

The extrinsic PsbO protein modulates the oxidation/reduction rate of the exogenous Mn cation at the high-affinity Mn-binding site of Mn-depleted PSII membranes

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Abstract The oxidation of exogenous Mn(II) cations at the high-affinity (HA) Mn-binding site in Mn-depleted photosystem II (PSII) membranes with or without the presence of the extrinsic PsbO polypeptide was studied by EPR. The six-lines EPR spectrum of Mn(II) cation disappears in the absence of the PsbO protein in membranes under illumination, but there was no effect when PSII preparations bound the PsbO protein. Our study demonstrates that such effect is determined by significant influence of the PsbO protein on the ratio between the rates of Mn oxidation and reduction at the HA site when the membranes are illuminated.

Keywords Photosystem II · PsbO extrinsic protein · High-affinity Mn-binding site · Mn(II) · Oxygen-evolving complex

Introduction

Oxygenic photosynthesis is accompanied by the water-oxidation to O₂-evolution, and is catalyzed by a catalytic metal cluster within the O₂-evolving complex (OEC) of photosystem II (PSII). The OEC is composed of four Mn cations, one Ca²⁺ cation, and five oxygen atoms

(Umena et al. 2011). The cluster is located on the luminal surface of the PSII reaction center (Seibert et al. 1987). Access to the site is limited by three peripheral extrinsic proteins (the Mn-stabilizing protein, PsbO, and the two Ca²⁺/Cl⁻-stabilizing proteins, PsbP and PsbQ), found in plants and green algae (reviewed in Bricker et al. 2012). All of these proteins shield and protect the Mn₄CaO₅ cluster from the bulk solution.

The Mn₄CaO₅ metal cluster can be extracted from the OEC by two methods: Tris treatment at alkaline pH and hydroxylamine treatment. Tris treatment extracts the Mn, Ca cation, and all three extrinsic proteins (PsbO, PsbP and PsbQ) from PSII (Yamamoto et al. 1981), whereas NH₂OH treatment produces Mn,Ca-depleted membranes with a single remaining PsbO extrinsic protein (Tamura and Cheniae 1987). It is known that the Mn cluster is rather stable in PSII membranes in the absence of the PsbP and PsbQ polypeptides, whereas the extraction of PsbO destabilizes the Mn cluster so that it slowly loses 2 Mn cations from the OEC in the dark (Ono and Inoue 1984). It is due to this effect that PsbO is called the “Mn-stabilizing protein”.

Mn-depleted PSII membranes have only one site (the high-affinity, Mn-binding site) for binding exogenous Mn(II) cations (Ono and Mino 1999). The Mn(II) ion bound to this site is oxidized by the Y_Z radical under illumination (Hoganson et al. 1989), and this reaction is the first step of the photoactivation (the process of Mn cluster reconstruction in Mn-depleted PSII membranes (Ono and Mino 1999)). We investigated Mn(II) cation oxidation at the HA site using EPR techniques and found that the presence of PsbO significantly changes the oxidation/reduction rate of Mn cations bound to the HA Mn-binding site.

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Materials and methods

PSII preparations

PSII-enriched membrane fragments (BBY-type) were prepared from market spinach leaves following Ghanotakis and Babcock (1983). The O₂-evolving activity of the native PSII membranes, measured polarographically, ranged from 450 to 550 μmol O₂ mg Chl⁻¹ h⁻¹, when 0.2 mM 2,6-dichloro-*p*-benzoquinone (Sigma-Aldrich) 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 buffer (pH 6.5). Chlorophyll concentrations were determined in 80 % acetone, according to the method of Porra et al. (1989). The concentration of PSII reaction centers (RC) is presented in μM on the basis of 250 molecules of Chl/RC of PSII sample (Ghanotakis et al. 1984; Xu and Bricker 1992).

2,6-dichlorophenolindophenol (DCPIP) reduction

The initial rate of DCPIP photoreduction (calculated for the deprotonated form of DCPIP) was determined spectrophotometrically during the first 15 s of illumination at 600 nm, using the molar extinction coefficient, $\epsilon=21.8 \text{ mM}^{-1} \text{ cm}^{-1}$ for calculations (Armstrong 1964).

