Requirements for the photoligation of Mn^{2+} in PS II membranes and the expression of water-oxidizing activity of the polynuclear Mn-catalyst

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Ligation of Mn^{2+} into the polynuclear Mn-catalyst of water oxidation was shown using PS II membranes depleted of their Mn and the 17, 23 and 33 kDa extrinsic proteins. This process specifically required light and Ca^{2+} concentrations of ~50 mM. Evidence was obtained indicating Mn^{2+}/Ca^{2+} competition for Ca^{2+} and Mn^{2+} binding sites essential for the photoligation of Mn. Photoligation of Mn did not result in an increase of water oxidation capacity; however, water oxidation capacity was expressed following dark reconstitution minimally with the 33 kDa protein. The results represent the first observation of photoactivation of water oxidation in a system that excludes simple light-driven Mn^{2+} transport across membrane(s).

Photoactivation *Manganese* *Oxygen evolution* *Photosynthesis* *Photosystem II* *Membrane protein*

1. INTRODUCTION

Many recent studies with PS II membranes have provided evidence that the expression of O_2 evolution capacity of the polynulear Mn-catalyst of water oxidation in photosynthesis can be manipulated by extraction/reconstitution of the 17, 23 and 33 kDa extrinsic proteins (reviews [1,2]). While it is clear that the 17 and 23 kDa proteins are not directly required for O_2 evolution [3], some evidence exists that the 33 kDa extrinsic protein is directly required [4]. In all cases, either partial or complete inactivation of the tetra-Mn catalyst results in the loss of water oxidation by the PS II trap/water oxidizing complex.

Previous studies with variously cultured algae [5], intact chloroplasts from leaves greened by widely spaced flashes [6,7], Tris-extracted chloroplasts [8], or leaves in which the tetra-Mn catalyst has been inactivated by NH_2OH [9,10], all indicate that light is required for appearance or reappearance of V_{O_2} [5–10] and ligation of Mn^{2+} as a tetra-Mn complex [5,9]. This light-dependent reappearance or appearance of O_2 evolution is independent of protein synthesis [5,9,10], photophosphorylation events ([5,7], see however [11]) and PS I ([5], see however [6]), and may require Ca^{2+} ([7,8], see however [9]) as well as stromal factors ([6], see however [8]). Such disparity of requirements might suggest differing light effects dependent on the complexity of the system studied. Indeed, it has been suggested [11,12] that photoactivation of O_2 evolution [5] simply reflects transport of Mn^{2+} across thylakoids to the apo-S-state complex.

Here we report requirements for photoactivation of the water-oxidizing complex in PS II membranes subjected first to extraction with $CaCl_2$ [4] and then to a reduced redox reagent [13,14] to solubilize the 17, 23 and 33 kDa extrinsic proteins [4] and ≥80% of the ~4 Mn/PS II trap [2,9,10,13,15]. The results show that photoligation of Mn^{2+} occurs in the absence of PS II extrinsic

Abbreviations: $DCIPH_2$, reduced form of 2,6-dichlorophenolindophenol; PS, photosystem; V_{O_2}, rate of O_2 evolution activity; TMF, Triton-membrane fragments

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proteins, but the expression of the catalytic activity of the photoligated Mn minimally requires the 33 kDa extrinsic protein. Rebinding of this 33 kDa protein can occur however with Mn-depleted membranes.

2. MATERIALS AND METHODS

Oxygen evolving PS II TMFs (TMF-2) were prepared [13] from wheat seedlings (*Triticum aestivum*) by modifications and combinations of procedures described in [16,17]. $CaCl_2$-TMF-2 was prepared as described [4], except the extraction with 1 M $CaCl_2$ was done twice. $DCIPH_2$-treated $CaCl_2$-TMF-2 was prepared by incubation of $CaCl_2$-TMF-2 (500 μg Chl/ml) for 1 h at 4°C in darkness in buffer A (15 mM NaCl, 0.4 M sucrose, 50 mM Mes-NaOH, pH 6.5) containing 500 μM DCIP and 2 mM Na ascorbate. Following incubation, the membranes were recovered by centrifugation (30000 × g, 10 min) and resuspended in buffer A.

