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Rapid Degradation of the Tetrameric Mn Cluster in Illuminated, PsbO-Depleted Photosystem II Preparations

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Abstract—A "decoupling effect" (light-induced electron transport without O_2 evolution) was observed in Ca-depleted photosystem II (PSII(−Ca)) membranes, which lack PsbP and PsbQ (Semin et al. (2008) *Photosynth. Res.*, **98**, 235-249). Here PsbO-depleted PSII (PSII(−PsbO)) membranes (which also lack PsbP and PsbQ) were used to examine effects of PsbO on the decoupling. PSII(−PsbO) membranes do not reduce the acceptor 2,6-dichlorophenolindophenol (DCIP), in contrast to PSII(−Ca) membranes. To understand why DCIP reduction is lost, we studied light effects on the Mn content of PSII(−PsbO) samples and found that when they are first illuminated, Mn cations are rapidly released from the Mn cluster. Addition of an electron acceptor to PSII(−PsbO) samples accelerates the process. No effect of light was found on the Mn cluster in PSII(−Ca) membranes. Our results demonstrate that: (a) the oxidant, which directly oxidizes an as yet undefined substrate in PSII(−Ca) membranes, is the Mn cluster (not the Y_Z radical or $P680^+$); (b) light causes rapid release of Mn cations from the Mn cluster in PSII(−PsbO) membranes, and the mechanism is discussed; and (c) rapid degradation of the Mn cluster under illumination is significant for understanding the lack of functional activity in some PSII(−PsbO) samples reported by others.

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Light drives the oxidation of water in plant photosynthesis, and O_2 evolution is a byproduct of the process. Water oxidation occurs within photosystem II (PSII) at a metal cluster composed of four Mn cations and one Ca^{2+} cation [1], which is part of the O_2-evolving complex (OEC). The Mn_4Ca cluster is shielded from the bulk solution by three peripheral extrinsic proteins (the Mn-stabilizing protein, PsbO, and the two Ca^{2+}/Cl^--stabilizing proteins, PsbP and PsbQ, found in plants and green algae) located on the luminal side of the PSII membrane (see [2] for a review). Extraction of PsbP and PsbQ from the OEC by high salt treatment is accompanied by the release of a calcium cation from the OEC [3]. Such Ca-depleted PSII preparations (PSII(−Ca)), which still contain the Mn_4 cluster, are unable to evolve O_2 [3]. However, if isolated in the absence of an exogenous chelator, PSII(−Ca) samples do not evolve O_2 but can oxidize an unknown substrate (possibly water), producing electrons for the reduction of an exogenous electron acceptor (2,6-dichlorophenolindophenol (DCIP)) on the reducing side of PSII [4]. This phenomenon was termed the "decoupling effect" [4, 5]. A similar light-induced electron transfer across PSII without O_2 evolution was also observed earlier in thylakoid membranes treated with the detergent lauroylcholine chloride [5].

PSII(−Ca) membranes obtained by treatment with 2 M NaCl still contain the PsbO extrinsic protein [3]. This protein stabilizes the Mn cluster since its extraction by $CaCl_2$-treatment of PSII allows for the release of two Mn ions over several hours of subsequent dark incubation [6]. Usually the extraction of the PsbO protein is performed by treatment of native PSII membranes with 1 M $CaCl_2$, and the release of this protein occurs along with the extraction

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FIK, fluorescence induction kinetics; OEC, oxygen-evolving complex; PSII, photosystem II; PSII(−Ca), Ca-depleted PSII; PSII(−PsbO), PsbO-depleted PSII; RC, reaction center; TMB, 3,3′,5,5′-tetramethylbenzidine.

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of PsbP, PsbQ, and the Ca^{2+} ion from the OEC [7]. In our previous study we observed that PSII membranes lacking the PsbP and PsbQ proteins can exhibit the decoupling effect mentioned above, depending upon how PSII(−Ca) membranes are prepared (i.e. plus or minus the presence of a chelator) and whether or not Ca^{2+} is specifically re-added to the membranes [4]. In the current study we examined how the extraction of the third extrinsic protein (PsbO) influences the decoupling effect. We found that PSII membranes, which have lost all of the extrinsic proteins as well as the Ca^{2+} ion (only one Ca in the OEC) (PSII(−PsbO)), cannot photoreduce the exogenous electron acceptor DCIP (i.e. the decoupling effect is not observed), and the reason for this is the rapid loss of Mn(II) from the Mn cluster as soon as the material is exposed to light. Our results show that: (a) the oxidant, which oxidizes an as yet undefined substrate (the ultimate source of electrons for DCIP reduction) in PSII(−Ca) membranes, is the Mn cluster (not the Y_Z radical or $P680^+$); (b) light causes the rapid release of Mn cations from the Mn cluster in PSII(−PsbO) membranes; and (c) the rapid degradation of the Mn cluster induced by light is significant for understanding discrepancies in previous EPR S2 signal observations (or lack of them) in PsbO-depleted PSII membranes. For example an EPR S2 signal coming from the Mn cluster has not always been detected in PSII(−PsbO) membranes [8-10], depending on the reported experimental conditions (i.e. Cl^- concentration, preliminary illumination, etc.).