Ca²⁺ depletion

Ca²⁺, PsbQ, and PsbP were removed from native PSII membranes using a buffer solution containing 2 M NaCl, 0.4 M sucrose, and 25 mM MES/NaOH (pH 6.5) (Ono and Inoue 1990). The PSII preparations were incubated in this buffer at 0.5 mg/ml Chl for 15 min under room light (4–5 μE m⁻² s⁻¹) and at room temperature (22 °C). The resulting material was washed twice with buffer A, resuspended in buffer A, and called PSII(-Ca) membranes.

Mn depletion by Tris treatment

Manganese-depletion was accomplished by incubating thawed PSII membranes (0.5 mg Chl/ml) in 0.8 M Tris-HCl buffer (pH 8.5) for 15 min in the light at room temperature. The membranes were then pelleted by centrifugation in an Eppendorf MiniSpin microfuge (12,000 rpm × 10 min), washed 3 times with buffer A to remove non-specific Mn(II), and finally re-suspended in buffer A. These membranes, which do not contain any extrinsic proteins (including PsbO polypeptide), Ca²⁺ ion, and the Mn catalytic cluster, are called PSII(-Mn/Tris) membranes. Residual Mn content in Mn-depleted PSII membranes after Tris treatment was 0.3 ± 0.1 Mn/RC.

Mn depletion by hydroxylamine treatment

Manganese-depletion was also accomplished by incubating thawed Ca-depleted PSII membranes (0.5 mg Chl/ml) for 5 min at 5 °C in the dark with 1 mM hydroxylamine in buffer A (Miyao and Inoue 1991). The membranes were then pelleted by centrifugation and washed 3 times with buffer A. These membranes, which do not contain two of the extrinsic proteins (PsbP and PsbQ), Ca²⁺ ion, and the Mn cluster, are called PSII(-Mn/NH₂OH) membranes. Residual Mn content in Mn-depleted PSII membranes after hydroxylamine treatment was 0.4 ± 0.1 Mn/RC.

Blocking of the high-affinity Mn-binding site with iron cations

PSII(-Mn/Tris) membranes (20 μg Chl/ml [0.08 μM]) were incubated in buffer A, containing 10 μM FeSO₄ under cool white fluorescent room light (4 μE m⁻² s⁻¹, PAR; 1-cm optical pathlength) for 3 min at room temperature (Semin et al. 2002). The membranes were then pelleted, washed once with buffer A, and subsequently resuspended in buffer A.

Determination of the Mn content in PSII samples

Mn assays were performed according to the colorimetric method suggested by (Semin and Seibert 2009) with minor modifications (Semin et al. 2013).

EPR measurements

EPR was used to detect the light-dependent oxidation of exogenous Mn(II) cations (MnCl₂·4H₂O) in different PSII preparations by measuring six-line spectra. PSII membranes (1 mg Chl ml⁻¹) were put into a flat-type quartz EPR cell (70 μl) in buffer A and illuminated directly in the cavity of an EPR spectrometer (RE1307, SCB AS, USSR) with continuous white light (1500 μE m⁻² s⁻¹). The EPR settings were: microwave power 20 mW, modulation amplitude 10 G, time constant 10 ms, sweep time 20 s, sweep width 1000G. All experiments we performed at 20 °C.

Kinetics of exogenous Mn(II) cations oxidation/reduction induced by turn on or turn off continuous white light (1500 μE m⁻² s⁻¹) were monitored using EPR spectrometer at room temperature. The composition of the sample: PSII(-Mn/Tris) membranes (1 mg Chl/ml [4 μM RC]); DCPIP, 40 μM; Mn(II), 4 μM; buffer A. The magnetic field was fixed on the third line of exogenous Mn(II) cation six-line EPR spectra. Other EPR settings were as follows: time constant, 10 ms; microwave power, 20 mW; field modulation amplitude, 10 G.

