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Title:

The Mehler reaction site is the Phylloquinone within Photosystem I

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ABSTRACT

Photosynthesis is a vital process, responsible for fixing carbon dioxide, and producing most of the organic matter on the planet. However, photosynthesis has some inherent limitations in utilizing solar energy. Up to a third of the energy absorbed is lost in the reduction of O₂ to produce the superoxide radical (O₂^{•−}), which occurs principally within photosystem I (PSI) *via* the Mehler reaction. Strikingly, the precise location as well as the evolutionary role of the reaction have long been a matter of debate. For decades, O₂ reduction was assumed to take place solely in the distal iron-sulfur clusters of PSI rather than within the two asymmetrical cofactor branches. Here we demonstrate that under high irradiance, O₂ photoreduction by PSI takes place at the phylloquinone of one of the branches (the A-branch). This conclusion derives from the light dependency of the O₂ photoreduction rate constant, and from the high rates of O₂ photoreduction in PSI complexes lacking iron-sulfur clusters and in a mutant PSI, in which the lifetime of this phyllosemiquinone state is extended 100-fold. On these grounds, we suggest that the Mehler reaction serves as a release valve, functioning only when needed, under conditions where both the distal iron-sulfur clusters of PSI and the mobile ferredoxin pool are over reduced.

SIGNIFICANCE STATEMENT

Photosynthesis is the process responsible for the oxygenation of the ancient anoxic atmosphere, and the transformation of inorganic carbon to most of the organic matter on Earth. However, it is less commonly appreciated that the appearance of oxygen in the atmosphere led to the unavoidable opposite process in which oxygen is consumed, thereby producing deleterious oxygen radicals such as the superoxide radical. For almost half a decade, the location of the main site of superoxide radical production in chloroplasts has been a matter of debate. We now provide conclusive evidence that it is located in the phylloquinones(s) within photosystem I.

INTRODUCTION

Under high irradiance, oxygenic phototrophs can use molecular oxygen (O_2) as an alternative sink for surplus electrons within the photosynthetic apparatus (1). Unfortunately, uncontrolled leakage of electrons to O_2 could decrease the quantum yield of photosynthesis and produce reactive oxygen radicals. As a result, the photosynthetic apparatus appears to have evolved strong regulation of such reactions in an aerobic environment (2).

The production of hydrogen peroxide (H_2O_2) in spinach thylakoid membranes under illumination was first reported by Alan Mehler (3). Later, the superoxide radical ($O_2^{\bullet-}$) was shown to be the primary product of this reaction (4). Nowadays, O_2 photoreduction to $O_2^{\bullet-}$ by a photosynthetic electron transfer (ET) chain is referred to as the true Mehler reaction, in order to distinguish it from a Mehler-like reaction (5, 6). Although some evidence indicates that $O_2^{\bullet-}$ production can take place in various locations (2, 7–9), photosystem I (PSI) seems to be the major site of this process (10, 11).

The PSI complex mediates a light-driven charge separation that is accompanied by plastocyanin (Pc) oxidation and ferredoxin (Fd) reduction (Fig. 1A). Two large membrane subunits, PsaA and PsaB together with a smaller extrinsic subunit PsaC coordinate the ET cofactors comprising six chlorophyll *a* (Chl) molecules, two phylloquinone (PhQ) molecules, and three [4Fe-4S] clusters. With the exception of the [4Fe-4S] clusters, all the other cofactors are organized within two asymmetric branches (named A- and B-). Both the A- and B-branches are active (12). One of the most critical differences between them is related to the redox properties of PhQs, where a higher potential quinone is located in the A-branch, while the B-branch has a lower potential quinone (Fig. 1A). Notably, the environment influences the properties of PhQ at the A_1 -sites, so that the E_m and lifetimes of the phyllosemiquinones ($PhQ^{\bullet-}$) are modulated *via* the identity of nearby amino acids. The most drastic effect published so far was due to the replacement of PsaA-Phe₆₈₉ with Asn (PsaA-F₆₈₉N mutant), which resulted in a ~2 orders of magnitude longer lifetime of $PhQ^{\bullet-}$ at the A-branch ($PhQ_A^{\bullet-}$) (from ~0.25 μ s to 17 μ s) (13), probably as a result of a ~125-mV upshift of the $PhQ_A/PhQ_A^{\bullet-}$ reduction potential (Fig. 1B).