MATERIALS AND METHODS

PSII preparations. PSII-enriched membrane fragments (BBY-type) were prepared from market spinach following the protocol of Ghanotakis and Babcock [11]. The O_2-evolving activity of the native PSII membranes, measured polarographically, ranged from 450 to 550 μmol O_2·mg chlorophyll $(Chl)^{-1}\cdot h^{-1}$, when 0.2 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) was used as an artificial electron acceptor. The preparations were stored at −80°C in buffer A containing 15 mM NaCl, 400 mM sucrose, and 50 mM MES/NaOH (pH 6.5). Chlorophyll concentrations were determined in 80% acetone according to the method of Arnon [12]. Samples were thawed in the dark at 5°C in a refrigerator for 70 min before treatment or measurement.

Ca^{2+}, PsbP, and PsbQ were removed from control PSII membranes using a buffer solution containing 2 M NaCl, 0.4 M sucrose, and 25 mM MES (pH 6.5) but no chelator [4]. The PSII preparations were incubated in this buffer at 0.5 mg/ml Chl for 15 min under room light (4-5 $\mu E\cdot m^{-2}\cdot sec^{-1}$) at room temperature (22°C). The resulting material was washed twice with buffer A, resuspended in buffer A, and called PSII(−Ca) membranes.

PsbO protein was extracted according to the method of Ono and Inoue [7] with minor modifications. PSII membranes (1 mg Chl/ml) were incubated in 1 M $CaCl_2$, 0.4 M sucrose, 15 mM NaCl, and 50 mM MES (pH 6.5) for 30 min in the dark at 5°C. The resulting material was washed twice with buffer A, resuspended in buffer A, stored at −80°C, and termed PSII(−PsbO) membranes.

Initial rate of exogenous DCIP photoreduction (1500 $\mu E\cdot m^{-2}\cdot sec^{-1}$ from a 150 W halogen lamp, 15 sec of illumination, 40 μM DCIP) was determined spectrophotometrically at 600 nm using an apparent molar extinction coefficient, $\varepsilon = 17.5\ mM^{-1}\cdot cm^{-1}$ (pH 6.5) for the rate calculations [13].

Determination of Mn content in PSII samples. Manganese assays were performed according to the method Semin and Seibert [14] with minor modifications. Samples (100 μg Chl) for Mn assay were suspended in 1 ml buffer A and then incubated with 50 mM $CaCl_2$ for 2 min in the dark at 5°C. After incubation the sample was centrifuged at 12,000 rpm for 5 min at 5°C in an Eppendorf tube, and the membrane pellet was resuspended in 90 μl HCl (0.6 M) at room temperature (22°C). Then the sample was stirred in the Eppendorf tube for 1 min using a Vortex mixer. Next 0.9 ml of distilled water was added to the membrane suspension, and finally the microfuge tube was recentrifuged for 3 min at 12,000 rpm. The supernatant (0.9 ml) was filtered through a 13-mm Acrodisc syringe filter containing a 0.2 μm nylon membrane (Pall Life Sciences, USA). Syringes and filters were pre-washed, first with 60 mM HCl and then with distilled water prior to the above-mentioned procedures. The filtrate (in a 1-ml glass or quartz cuvette) was mixed consecutively with 40 μl of 2 M NaOH, 40 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (100 mg TMB in 100 ml of 0.1 M hydrochloric acid), and 40 μl of 5.3 M phosphoric acid. A control sample was also prepared by stirring 0.1 ml 0.6 M HCl in a Eppendorf tube for 1 min using a Vortex mixer, followed by the addition of 0.9 ml distilled water. After the control sample was centrifuged for 3 min in a microfuge at room temperature, 0.9 ml of the mixture was removed with a plastic syringe and passed through a prewashed Acrodisc syringe filter into a cuvette. After that the reagents (NaOH, TMB, and phosphoric acid) were added at the same volume and in the same order as above. The absorbance at 450 nm was used to calculate the Mn(II) concentration in the samples ($\varepsilon = 34\ mM^{-1}\cdot cm^{-1}$ [15]), taking into account the dilution factor and volume of extract adsorbed by the filter during the filtration. The Mn content in all PSII samples was calculated on the basis of 250 molecules of Chl/reaction center (RC) [16].