Sample characterization

SDS-urea-PAGE was performed in the presence of 6 M urea and 2 % SDS. Aliquots of PSII membranes (15 µg Chl) were loaded on the gel. The gels were stained with Coomassie blue R-250.

Results

Mn-depleted PSII without PsbO The EPR spectra in Fig. 1 show the interactions of Mn(II) cations with PSII(-Mn/Tris) membranes in the dark and under illumination. These PSII membranes don't contain PsbO. Mn(II) cations (4 µM) in buffer A (pH 6.5) without PSII membranes exhibit the usual six-line EPR spectrum (sample 1, Fig. 1). Addition of PSII(-Mn/Tris) membranes to this solution at a concentration of 4 µM (the ratio of Mn(II):RC is 1:1) is accompanied by some decrease in the spectrum amplitude (about 25 %, sample 2, left; Fig. 1), possibly due to firm binding of part of the Mn(II) cations by amino acids residues at the membrane surface.

Under illumination the six-line spectrum of Mn(II) disappears (sample 2, right; Fig. 1) due to the oxidation of the manganese cations. The spectrum of this sample was measured without the addition of the exogenous electron acceptor, DCPIP, whereas the next sample (sample 3, left; Fig. 1) was measured with DCPIP (40 µM) present. DCPIP addition doesn't affect the Mn(II) EPR spectrum, and illumination causes the disappearance of the 6-lines spectrum as seen in the previous sample (sample 2). It is interesting that switching off the light is accompanied by a rapid, dark reduction of the Mn(III) pool (sample 4, left; Fig. 1; see below for more explanation). This result demonstrates that under illumination two reactions involving Mn cations occur—the light-induced oxidation of Mn(II) cations and the reduction of the Mn(III) cations. Thus, we can observe the competition between the Mn(II) oxidation and the Mn(III) reduction processes by measuring the amplitude of the six-line EPR signal. To determine the rates of Mn(II) oxidation/Mn(III) reduction, we measured the kinetics of the disappearance of the six-line spectrum after the light is turned on and its appearance after light is turned off (Fig. 2a). We obtained $t_{1/2}=90$ ms for the Mn(II) oxidation rate and $t_{1/2}=300$ ms for the Mn(III) reduction rate. These results clearly demonstrate that the rate of Mn(II) oxidation is significantly higher than the rate of Mn(III) dark reduction. Oxidation of Mn(II) cations occur via the high-affinity (HA), Mn-binding site by Y_Z^* reduction as demonstrated in two samples (Fig. 3, samples 1 and 2). In the first sample (sample 1, Fig. 3) the HA site was occupied by Mn cation (we used Ca-depleted PSII membranes). No Mn(II) oxidation was observed in this sample under illumination. In the second

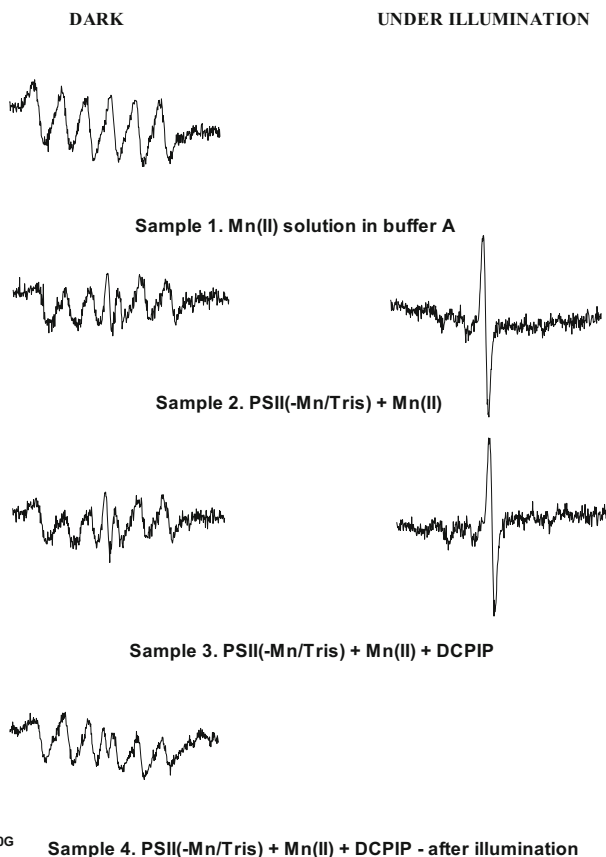


Fig. 1 EPR spectra of Mn-depleted PSII membranes prepared by Tris treatment. Concentrations: PSII(-Mn/Tris), 1 mg Chl/ml (4 µM RC); DCPIP, 40 µM; Mn(II), 4 µM