For photoactivation, the $DCIPH_2$-treated $CaCl_2$-TMF-2 was suspended (250 μg Chl/ml) in 0.4 ml of buffer A containing 2 mM $MnCl_2$, 50 mM $CaCl_2$ and 100 μg of 33 kDa protein unless otherwise noted. This suspension in a 10 ml beaker was illuminated (24 $\mu E/m^2$ per s) from above at 4°C for various durations. Following illumination, O_2 evolution activity was directly determined polarographically (5 μg Chl/ml) in assay buffer [13] containing 1.0 mM $CaCl_2$ from addition of the variously incubated $DCIPH_2$-treated $CaCl_2$-TMF-2.

The 33 kDa PS II protein was obtained by two successive extractions of wheat 17 and 23 kDa depleted TMF-2 (500 μg Chl/ml) with 1 M $CaCl_2$ in buffer A for 1 h at 4°C. The combined supernatants were concentrated and desalted (Amicon PM 30) then dialyzed vs 10 mM Mes-NaOH, pH 6.5, before centrifugation (30000 × g, 30 min) and concentration by lyophilization. The ratio of absorbance at 276 (peak):260:250 nm (trough) was 1.0:0.57:0.37, similar to values reported by Kuwabara and Murata [18] for highly purified 33 kDa protein. SDS-PAGE analyses of the 33 kDa fraction revealed no significant contamination by other proteins. SDS-PAGE analyses were carried out using a 5% stacking and a 12% polyacrylamide running slab gel.

3. RESULTS AND DISCUSSION

Table 1 shows the effects of $MnCl_2$, $CaCl_2$ and the 33 kDa extrinsic protein on O_2 evolution capacity of $DCIPH_2$-treated $CaCl_2$-TMF-2 incubated for 20 min in either weak light or darkness. Note: (i) $DCIPH_2$ treatment diminished O_2 evolution capacity of 33 kDa reconstituted $CaCl_2$-TMF-2 by about 87%; (ii) dark incubation of $DCIPH_2$-treated $CaCl_2$-TMF-2 at any condition shown did not cause any significant increase in O_2 evolution capacity. In contrast, light incubation in the presence of $MnCl_2$ and $CaCl_2$ resulted in 3.3–3.5-fold increases in V_{O_2} if the 33 kDa protein was present during the light incubation (condition D) or added following light incubation but before assay of V_{O_2} (condition C). Omission of either $MnCl_2$ or $CaCl_2$ caused appreciably less light-dependent recovery of V_{O_2}. The requirements for both $MnCl_2$ and $CaCl_2$ for light-dependent recovery of V_{O_2} are qualitatively similar to observations reported in [6–8] in different type

Table 1

Effects of $MnCl_2$, $CaCl_2$ and the 33 kDa extrinsic protein on photoactivation of oxygen evolution in $DCIPH_2$-treated $CaCl_2$-TMF-2

Additions	Incubation condition	
	Dark (μmol O_2/mg Chl per h)	Light (μmol O_2/mg Chl per h)
(A) None	25 (24)[a]	26 (24)[a]
(B) Plus $MnCl_2$	30 (29)	47 (41)
(C) Plus $MnCl_2$, $CaCl_2$	30 (29)	86 (46)
(D) Plus $MnCl_2$, $CaCl_2$, 33 kDa protein	29 (28)	92 (88)

[a] Numbers in parentheses represent rates of O_2 evolution without addition of the 33 kDa protein before assay following the various incubations

Results expressed as μmol O_2/mg Chl per h. The $DCIPH_2$-treated $CaCl_2$-TMF-2 (250 μg Chl/ml) was incubated for 20 min in buffer A containing where noted 2 mM $MnCl_2$, 50 mM $CaCl_2$ and 250 μg 33 kDa protein/ml. $CaCl_2$-TMF-2 gave 200 μmol O_2/mg Chl per h on addition of the 33 kDa protein. Where indicated, PS II membranes (5 μg Chl) were incubated for 2 min with 10 μg 33 kDa protein in the polarograph vessel (1.0 ml) before onset of assay of V_{O_2}

chloroplast systems inactive in O_2 evolution. An addition of 50 mM $CaCl_2$ to the assay medium enhanced the V_{O_2} values in conditions B and D by 50 and 10%, respectively.