Fig. 1. Diagram of electron transfer in Photosystem I of wild type (A) and PsaA-F₆₈₉N (B) with lifetimes and E_m of cofactors according to (14); blue lines represent E_m of O₂/O₂^{•−} in water (−160 mV) and dimethylformamide (−500 - −600 mV) (15). Modified from (2).

Oxygen photoreduction in PSI is generally thought to occur in the F_A/F_B clusters. This common belief stems from the fact that, in isolated thylakoid membranes, F_A/F_B are the terminal cofactors, and they reduce O₂ under steady-state illumination in the absence of Fd (11, 16–18). However, the primary site of O₂ photoreduction in thylakoid membranes was shown to be the heterodimer PsaA/PsaB, rather than PsaC carrying F_A/F_B clusters (11). A contribution of PhQs to the Mehler reaction was first suggested by (19) and a first experimental evidence for the involvement of PhQs in O₂ photoreduction under steady-state illumination was provided by (20). However, a contribution of PhQ at the A- and B-branches as well as contribution of [4Fe-4S] clusters, especially the intermediate F_X cluster, are still open questions. The mechanisms underlying the O₂^{•−} production within PSI occurring concomitantly with electron transfer to Fd and then to NADP⁺ and with varying irradiance are still unclear.

The aim of the present study was to identify where and how photoreduction of O₂ occurs within PSI. Towards that goal, we measured the apparent second-order rate constant of O₂ photoreduction by PSI complexes (k_2) as a function of irradiance. We investigated intact fully mature PSI complexes isolated from a model organism, green alga *Chlamydomonas reinhardtii*, and PSI complexes lacking either the F_A/F_B (F_X-core), or all three [4Fe-4S] clusters (A₁-core). In addition, we investigated PSI complexes harboring the PsaA-F₆₈₉N mutation, which results in a long-lived PhQ_A^{•−} (13). Our results strongly support the conclusion that PhQs, especially PhQ_A, are the major site of O₂ photoreduction within PSI under high irradiance.

RESULTS

Under continuous illumination, O₂ photoreduction is the rate-limiting step of electron transfer

Identification of the sites of O₂ photoreduction within PSI under steady-state illumination requires the ET to O₂ to be the rate-limiting step of total ET. This can be accomplished by providing an efficient electron donor to P₇₀₀⁺. Here, we used excess Pc to reduce P₇₀₀⁺ in isolated fully mature intact PSI complexes. In order to verify the Pc concentration, we used the approach described in (20) for cyanobacterial PSI complexes and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as an electron donor to P₇₀₀⁺. Since increasing the concentrations of Pc in this model did not affect the O₂ photoreduction rate (Fig. 2A), we conclude that the O₂ photoreduction is indeed the rate-limiting step of the overall reaction. The same result was observed in our system when Pc was replaced by TMPD (Fig. S1). Although the rate constant for TMPD ($3.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (21)) is ~ four orders of magnitude lower than that of Pc, the rates of O₂ photoreduction were the same. Neither did increasing the concentration of electron donor affect the rate of O₂ photoreduction when all the [4Fe-4S] clusters were removed (Fig. S2), although the

basal rate was higher (see further). In contrast, when MV, an efficient redox-mediator from PSI to O_2 , was added to intact PSI complexes, the rate of O_2 photoreduction did increase with increasing Pc concentration (Fig. 2A). Thus, we can conclude that the electron donation to P_{700}^+ from Pc/TMPD was the rate-limiting step only in the presence of efficient electron acceptors such as MV.

Fig. 2. Effect of plastocyanin concentration on the O₂ photoreduction rate in the absence or presence of MV at 50 μM (A) and effect of irradiance on the apparent second order rate constant of O₂ photoreduction by PSI in the absence of MV (B) and in the presence of MV at 4 μM (light blue line) and 50 μM (dark blue line) (C); Panel B, the O₂ photoreduction rate (dashed lines) is shown for comparison; Panel C, the intact PSI (green line) is shown for comparison. The experiments conditions were: Mature wild type PSI complexes, 10 nM (B and C) or 15 nM (A); Asc, 10 mM; catalase, 500 U ml⁻¹ (B and C); Pc, varied (A) or 5 μM (B and C); initial O₂ concentration, 10-30 μM for measurement of apparent k_2 (B and C) and 250 μM for measurement of O₂ photoreduction rate (A and black dashed lines on panel B); irradiance, 2200 μmol photons m⁻² s⁻¹ (A). Values are mean for 7-10 replicates for k_2 and 3-4 replicates for rates; ±SE.