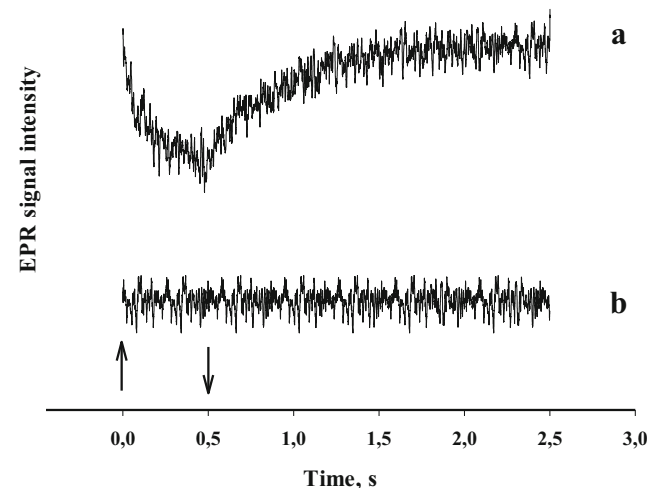


Fig. 2 The kinetic of disappearance and appearance of exogenous Mn(II) six-line spectrum in Mn- depleted PSII membranes without PsbO (a) and with PsbO (b) after the light was turned on (first arrow) and turned off (second arrow). Concentrations: PSII(-Mn/Tris), 1 mg Chl/ml (4 µM RC); DCPIP, 40 µM; Mn(II), 4 µM. Details of EPR measurements are presented in Materials and Methods section

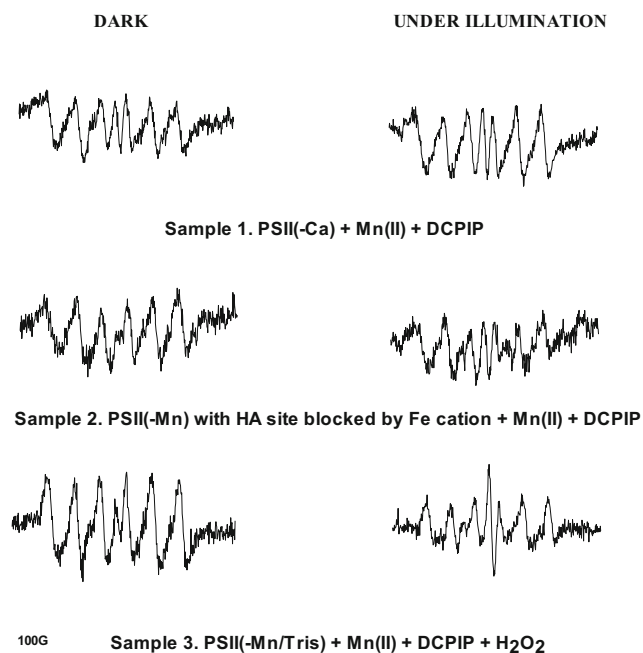


Fig. 3 Oxidation of Mn(II) in different PSII membranes. Concentrations: PSII(-Ca), PSII(-Mn), or PSII(-Mn/Tris), 1 mg Chl/ml (4 μ M RC); DCPIP, 40 μ M; Mn(II), 4 μ M; H₂O₂, 3 mM