Fluorescence induction kinetics (FIK). FIK were measured under continuous saturating actinic light (3000 $\mu E\cdot m^{-2}\cdot sec^{-1}$) using a Hansatech Instruments, Ltd., portable Plant Efficiency Analyser (UK). The fluorimeter employed LEDs as the source of excitation light (λ_{max} = 650 nm; spectral range, 580-710 nm). The time resolution of the fluorescence detection system was 10 μsec (within the initial 2 msec), 1 msec (within the

time interval 2 msec to 1 sec), and 100 msec (at a time interval >1 sec). The fluorescence signal 50 μsec after the start of the actinic light was defined as F_0. The total period illumination was 2 sec. PSII membranes were suspended in buffer A at a concentration of 25 μg Chl/ml. A logarithmic time scale is used in the figure as commonly employed for the presentation of FIK data.

RESULTS AND DISCUSSION

Extraction of the PsbP and PsbQ extrinsic proteins, together with the Ca^{2+} ion from the OEC in the absence of a chelator, is accompanied by strong inhibition of O_2 evolution (by up to 86%) but rather weak inhibition of DCIP reduction function (the residual activity of the latter was 65% in the absence of added Ca^{2+}) (Table 1). These data are consistent with previously reported results [4]. However, if the PsbO extrinsic protein is extracted together with the PsbP and PsbQ proteins (after treatment with 1 M $CaCl_2$), the PSII complexes lose their ability to reduce DCIP. The residual O_2-evolution and DCIP-reduction activities in PSII(−PsbO) membranes are in fact roughly the same, about 12 and 7%, respectively (Table 1). This result indicates the strong effect of the PsbO protein on oxidation of the unknown substrate mentioned in the introductory section (i.e. the source of electrons for the DCIP reduction) in PSII(−PsbO) membranes, because the only protein difference between the PSII(−Ca) sample (exhibiting the decoupling effect) and the PSII(−PsbO) membranes (no decoupling) is the absence of PsbO protein in the latter samples.

It is known that the PsbO protein does not participate in the coordination of either the Mn or Ca ions [7]. However, its extraction has a strong effect on the stability of the Mn cluster since Miyao and Murata [6] and Ono and Inoue [17] reported dark incubation of PSII(−PsbO) membranes leads to the loss of two Mn ions after several hours. Considering the Mn-stabilizing effect of this protein, we suggest that the extraction of PsbO is accompanied by an enhanced sensitivity of the Mn cluster when PSII(−PsbO) membranes are exposed to light for a short period of time. In Table 2 we report the results of several experiments in which we measured Mn contents in different PSII membranes treated in different ways before illumination. Darkness (up to 1 h; Table 2) alone did not alter the Mn content of PSII(−PsbO) membranes. However, after brief illumination (15 sec) under conditions used to determine the rate of DCIP reduction (1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$ from a 150 W halogen lamp, 15 sec of illumination, 40 μM DCIP), the PSII centers lost most of their Mn ions – about 3 Mn/reaction center (RC) (Table 2).

The most likely explanation for the rapid degradation of the Mn cluster after illumination in the presence of an electron acceptor is the fact that PSII(−PsbO) preparations do not contain any of the extrinsic proteins that normally shield the Mn cluster from the bulk solution. Therefore, the Mn cluster is fully exposed to exogenous reductants, which can reduce and then release Mn(II) ions from the cluster [18]. In our experiments we used DCIP as an electron acceptor. Part of the DCIP is reduced at the beginning of the illumination period, and the resultant $DCIPH_2$ generated can itself affect the state of the Mn cluster. To confirm this hypothesis, we studied the effect of reduced DCIP on the Mn content on the unprotected Mn cluster. $DCIPH_2$ was generated by reducing DCIP during the illumination of native PSII membranes, followed by pelleting of the membranes. The resulting supernatant was used subsequently as $DCIPH_2$ reagent. Dark incubation of PSII(−PsbO) membranes with this $DCIPH_2$ solution rapidly (15 sec) released the majority of the Mn ions (about 3 Mn/RC; Table 2) from the sample, which had not previously been exposed to light. However, the interaction of the reduced electron acceptor with the Mn cluster is not the only reason for loss of the cluster since the illumination of PSII(−Ca) membranes in the presence of DCIP (Table 2) does not degrade the Mn cluster (the Mn cluster is also accessible