Our working hypothesis is that under steady-state illumination, there are multiple oxygen photoreduction sites in PSI, each catalyzing a mechanistically independent reaction associated with a discrete rate constant. The accumulation of electrons on these sites changes in response to irradiance. In other words, each site reaches maximal activity at a different specific irradiance. Therefore, if the apparent second-order rate constant of O₂ photoreduction by PSI (k_2) is not the same at all light levels, but is rather a function of irradiance, it means that multiple oxygen photoreduction sites are involved. A physical model to explain this behavior would be to postulate that a site must be reduced in order to be able to transfer an electron to O₂. If there are few electron acceptors, electrons become “stuck” in the system, with the terminal sites filling up first. Hence, electrons start to accumulate at the F_A/F_B centers under low irradiance and then, with increasing irradiance, they begin to occupy the F_X site and then A₁-sites.

Determination of the second-order rate constant of O₂ photoreduction in PSI

The exact rate constant of the Mehler reaction is not currently known and estimates for the kinetic parameters for O₂ photoreduction by PSI range from ~10³ M⁻¹ s⁻¹ (22) to 10⁷ M⁻¹ s⁻¹ (23). The mid value (7 × 10⁴ M⁻¹ × s⁻¹) was obtained by kinetic modeling of charge recombination kinetics observed after a light flash (24). We measured the apparent k_2 for intact fully mature PSI complexes purified from the wild type (WT) strain (PSI^{WT}), under conditions where O₂ was the principal electron acceptor. In this case, k_2 was denoted as $k_2^{O_2}$. The apparent $k_2^{O_2}$ varied slightly between different PSI preparations and was estimated to be ~3.5 × 10⁵ M⁻¹ s⁻¹ at the highest irradiance tested (2200 μmol photons m⁻² s⁻¹). Fig. 2B shows the dependency of the apparent $k_2^{O_2}$ on irradiance as measured in a typical PSI preparation. The apparent $k_2^{O_2}$ clearly increased with irradiance up to 2200 μmol photons m⁻² s⁻¹. The O₂ photoreduction rate measured at atmospheric pressure of O₂ as a function of irradiance resembles that of $k_2^{O_2}$ (Fig. 2B, dashed line), as expected.

Saturating concentrations of Methyl Viologen (MV) *de facto* produce a single O₂ reducing site

As F_A/F_B are oxidized completely by an excess of MV, the effect of irradiance was examined in the presence of MV at saturating concentration (50 μM). In this case, the measured constant was denoted as k_2^{MV} since MV was the principal electron acceptor from PSI and was constantly recycled by reduction of O₂. Indeed, the apparent k_2 and O₂ uptake rates were higher in the presence of MV (Fig. 2A and 2C), reflecting higher PSI turnover. While direct O₂ photoreduction by PSI cofactors can take place at the same

time as MV reduction, it will be largely suppressed at saturating MV concentrations. In contrast to behavior of $k_2^{O_2}$, the values of the apparent k_2^{MV} reached a plateau at 220 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 2C, dark blue circles), demonstrating that in the presence of high concentrations of MV, there is *de facto* a single O_2 photoreduction site. However, when MV was added at a 12-fold lower concentration (4 μM), nearing the $K_m(\text{MV})$ value determined in separate experiments (Fig. S3), k_2^{MV} increased with increasing irradiance and did not reach a plateau (Fig. 2C, light blue circles), *i.e.*, the behavior of k_2^{MV} at 4 μM MV was more similar to that of $k_2^{O_2}$. Since at concentrations around the K_m , MV would account for only about half of the maximal rate, we conclude that electrons are still accumulating at intermediate PSI cofactors and are available for direct reduction of O_2 . In order to negate the possibility that the saturation behavior of k_2^{MV} at 50 μM MV is due to limitations in re-reduction of P_{700}^+ , we replaced Pc by TMPD, a less efficient electron donor than Pc. In the presence of TMPD, the apparent k_2^{MV} was lower but still reached a plateau at the same irradiance (Fig. S4), confirming that the irradiance dependence of the apparent k_2^{MV} is not influenced by processes taking place at the donor side of PSI.