sample (sample 2), the HA site in the Mn-depleted PSII was blocked by a Fe cation, which prevents the donation of electrons from Mn(II) cations to Y_Z^* through this site (Semin et al. 2002). In this sample similar to sample 1 (Fig. 3), no oxidation of Mn(II) occurred under illumination (sample 2, compare left and right; Fig. 3). The following experiments confirmed the conclusion that the disappearance of the six-line spectrum, when PSII(-Mn/Tris) membranes were illuminated, is caused by the higher rate of Mn(II) oxidation as compared to the rate of Mn(III) reduction. It is known that the rate of Mn(II) oxidation at the HA site increases significantly in the presence of H₂O₂ due to the fast reduction of Mn(III) by H₂O₂ (Boussac et al. 1986; Inoue and Wada 1987). Indeed, a significant increase in the electron transfer rate from the donor side to the artificial electron acceptor, DCPIP, occurs in the presence of H₂O₂ (Table 1). We used this effect of H₂O₂ to increase the rate of Mn(III) reduction. The addition of hydrogen peroxide to the sample containing PSII(-Mn/Tris) membranes, Mn(II) cations, and DCPIP causes the appearance of the six-line spectrum (sample 3, right; Fig. 3 compare with sample 3 in Fig. 1) in the light since the rate of Mn(III) reduction becomes comparable with the rate of Mn(II) oxidation.

Mn-depleted PSII with PsbO We observed a quite different effect of light on the six-line EPR spectrum of Mn(II) cations in Mn-depleted PSII membranes containing PsbO polypeptide (PSII[-Mn/NH₂OH] membranes) compared with PSII(-Mn/

Table 1 The rate of DCPIP reduction in Mn-depleted PSII membranes with or without the extrinsic PsbO polypeptide in the presence of the following electron donors: Mn(II) cations or Mn(II) cations in the presence of H₂O₂

Sample Addition	Rate of DCPIP reduction (μ mol•mg Chl ⁻¹ •h ⁻¹)	
	PSII(-Mn/Tris)	PSII(-Mn/NH ₂ OH)
0	5±0.4 (5 %) ^a	2±0.3 (2 %)
MnCl ₂	8±0.5 (7 %)	4±0.5 (3 %)
MnCl ₂ +H ₂ O ₂	122±2 (110 %)	67±2 (60 %)

^a 100% - the rate of DCPIP reduction by native PSII membranes (112±2 μ mol DCPIP•mg Chl⁻¹•h⁻¹). Concentration of components: 20 μ g Chl/ml PSII membranes (0.08 μ M RC); 3 mM H₂O₂; 4 μ M MnCl₂; 40 μ M DCPIP

Tris), which don't contain PsbO. The presence of the PsbO protein in the membranes treated with NH₂OH and the absence of this extrinsic protein in PSII(-Mn/Tris) membranes was shown by gel electrophoresis (Fig. 4). Electrophoretogram is presented on the Fig. 4a while optical density traces from SDS-PAGE of PSII preparations is shown on Fig. 4b. Our results clearly show that Tris extracts PsbO protein whereas hydroxylamine doesn't. Extraction of all extrinsic proteins (PsbO, PsbP and PsbQ) by Tris and two extrinsic proteins (PsbP and PsbQ) by NH₂OH have been shown correspondingly by Yamamoto et al. (1981) and Tamura and Cheniae (1987). The EPR results are presented in Fig. 5. Interestingly, the mixing of the Mn(II) solution (this EPR spectrum is presented on Fig. 5, left; sample 1) with the PSII(-Mn/NH₂OH) membranes is not accompanied by the appearance of the EPR-silent portion of Mn cations (compare sample 1 with sample 2, left; Fig. 5 with samples 1 and 2 in Fig. 1). This result differs from that obtained with PSII(-Mn/Tris) membranes, where we observed the appearance of an EPR-silent portion of the Mn cations. Possibly this data is evidence that part of exogenous Mn(II) cations in PSII(-Mn/Tris) (without PsbO protein) sample are ligated by amino acid residues participating in the binding of PsbO to the PSII membrane. In PSII(-Mn/NH₂OH) membranes, these amino acids residues are blocked by PsbO. Another interesting fact was also discovered. Opposite to PSII(-Mn/Tris) preparation (sample 3, Fig. 1), illumination of PSII(-Mn/NH₂OH) membranes (4 μ M RC) containing exogenous 4 μ M Mn(II) and DCPIP is not accompanied by the disappearance of the six-line spectrum (sample 3, Fig. 5, compare with sample 3, Fig. 1). In fact, EPR signal of exogenous Mn(II) doesn't change its intensity after switching on light (compare the right and left spectra of sample 3, Fig. 5). Correspondingly, when we measured the kinetic of Mn(II) oxidation in PSII(-Mn/NH₂OH) membranes turning on light we didn't observe Mn(II) oxidation (Fig. 2b) and accordingly we didn't observe the reduction of Mn cations (Fig. 2b). One could say that PSII(-Mn/NH₂OH)

