect of the PsaA-L722T mutation upon the back-reaction rate in PS1 loaded with PQ_t although it was a bit smaller (Table S1 of the Supporting Information).

Role of Protonation of the Quinone and Its H-Bond Donor. Although reduction of PQ would be relatively easy for the ET chain of PS1, the transfer of a proton to the semiquinone must precede (or occur concomitantly with) the second ET, as reduction of the semiquinone anion would be too unfavorable.[27] For example, full reduction of the Q_B^- semiquinone to a quinol in type 2 RCs requires proton transfer,[63−67] and there is discussion about whether the second ET event is a proton-coupled ET.[68] The double reduction of Q_A demonstrated in type II RCs some time ago[69−72] is likely more analogous to photoinactivation of PS1 in the *menD1/menB* mutants. However, this seemed to occur only under extreme conditions (e.g., in the presence of strong reductants and low-potential electron transfer mediators, inhibitors, etc.). We do, however, expect a protonation event to be an essential and likely rate-limiting step in the PQ double-reduction process in *menD1/menB* PS1. Although the importance of protonation is a general feature of redox enzymes using quinones as electron donors and acceptors, it is likely to be especially important in PS1, which has evolved to inhibit protonation of its quinones, as double reduction of the quinone would inactivate it as an ET cofactor (see discussion below and Discussion in ref 55). We have performed two different experiments to examine the role of protonation in this process.

In the first experiment, we lowered the pH to increase the local H^+ concentration. This resulted in faster photoinactivation, consistent with a protonation event (or events) limiting the rate of the double-reduction process. The second experiment was more subtle. In this, we used a point mutation in the PhQ_A-binding site that results in faster ET from PhQ_A to F_X, PsaA-L722T.[10] On the basis of our analysis of this mutant and work performed with a similar mutant (PsaA-L722W) in *Synechocystis* PCC 6803,[55,73] the H-bond donated to PhQ from the peptide N atom of residue 722 of PsaA (Leu in WT PS1) seems to be weakened in the substitution mutants, perhaps because of steric constraints. Srinivasan et al.[55] have suggested that one role of the H-bond may be to block protonation of the PhQ_t thereby preventing its double reduction and inactivation of one of the branches. Contrary to the expectation of this hypothesis, we did not see significant photoinactivation of PsaA-L722T PS1 containing PhQ_t but there could be several reasons for this (e.g., different species, different mutation, an overly short experimental timeline, etc.).

Weakening the H-bond to the quinone could have two different effects, which would be partially compensatory: destabilizing the semiquinone anion state (PhQ^-), thereby accelerating its reoxidation by F_X, and increasing the rate of protonation of the semiquinone. In the experiment described by Srinivasan et al.,[55] illumination of PsaA-L722W PS1, which had been frozen after reduction by dithionite in the dark, resulted in loss of the transient flash-induced $P_{700}^+A_1^-$ EPR signal. This was interpreted as double reduction of the quinone; although never directly tested, this hypothesis seems to be a reasonable explanation. In the case described here, the quinone in the A_1 site was changed from PhQ to PQ_t drastically slowing the rate of semiquinone reoxidation.[32,33,51] In the *menD1/psaA-L722T* double mutant PS1, we did see more rapid photoinactivation than in the *menD1* mutant. Importantly, the effect of the mutation on photoinactivation was more pronounced at higher pH values, where protonation would be more limiting. The mutation may therefore lower an energy barrier encountered during the transfer of a proton to the semiquinone, which is consistent with the hypothesis of Srinivasan et al.[55] Altogether, we feel that these data serve as compelling evidence for the role of protonation in the double reduction of PQ by PS1.