Stripping PSI of its [4Fe-4S] clusters enhance O_2 photoreduction

The hypothesis that there are multiple sites within PSI for O_2 photoreduction can be tested by removal of the terminal cofactors. This should remove the competition for electrons and favor reduction of O_2 by upstream cofactors (*i.e.*, F_X , PhQ_A , and PhQ_B). To test the role of F_X and PhQ in O_2 photoreduction, we sequentially stripped PSI of the [4Fe-4S] clusters. Initially, the subunit PsaC containing the F_A/F_B clusters was removed, resulting in a PSI complex, in which the terminal cofactor is F_X (F_X -core complexes). The behavior of the apparent $k_2^{O_2}$ in the F_X -core complexes was rather similar to that of the intact complexes (Fig. 3A, yellow circles *vs.* green squares), especially at high irradiance, with no stimulation of O_2 photoreduction observed. This result suggests that F_X plays a minor role in O_2 photoreduction in PSI. Removal of F_A/F_B actually decreased O_2 reduction at low irradiances. This result supports the contention that F_A/F_B plays a role in O_2 reduction in low light. As the next stage, we removed F_X , leaving the bound PhQ molecules at the A_1 -sites as the terminal cofactors (A_1 -core complexes). In contrast to the results obtained with the F_X -core, A_1 -core complexes showed markedly higher rates of O_2 photoreduction at irradiance above 350 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 3A, blue triangles *vs.* green squares). Interestingly, O_2 photoreduction in the A_1 -core complexes in low light is faster than in F_X -core complexes. This indicates that F_X is a poor reductant of O_2 . Taking together, these results demonstrate that none of the [4Fe-4S] clusters is essential for photoreduction of O_2 by PSI. Furthermore, they indicate that the PhQ s are the moieties responsible for O_2 photoreduction in intact PSI in high light.

Fig. 3. Effect of irradiance on the apparent rate constant of O₂ photoreduction by F_X- and A₁-core complexes (A) and by the PsaA-F₆₈₉N mutant (B). Intact wild type PSI (green line) is shown for comparison. The experiments conditions were: F_X-core, A₁-core complexes, PSI^{AFN}, 10 nM; TMPD, 1 mM; Asc, 10 mM; catalase, 500 U ml⁻¹; initial O₂ concentration, 10-30 μ M. Values are mean for 7-10 replicates; $\pm S_E$.

A mutant PSI^{AFN}, in which PhQ_A⁻ has a longer lifetime, demonstrates dramatically enhanced O₂ photoreduction

One might argue that the results with the A₁-core complexes were merely due to unspecified damage caused by the removal of the [4Fe-4S] clusters. In order to test our hypothesis in intact PSI particles, we made use of the PsaA-F₆₈₉N mutant, in which PhQ_A⁻ has a lifetime about two orders of magnitude longer than in the WT (13). Slowing down ET from PhQ_A should increase the chances that O₂ can catch electrons from PhQ_A⁻ *en route* to downstream cofactors, obviating the need to remove them. The behavior of charge recombination kinetics in PSI complexes from this mutant (PSI^{AFN}) more closely resembled that of WT A₁-core complexes than intact PSI^{WT} (Fig. S5, Petrova, in preparation) showing a diminished population with the reduced F_A/F_B. Here, we used mature PSI^{AFN} complexes capable of reducing NADP⁺ in the presence of Fd and FNR under steady-state illumination (Table S1). However, the rates of NADP⁺ reduction in PSI^{AFN} complexes were 2-5 times lower than in PSI^{WT}. Our hypothesis predicts faster rates of O₂ reduction in the PsaA-F₆₈₉N mutant than in intact PSI^{WT} complexes. Indeed, at intensities above 350 $\mu mol\ photons\ m^{-2}\ s^{-1}$, the values of the apparent $k_2^{O_2}$ in PSI^{AFN} were markedly higher than those of intact PSI^{WT} (Fig. 3B, pink circles vs. green squares) and the curve resembled that of WT A₁-core complexes (Fig. 3A, blue triangles) more than WT intact PSI.