Table 1. Rates of light-induced O_2 evolution and DCIP reduction in different PSII preparations

Sample	Functional activity			
	Oxygen evolution, %		DCIP reduction, %	
	$-Ca^{2+}$	$+Ca^{2+b}$	$-Ca^{2+}$	$+Ca^{2+b}$
PSII	100[a] ± 4	107 ± 3	100[a] ± 3	106 ± 3
PSII(−Ca)	14 ± 1	72 ± 2	65 ± 1	80 ± 1
PSII(−PsbO)	12 ± 1	16 ± 1	7 ± 1	9 ± 1

[a] 100% values correspond to 450-550 μmol $O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$ and 140-150 μmol $DCIP \cdot mg\ Chl^{-1} \cdot h^{-1}$. No chelator was present during preparation of the sample.

[b] Membranes (20 μg Chl/ml) were incubated with 30 mM $CaCl_2$ in the dark for 10 min at 5°C before measurement.

Table 2. Light effects on the Mn content of native, Ca-depleted, and PsbO-depleted PSII membranes

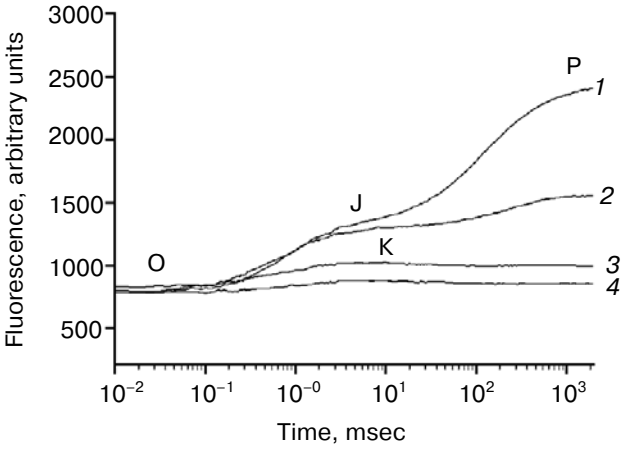
Sample	Conditions	Mn content per RC
PSII	dark	4.0 ± 0.2
	light + 40 μM DCIP	4.0 ± 0.1
PSII(−PsbO)	dark	4.0 ± 0.2
	light	3.0 ± 0.4
	light + 40 μM DCIP	1.2 ± 0.2
	light + 40 μM DCIP + + 500 mM NaCl	2.7 ± 0.2
	light + 40 μM DCMU	3.3 ± 0.3
	light + 1 μM DCBQ	2.5 ± 0.1
	light + 200 μM DCBQ	2.6 ± 0.1
	dark + $DCIPH_2$ (15 sec)	1.2 ± 0.1
PSII(−Ca)	dark	4.0 ± 0.2
	light + 40 μM DCIP	4.0 ± 0.1

Notes: Dark, 1 h; light, 1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$ for 15 sec. $DCIPH_2$, 20 μg Chl/ml native PSII membranes were incubated with 40 μM DCIP under the same light (1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$) but for 3 min. Then the membranes were pelleted, and the supernatant was used as the source of reduced DCIP.

to $DCIPH_2$ reduced during illumination or to endogenous reductants like H_2O_2 when PsbO is present). This implies that destabilization of the Mn cluster upon PsbO extraction makes the Mn cluster more sensitive to exogenous/endogenous reductants. Indeed, the addition of Cl^- anions (500 mM), a well known stabilizing factor in the absence of PsbO [6], delays but does not totally inhibit the degradation process (Table 2).

The loss of Mn from the cluster was also observed in the presence of DCBQ, another exogenous electron acceptor (Table 2), but the rate of Mn release was lower (2.5 ± 0.1 Mn/RC remained after 15 sec of illumination, twice that observed with DCIP). This can be explained by the hydrophobic nature of DCBQ, which can delay diffusion of reduced DCBQ from the acceptor side of the PSII(−PsbO) membrane to the donor side, or by its lower affinity for interaction with the Mn-cluster. Illumination of PSII(−PsbO) membranes without DCIP, but in the presence of DCMU (allowing only one turnover of the RC) or in the absence of DCMU (allowing at least three turnovers), is also accompanied by Mn extraction but with lower efficiency (the residual Mn values are 3.3 and 3.0 Mn/RC, respectively). The explanation for Mn-release in this case is probably related to the same reductant-dependent mechanism. H_2O_2 (also a reductant for oxidized Mn-ion states), generated on the donor side of PSII in the light when the OEC is damaged (see [19] for a review), might also participate in the reduction of Mn ions and their release from the OEC in the absence of an exogenous electron acceptor.