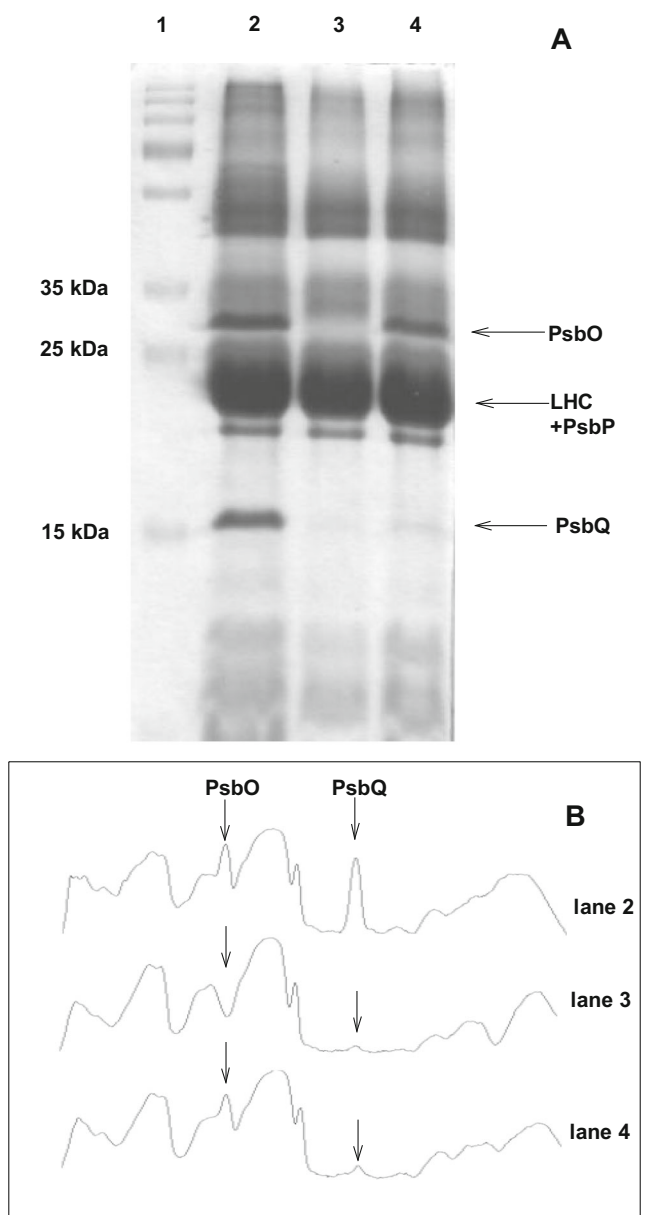


Fig. 4 Effect of Mn depletion by Tris or NH_2OH on the extrinsic proteins in PSII preparations. **a.** Polyacrylamide gel electrophoretogram of PSII membranes. The samples were treated with 2.0 % SDS. Lane 1, protein standards; lane 2, control PSII membranes (BBY type); lane 3, Mn-depleted PSII by Tris treatment; lane 4, Mn-depleted PSII by NH_2OH treatment. PS II extrinsic proteins are labeled to the right. **b.** Optical density traces from SDS-PAGE of PSII preparations showing loss of the PsbO subunit after Tris treatment