O₂ photoreduction does not compete with NADP⁺ reduction

One might question the relevance of these *in vitro* results to the situation *in vivo*, where electron acceptors such as Fd are available to PSI. In order to assess the impact of such acceptors upon O₂ photoreduction by PSI, we repeated the experiment with the addition of Fd, FNR, and NADP⁺, *i.e.* under conditions, which simulate the situation *in vivo*. The presence of FNR and excess NADP⁺ provided an efficient electron sink for Fd, as evidenced by the measured NADP⁺ photoreduction rate (Fig. 4B).

Photoreduction of O_2 by components of the thylakoid membrane has been shown to occur concomitantly to reduction of $NADP^+$ (18). Furthermore, Fd also contributes to O_2 reduction, especially in the absence of $NADP^+$. Hence, the apparent k_2 in the presence of Fd (k_2^{Fd}) should be the sum of k_2 for ET from PSI to O_2 either directly or *via* Fd. Indeed, in the presence of Fd alone, the values of the apparent k_2^{Fd} were higher than $k_2^{O_2}$ (Fig. 4A), suggesting a strong contribution of Fd to the total O_2 reduction under such conditions. However, in the presence of FNR and $NADP^+$, we observed similar rates of O_2 photoreduction at high irradiance as in the absence of Fd/FNR/ $NADP^+$ (Fig. 4A, violet circles vs. green squares). Hence, electron flow to $NADP^+$ probably decreases the Fd-dependent O_2 reduction by inhibiting the accumulation of reduced Fd. Interestingly, in contrast to the situation at high irradiance, the presence of Fd/FNR/ $NADP^+$ slows down O_2 photoreduction at low irradiance, indicating that $NADP^+$ reduction also decreases the accumulation of electrons on [4Fe-4S] clusters. The similarity in behavior of k_2^{Fd} in the presence of FNR/ $NADP^+$ and $k_2^{O_2}$ at high irradiance strongly suggests that PhQs at the A_1 -sites represent the major site of O_2 photoreduction in PSI *in vivo* under high light.

Fig. 4. Effect of irradiance on the apparent rate constant of O_2 photoreduction in the presence of Fd and Fd + FNR + $NADP^+$ (A) and on the rate of $NADP^+$ reduction (B); the intact PSI (green line) is shown for comparison. The experiments conditions were: Mature wild type PSI complexes, 10 nM; Pc, 5 μM ; Asc, 10 mM; catalase, 500 U/ml; where indicated Fd, 5 μM ; FNR, 200 nM; $NADP^+$, 1 mM; initial O_2 concentration, 10-30 μM . Values are mean for 7-10 replicates for k_2 and 5 replicates for rates; $\pm S_E$.

DISCUSSION

In this study, we measured the rates of O_2 photoreduction by various PSI complexes and demonstrated that the apparent rate constant of this reaction depends on irradiance (Fig. 2A). This observation could be explained by either *i*) the presence of a single O_2 reduction site (*i.e.* F_A/F_B), that attains full activity under high irradiance, or *ii*) the presence of multiple O_2 reduction sites within PSI, which are activated under different light regimes. Our data are much more consistent with the latter model.

F_A/F_B is neither the sole nor the major O₂ photoreduction site within PSI

The observation that the removal of F_A/F_B slows the O₂ reduction rate at low irradiances indicates their responsibility for the process under low light (Fig. 3A). The results obtained following MV addition to the intact PSI complexes (Fig. 2C) demonstrate that there is unlikely to be a single O₂ photoreduction site in PSI. With saturating concentrations of MV, which *de facto* simulate the behavior of a single site of O₂ reduction, k_2^{MV} reached a plateau at an irradiance of 220 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Since in intact PSI, MV accepts electrons exclusively from F_A/F_B, and not from intermediate cofactors, this value represents the optimal irradiance for activating F_A/F_B. In other words, if k_2 increases any further in the absence of MV, it must indicate reduction of O₂ on upstream cofactor(s). Moreover, the contribution of upstream cofactor(s) to O₂ reduction must depend on the rate of electron withdrawal from PSI by available acceptors, since a k_2^{MV} plateau was no longer observed when lower concentrations of MV were provided (Fig. 2C) and the behavior was similar to that of $k_2^{\text{O}_2}$ at higher irradiances.