Mechanism of Double Reduction of PQ by PS1. There are several ways in which PQ could be reduced to PQH_2 by PS1. One could imagine dissociation of plastosemiquinone from the A_1 site (more likely as $PQH^\bullet$, rather than the anionic form), followed by a dismutation reaction outside of PS1 ($2PQH^\bullet \rightarrow PQ + PQH_2$). This seems very unlikely to us for the following reasons. First, the dismutation reaction would produce PQ and PQH_2 in equal proportion, yet we saw very little PQ and mostly PQH_2 after photoinactivation (Figure 3). Second, it was necessary to concentrate the particles 20-fold by microfiltration before solvent extraction, to keep the volume of the extract sufficiently small. If quinol were produced outside of PS1, we would expect to lose >90% of it in the filtrate, but we recovered ∼75% of the quinone content as PQH_2 and ∼15% as PQ in the extract of the concentrated particles. Thus, we can eliminate this possibility and focus on mechanisms in which PQH_2 is generated inside PS1.

There are two main ways in which one could imagine PQ being doubly reduced at the A_1 site. The first resembles the mechanism by which Q_A is likely doubly reduced in type II RCs.[74,75] In this model, the first charge separation produces $P_{700}^+PQ^-$, and then a second charge separation occurs on the same branch (after P_{700}^+ is re-reduced) to generate $P_{700}^+A_0^-PQ^-$. The transfer of an electron and a proton to the semiquinone then leads to $P_{700}^+A_0PQH_2$. There are two main problems with this model. The first is that the semiquinone state is short-lived (lifetime of ≈8−9 μs in *menD1* PS1),[32] making it unlikely to persist until the next flash elicits a second CS. The second difficulty is that the current model of charge separation in PS1 assigns the $ec2^+ec3^-$ pair as the first charge-separated state,[56,76] and the negative charge on PQ^- would likely prohibit CS on the same branch as the semiquinone. In fact, in a point mutant of PS1 in which the lifetime of PhQ_A^- is

increased 100-fold, one sees redirection of CS to the B-branch (S. Santabarbara, F. Rappaport, and K. E. Redding, manuscript in preparation), consistent with this notion. However, protonation of the plastosemiquinone before the second CS would alleviate both of these problems. Thus, we cannot exclude this possibility.

In the second model, the FeS clusters are involved in the double-reduction process. There are two different ways to imagine this occurring. In the first scenario, CS generates $P_{700}^{+}PQ^{-}$, and then a second charge separation occurs on the other branch (for the reasons mentioned above) to generate $P_{700}^{+}PQ_A^{-}PQ_B^{-}$. Then a dismutation reaction occurs, likely by way of an intermediate in which F_X is reduced and one of the quinones is protonated $[P_{700}^{(+)}PQH^{\bullet}F_X^{-}]$. ET from the FeS cluster to the semiquinone (and further protonation) would result in production of PQH_2 in one of the A_1 sites. In the second scenario, the first CS results in ET all the way to the terminal FeS clusters, and the second CS generates $P_{700}^{+}PQ^{-}F_{(A/B)}^{-}$. ET from the terminal clusters (via F_X) to the semiquinone would then yield PQH_2. Thus, these are just two variations on the same theme, in which a reduced FeS cluster reduces a semiquinone to quinol. We must note that our experiments do not allow us to distinguish between these general scenarios. However, we do not favor the first scenario for two reasons. First, it suffers from the same problem as the first model (short lifetime of the $P_{700}^{+}PQ^{-}$ state compared to that of $P_{700}^{+}FeS^{-}$); it is much more likely that the second CS event would take place in the context of a reduced FeS cluster than a semiquinone. Second, the first scenario requires use of both branches, but a branch is effectively dead once PQH_2 is generated in it. Thus, although the first scenario might occasionally be operative (in RCs with two active branches), it cannot explain photoinactivation beyond 50% (i.e., all RCs having one PQ and one PQH_2).