membranes are not able to oxidize Mn(II) cations under illumination. However, results presented in the Table 1 show that Mn(II) cations are oxidized by PSII(-Mn/ NH_2OH) membranes under illumination, although with a rate half that of PSII(-Mn/Tris) membranes. Therefore, we believe that the lack of a light effect on the six-line EPR spectrum of Mn(II) in PSII(-Mn/ NH_2OH) membranes is due to a change in the ratio between the rate of Mn(II) oxidation and the rate Mn(III)

reduction (under the influence of PsbO). Accordingly we observed that PsbO decreases the rate of Mn(II) oxidation (see Table 1) and increases the rate of Mn(III) reduction. To provide continuous Mn(II) oxidation, we added the artificial electron acceptor, DCPIP. It is reasonable to suggest that the reduced DCPIP molecule can participate in the reduction of Mn(III) cations. Therefore, we studied the light effect on Mn(II) content in the absence of DCPIP (sample 2, right; Fig. 5). We found that under illumination, the intensity of the six-line spectrum of Mn(II) was decreased (compared with sample 3 under illumination in the presence of DCPIP). This result shows that 1) under illumination Mn(III) cations are reduced partially by the reduced molecules of the artificial electron acceptor (DCPIP_2); 2) the absence or presence of the six-line spectrum of exogenous Mn(II) cations in Mn-depleted PSII preparations depend on the ratio between the oxidation/reduction rates of Mn cations under illumination. Mn(II) ions in a mixture of PSII(-Mn/Tris) with PSII(-Mn/ NH_2OH) membranes are available for oxidation as shown in Fig. 5 (sample 4). Here a mixture of both types of Mn-

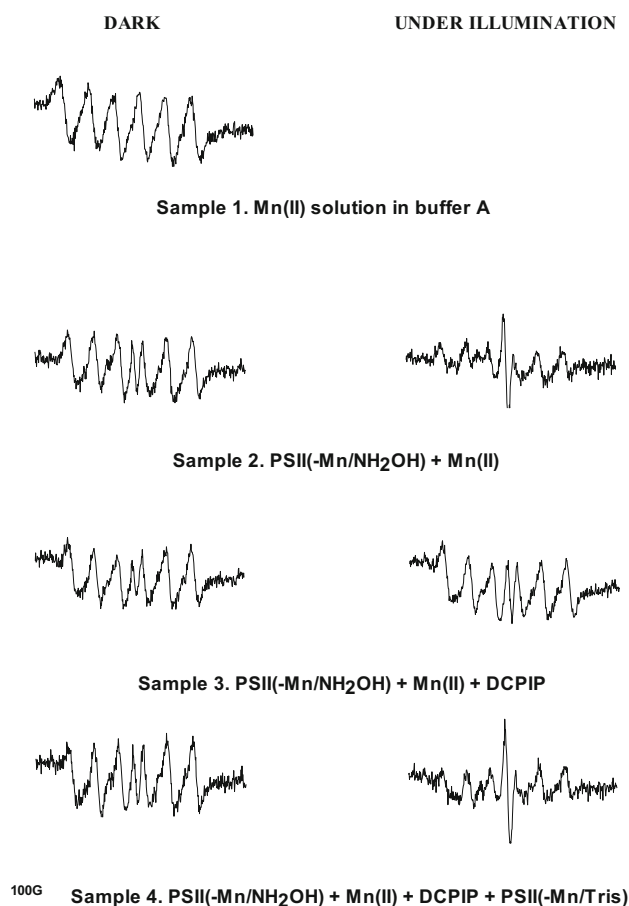


Fig. 5 EPR spectra of Mn-depleted PSII membranes prepared by NH_2OH treatment. Concentrations: PSII(-Mn/ NH_2OH), 1 mg Chl/ml (4 μM RC) in samples 1–3; DCPIP, 40 μM ; Mn(II), 4 μM . Concentrations of PSII(-Mn/ NH_2OH) and PSII(-Mn/Tris) membranes in sample 4 were 0.5 mg Chl/ml (2 μM RC)

depleted membranes (with and without PsbO) showed the oxidation of exogenous Mn(II) cations under illumination.