Under high irradiance, PhQ_A is the major site of O₂ photoreduction in PSI

In order to localize the site(s) of O₂ photoreduction in PSI, we removed the [4Fe-4S] clusters sequentially. Stripping PSI of F_A/F_B lowered O₂ photoreduction rates at low irradiances but had little effect at high irradiances (Fig. 3A). The additional removal of F_X resulted in a significant increase in $k_2^{\text{O}_2}$ values at irradiances exceeding 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. These results demonstrate that PhQs at the A₁-sites, and not F_X, are the major sites of O₂ reduction in intact PSI under high irradiance. The same conclusion regarding the contribution of PhQs to O₂ photoreduction was reached independently *via* experiments using the mature PSI particles from PsaA-F₆₈₉N mutant, in which the lifetime of PhQ_A^{•-} is increased from ~0.2 μs to ~17 μs (13). At irradiances higher than 350 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the $k_2^{\text{O}_2}$ values for PSI^{AFN} complexes were significantly higher than those measured for the PSI^{WT} complexes (Fig. 3B). Our interpretation is that the longer lifetime of PhQ_A^{•-} increases the chances of this semiquinone reacting with O₂. Note that the competing hypothesis that [4Fe-4S] cluster(s) are the primary site(s) of O₂ reduction at high irradiance, would have predicted the opposite result, as the population with reduced clusters is diminished in this mutant (Fig. S5, Petrova, in preparation). This is consistent with our observation of a 2-5 times lower rate of NADP⁺ photoreduction under steady-state illumination (Table S1) as well as a decrease in MV-driven O₂ reduction rates (Fig. S7) by PSI^{AFN} than by PSI^{WT} complexes.

Santabarbara reported that the lifetime of PhQ_B^{•-} in PsaA-F₆₈₉N mutant is the same as in WT (13) and is ~one order of magnitude lower than the lifetime of PhQ_A^{•-} in WT. This decreases the chances of PhQ_B^{•-} being an efficient contributor to O₂ photoreduction in intact PSI. Taking all these points into consideration, we hypothesize that the results presented in Fig. 3B, demonstrate that PhQ_A^{•-} rather than PhQ_B^{•-} is responsible for O₂ photoreduction.

O₂ photoreduction does not compete with NADP⁺ reduction

Our data revealed that at high irradiance, the major contribution of PhQs within PSI to O₂ photoreduction is valid even when Fd and NADP⁺ reduction takes place, *i.e.* under conditions simulating physiological conditions. Addition of Fd alone significantly increased the rates of O₂ reduction (Fig. 4A). This partially imitates the situation *in vivo* when the stromal NADP⁺ pool is in the reduced state (*e.g.*, due to a retarded Calvin cycle at limiting CO₂). However, since we used Fd at much higher levels relative to PSI than present *in vivo* (500:1 ratio vs. 5:1 ratio in spinach chloroplast; (25)), care should be taken when extrapolating this result to the *in vivo* situation. It is likely that the addition of FNR and NADP⁺ minimized the Fd-dependent O₂ reduction by decreasing the accumulation of reduced Fd. These observations are in good agreement with previously reported conclusions (18, 26). The results of our experiments indicated saturation of NADP⁺ photoreduction at high irradiances (Fig. 4B). This provides insights into the *in vivo* situation, since these are conditions, when the Mehler reaction plays a physiological role in protecting the photosynthetic apparatus from over-reduction of the ET chain and when the PhQs pass electrons to O₂. This can suggest a crucial role for PhQs, and specifically PhQ_A (see above), in O₂ photoreduction in PSI *in vivo*.

Here we used PSI complexes isolated from *C. reinhardtii*. The acceleration of H₂O₂ in the chloroplasts as well the increase in enzymatic antioxidant capacity was shown to occur when *C. reinhardtii* cells were subjected to high light (27). However, the physiological significance of the true Mehler reaction in angiosperms is much well recognized. Since the composition of the ET cofactors in PSI is highly conserved through all green lineages, O₂ photoreduction by PhQs represents a universal mechanism of the Mehler reaction.