We routinely observed a small but measurable decrease (<10%) in the amplitude of the P_{700}^{+} bleaching signal in the presence of the native PhQ. This initial decrease, which recovers by at least ~50% after 20 min in the dark, is likely due to the limitation of the donors (ascorbate and PMS). Thus, the "recovery" observed after the train of flashes would be due to recharging of PMS by ascorbate. If a significant fraction of the donor to P_{700}^{+} is not itself reduced, then one would expect the accumulation of RCs maintaining P_{700}^{+} over time. This will in turn retard the double-reduction process. Use of a faster donor to P_{700}^{+} would then be expected to accelerate the photoinactivation process by keeping more PS1 RCs reduced (quenching the P_{700} cation), thus leading to more charge separations. This prediction was realized when we added Pc as an electron donor (Tables S2 and S3 of the Supporting Information).

The overall process of complete photoinactivation of *menD1/menB* PS1 is expected to be very inefficient for several reasons and might be multiphasic, as well. Generation of the state(s) leading to double reduction would be a rare event, as described above. A large portion of the RCs would back-react within the 66 ms between flashes. Moreover, the fraction of RCs with oxidized P_{700} likely increases as the experiment progresses, further lowering the efficiency of double reduction, as discussed above. We have attempted to fit the photoinactivation time course to the simplest model. In this model, there are three different types of PS1 RCs: A (with two active quinones), B (with one active quinone), and C (with no active quinones). At the start, all RCs are type A. With each flash, there is a certain probability (P_{AB}) that a type A RC will be converted to a type B RC, and a similar probability (P_{BC}) that a type B RC will be converted to a type C RC. We assume that the PQ-loaded PS1s are perfectly bidirectional (i.e., going through the A-branch 50% of the time) and that the two sides are not significantly different. (This is probably close to the truth, as the energetic differences produced by exchanging quinones are much larger than those produced by the differences between the two quinone sites.) Thus, the P_{700}^{+} bleaching signal produced by a type B RC would be half of that of a type A RC, and the type C RC would produce no signal. With all these simplifications, we can satisfactorily model the data at pH 5.5 using PMS as donor with a P_{AB} of $\approx 1.6 \times 10^{-4}$ and a P_{BC} of $\approx 2 \times 10^{-5}$ (Figure S4 of the Supporting Information). While this model is certainly oversimplified, it does provide a ballpark estimate of the efficiency of double reduction with each flash. However, this should be recognized as a gross underestimate of the inherent efficiency of the process, for the reasons discussed above.

Double reduction of the native quinone (PhQ) in PS1 had been reported previously,[77,78] but only under reducing conditions created by addition of dithionite, typically at high pH. To explain how PhQ was reduced to $PhQH_2$ in the presence of ditionite, Sétif and Bottin[78] proposed a reaction scheme very similar to the one that we favor. These authors also noted that the process of double reduction may be very slow and inefficient as the quinol form progressively accumulates. While we doubt that the process we describe here is fundamentally different from the double reduction of PhQ in the presence of dithionite, the quantitative measurement of PQ reduction in the absence of a strong exogenous reductant and the use of saturating flashes permit a more quantitative analysis of the process and allow one to make conclusions about the origins of the electrons used to double reduce the quinone with more confidence.

■ ASSOCIATED CONTENT

Supporting Information

Nanosecond pump−probe measurements (Figure S1), the requirement of light for inactivation at pH 5.5 (Figure S2), the resistance to further photoinactivation in PhQ-reconstituted samples (Figure S3), comparison of the photoinactivation rate to calculated data using a model of photoinactivation described in the text (Figure S4), charge recombination rates of all PS1 samples used in this study (Table S1), extents of photoinactivation under all conditions described in the text (Table S2), and the effect of increasing the repetition rate of the actinic flashes and of using a faster electron donor to P_{700}^{+} (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CS, charge separation; ET, electron transfer; HPLC, high-performance liquid chromatography; Pc, plastocyanin; PhQ, phylloquinone; $PhQH_2$, phylloquinol; PMS, phenazine methosulfate; PQ, plastoquinone; PQ^- or $PQH^\bullet$, plastosemiquinone (anionic or neutral form); PQH_2, plastoquinol; PS1, photosystem 1; PS2, photosystem 2; RC, reaction center.

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