Discussion

Our results demonstrate that the Mn(II) cation bound to the HA Mn-binding site in Mn-depleted PSII membranes is oxidized in the light, but this oxidation reaction competes with Mn(III) reduction. PsbO has a strong effect on the oxidation/reduction rates. Without PsbO the rate of Mn(II) oxidation is much higher than the rate of Mn(III) reduction. In the presence of PsbO, the rate of Mn(II) oxidation decreased significantly (Table 1), while the Mn(III) reduction rate increased as manifest by the absence of a light effect on the six-line EPR spectrum of Mn(II) cations (sample 3, Fig. 5). As a result the six-line EPR spectrum of Mn(II) cations in illuminated membranes disappears in the absence of PsbO, but doesn't disappear when the PsbO protein is present in the PSII(-Mn) preparation. Burnap et al. (1996) concluded that the rate of Mn(II) oxidation at the HA site is higher in the absence of PsbO, which is consistent with our EPR data. Moreover, we have shown by measuring DCPIP reduction in the presence of electron donors (Mn(II) or Mn(II)+H₂O₂) that PsbO significantly inhibits DCPIP reduction. However, Hoganson et al. (1989), measuring the rate of Y_Z[•] reduction in illuminated Mn-depleted PSII membranes, have shown that PsbO doesn't influence the rate of Mn(II) cation oxidation. However, it is difficult to compare our data with the results of Hoganson et al. due to very different experimental protocols: 1) Hoganson et al. (1989) studied the oxidation of Mn(II) cations measuring Y_Z^{ox} reduction by Mn(II). We measured Mn(II) oxidation by determining the content of Mn(II), which means that Hoganson with colleagues measured the rate of electron transfer between Mn(II) and Y_Z, whereas we measured the content of Mn(II) as the sum of two processes, i.e., the oxidation and reduction of Mn cations; 2) Hoganson measured the Y_Z reduction rate after a flash, whereas we measured Mn(II) oxidation during steady-state illumination.

The rate of Mn(II) oxidation by PSII(-Mn/Tris) membranes ($t_{1/2}$ =90 ms) determined in our experiments in fact should be higher. As we discussed above the light-induced rate of Mn(II) cation oxidation at the HA Mn-binding site is determined by two competitive processes (oxidation and reduction). Thus, we determined the apparent rate of Mn(II) oxidation. Moreover, at least in PSII(-Mn/Tris) membranes, the oxidation/reduction rates determined by EPR can be underestimated due to the existence of an EPR-silent fraction of Mn cations. The reduction process is realized in the presence of reductant(s). One of the main reductants in our experiments is DCPIPH₂ (sample 2, Fig. 5), and our results are consistent with those of Chen et al. (1995), who have shown susceptibility of photoligated Mn to reductants, including

DCPIPH₂. But other reductants of unknown nature may also take part in this process.

The mechanism of PsbO modulation of the oxidation/reduction processes with Mn cations bound to the HA site in Mn-depleted PSII membranes is not clear. Possibly PsbO hinders the accessibility of Mn(II) cations to the HA site. This suggestion would explain the decrease in the Mn(II) oxidation rate in the presence of PsbO. However, PsbO doesn't influence the accessibility of DCPIPH₂ to Mn(III) at the HA site and its reduction (Fig. 5, samples 2,3). Another possible PsbO effect might be the influence of this polypeptide on the redox potential of the Mn(II)/Mn(III) couple at the HA site, which depends on the binding affinity. If PsbO increases the redox potential, it should result in a decrease in the oxidation rate of Mn(II) and an increase in the rate of Mn(III) reduction by DCPIPH₂.

Our data demonstrate that PsbO modulates the rate of the oxidation/reduction processes associated with Mn cations bound at the HA site. These data can be of interest in studying photoactivation *in vivo* or *in vitro* since the first step of the photoactivation process is the oxidation of the Mn(II) cation on the HA Mn-binding site after Mn depletion of the PSII membranes. Burnap et al. (1996) in fact showed that in the absence of PsbO, the quantum yield of photoactivation *in vivo* increases.

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