Damage to the Mn cluster during illumination is supported by measurements of chlorophyll a FIK (figure). In native PSII samples, the FIK curve (figure, curve *1*) exhibits three characteristic points corresponding to F_0 (point O), the yield of fluorescence when Q_A is reduced (point J), and the F_{max} level (point P), where the plastoquinone pool (which quenches fluorescence in the oxidized form [20]) is also reduced. Damage to the Mn cluster significantly changes the FIK curve, where a new peak, K, close to point J seen in native PSII membranes appears, and also indicates Q_A reduction [21]. However, the fluorescence yield subsequently decreases after peak K since there is no additional electron transfer from the donor side of PSII to continue to reduce Q_A, and Q_A is rapidly reoxidized by Q_B [21]. The extraction of PsbP and PsbQ together with Ca^{2+} in PSII(−Ca) membranes does not change the shape of the FIK curve, although it does reduce its extent (figure, curve *2*; [4]). This result shows that PSII(−Ca) membranes are capable of electron transport across PSII as are native PSII membranes (see Table 1). In contrast the FIK curve of PSII(−PsbO) membranes clearly shows the inhibition of electron transfer from the OEC to Q_A since peak K is present. This means that Q_A is



Chlorophyll a fluorescence induction curves for dark adapted PSII membranes (25 μg Chl/ml) in buffer A: *1*) native PSII membranes; *2*) PSII(−Ca) membranes; *3*) PSII(−PsbO) membranes (exposed to 3000 $\mu E \cdot m^{-2} \cdot sec^{-1}$ for 1 sec in the absence of an artificial electron acceptor); *4*) PSII(−PsbO) membranes (incubated for 15 sec with 40 μM DCIP under 1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$ of light followed by pelleting and resuspension in buffer A)

reoxidized by Q_B but without the subsequent reduction of oxidized Q_A by electrons from the OEC (figure, curves *3* and *4*). In fact, the fluorescence yield declines after the first maximum in the region of peak K is reached, similar to the FIK curve of Mn-depleted PSII membranes [21]. This result indicates damage to the Mn cluster in the samples shown in curves *3* and *4* and is consistent with the results in Table 2. Curve *3* corresponds to the FIK of PSII(−PsbO) samples, when the damage to the Mn-cluster occurs under actinic light exposure (3000 $\mu E \cdot m^{-2} \cdot sec^{-1}$) in the absence of an artificial electron acceptor. In this experiment 2.1 Mn/RC were lost (note that the data in Table 2 for a similar experiment (light in the absence of DCIP) employed a lower light intensity). Curve *4* reflects the FIK of PSII(−PsbO) samples that were previously incubated for 15 sec of illumination (1500 $\mu E \cdot m^{-2} \cdot sec^{-1}$ of halogen lamp light) with 40 μM DCIP followed by a centrifugation step to remove the DCIP.

Thus, PSII(−PsbO) membranes cannot support the photooxidation of the endogenous substrate (probably water [4]), in contrast to PSII(−Ca) membranes, due to the rapid reduction and release of Mn cations from the OEC in the former during the initial stage of illumination. This means that the oxidation of substrate (the source of electrons for the reduction of DCIP observed as the decoupling effect) by PSII(−Ca) membranes is catalyzed by the Mn cluster and not the Y_Z radical or $P680^+$ (i.e. the substrate does not donate directly to either the Y_Z radical or $P680^+$). Our results also show that the Mn cluster in the OEC becomes particularly sensitive to light-generated reductants in PSII membranes when the Mn-stabilizing PsbO protein is not present. Destruction of the Mn cluster when light is present (loss of Mn is much slower in PSII(−PsbO) membranes that are not exposed to light), especially at low Cl^- concentration, can now explain some of the discrepancies in previous reports of light-dependent functional parameters of PSII(−PsbO) membranes (e.g. why the S2 EPR signal arising from the native Mn cluster is not always detected in PsbO-depleted PSII membranes [8-10]). Therefore, the rapid loss of Mn ions from the OEC of illuminated PSII(−PsbO) membranes, especially in the presence of exogenous electron acceptors, is essential for understanding the reason for the lack of functional activity in some PSII(−PsbO) samples reported in the literature.

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