MATERIALS AND METHODS

Strains, growth, thylakoid membrane and PSI isolation

C. reinhardtii strains with His₆-tag added to the N-terminus of the PsaA protein (PBC1 pKR152 for WT and PBC1 pKR411 for the strain bearing a mutation in codon 689 of *psaA* exon 3 (*psaA-3*)) were grown in TAP medium at 25 °C with constant air flow under continuous white light (~70 μmol photons m⁻² s⁻¹). The culture was harvested, and thylakoid membranes and mature PSI complexes were purified as described in (28). The fraction corresponding to the mature PSI complexes was collected, concentrated, and stored at -80 °C. The maturity of the complexes was confirmed (Table S1).

Expression and purification of recombinant proteins

The recombinant *C. reinhardtii* Pc, Fd and ferredoxin-NADP⁺ oxidoreductase (FNR) were heterologously expressed in *Escherichia coli* BL21 cells, and purified as described in (28) and (29).

Determination of P_{700}^+

The concentration of photo-oxidized P_{700}^+ was determined by measuring light minus dark absorption changes at 698 nm ($\epsilon = 100 \text{ mM}^{-1} \times \text{cm}^{-1}$)(30). The assay medium contained 30 $\mu\text{g Chl ml}^{-1}$ PSI, 20 mM NaCl, 5 mM MgCl_2 , 10 mM Asc, 10 nM Pc, 50 μM methyl viologen (MV), 0.03% β -DDM, 20 mM Tricine-NaOH (pH 7.5) was placed in spectrophotometer (Cary 50, Varian) and illuminated with saturating white light (Intralux 5000, Volpi).

Measurement of O_2 concentration changes using MIMS

Changes in O_2 (the stable isotope $^{16}\text{O}_2$) concentration were measured with a Membrane Inlet Mass Spectrometer (MIMS, QMS 200 M1; Pfeiffer Vacuum) according to (31); the data collection frequency was 1 s. Measurements were conducted under constant stirring at 21°C. The signal was calibrated by calculating the difference in values obtained in air saturated buffer (containing 250 $\mu\text{M O}_2$) and in O_2 -depleted buffer after removal of O_2 by glucose (10 mM), glucose oxidase (30 U ml^{-1}), and catalase (500 U ml^{-1}). A suspension of PSI complexes in buffer containing 20 mM NaCl, 5 mM MgCl_2 , 0.03% β -DDM, 20 mM Tricine-NaOH (pH 7.5), and electron donors and acceptors as indicated in figure legends, was placed in a sealed cuvette and illuminated by red light provided by a Dual-PAM (Walz GmbH).

In preliminary experiments, using the approach described in (20, 32), we confirmed that light-dependent O_2 consumption resulted from direct ET from PSI cofactors to O_2 (Table S2 and Table S3). The light-induced rate of O_2 consumption (V_{O_2}) was calculated by subtraction of the dark slope from the light slope.

In experiments aimed for determination of k_2 , the O_2 concentration in the reaction medium was decreased to 10-30 μM by N_2 flushing of the headspace over the suspension in the sealed vessel, followed by transfer of the suspension by syringe to the air-depleted sealed cuvette containing the MIMS inlet probe and stir bar. It was necessary to restrict the O_2 concentration because k_2 can only be determined if the rate of the reaction depends on the concentration of O_2 (Fig. S6). In these experiments, catalase (500 U ml^{-1}) was added to prevent accumulation of H_2O_2 , which is decomposed by [4Fe-4S] clusters to give a highly reactive hydroxyl radical capable of inhibiting PSI. In addition, accumulated $\text{H}_2^{16}\text{O}_2$ molecules (mass 34) could potentially interfere with the $^{16}\text{O}_2$ (mass 32) signal, and introduce errors. The rate constant was calculated as described in Appendix.

Preparation of F_X -core and A_1 -core

The F_X -core complexes were obtained by incubating PSI complexes in buffer containing 6.8 M urea (33). The A_1 -core complexes were obtained by incubating F_X -core complexes in buffer containing 3.4 M urea and 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$. The treatment efficiency was followed by monitoring the acceleration

of charge recombination kinetics from ca. 50 ms in the intact PSI complexes to 1 ms and 10 μ s in F_X-core and A₁-core respectively (Fig. S5).

NADPH measurement

Light induced NADPH production was detected by a Dual-PAM equipped with DUAL-ENADPH and DUAL-DNADPH modules. The signal was calibrated by adding a known concentration of NADPH.

Chlorophyll extraction and measurement

Chl concentration was determined spectrophotometrically after extraction in 96% ethanol according to (34).

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