Fig.1 shows the time course obtained for the photoactivation of $DCIPH_2$-treated $CaCl_2$-TMF-2 in the presence of optimal concentrations of $MnCl_2$, $CaCl_2$ and the 33 kDa proteins. The data tend to suggest more complicated kinetics than previously observed for photoactivation of V_{O_2}. In all of the previous studies of photoactivation, the conversion of inactive apo-S-state centers to active Mn-S-state centers showed apparent homogeneous first-order kinetics with half-times ranging from ~23 s [5] to 10 min [6–8] and to 50 min [9]. The overall half-time (10 min) for this photoactivation (fig.1) is not inconsistent with values reported for broken [8,11] or intact chloroplasts [6,7]. However, we observed maximally here (4°C) about 50% photoactivation of the S-state centers originally present in $CaCl_2$-TMF-2 as judged by the 33 kDa reconstituted V_{O_2} values before $DCIPH_2$ treatment and after $DCIPH_2$ treatment and subsequent photoactivation. Similar incubations at 23°C yielded maximally about 75% photoactivation of the centers. This overall limited conversion of centers is not a consequence of photoinhibition of the donor side of PS II traps [9] (not shown).

The pH dependency for photoactivation of $DCIPH_2$-treated $CaCl_2$-TMF-2 shows a maximum between pH 6.2 and 6.5 and a significant decline at >pH 7.0 (fig.2). As shown, similar incubations at any of the pH values but in dark, resulted in no change in O_2 evolution activity. This pH dependency is similar to those for the stability of the tetra-Mn complex, O_2 evolution activity assayed with 33 kDa protein, and the inactivation of tetra-Mn complex in $CaCl_2$-TMF-2 by NH_2OH (unpublished), but is different to the pH optimum (pH 7.8) reported with Tris-treated chloroplasts [19].

Fig.3 records the dependency of photoactivation on $MnCl_2$ and $CaCl_2$ concentrations. Irrespective of $MnCl_2$ concentration little photoactivation occurred in the absence of $CaCl_2$ (fig.3A). In the presence of 50 mM $CaCl_2$, half-maximal and maximal yields of photoactivation were obtained at about 0.25 and 1 mM $MnCl_2$, respectively. Under similar conditions, but with $DCIPH_2$-treated $CaCl_2$-TMF-2, the half-maximal rate of Mn^{2+} photooxidation (23°C) was obtained at 0.3 mM $MnCl_2$; thus, in both cases, the Mn^{2+} requirements are significantly greater (30–300-fold) than for photoreactivation of V_{O_2} in Tris-inactivated grana preparations [19], photoactivation of V_{O_2} in intact chloroplasts from flash greened wheat leaves [7] and for photooxidation of Mn^{2+} by NH_2OH-Tris washed PS II membranes [20]. These differing Mn^{2+} requirements may reflect differences in the

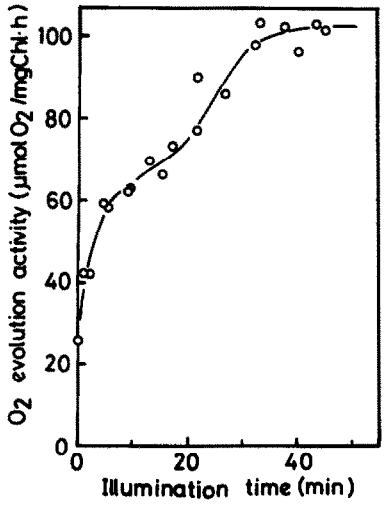

Fig.1. Time course of photoactivation of V_{O_2} by $DCIPH_2$-treated $CaCl_2$-TMF-2. See section 2 for details.

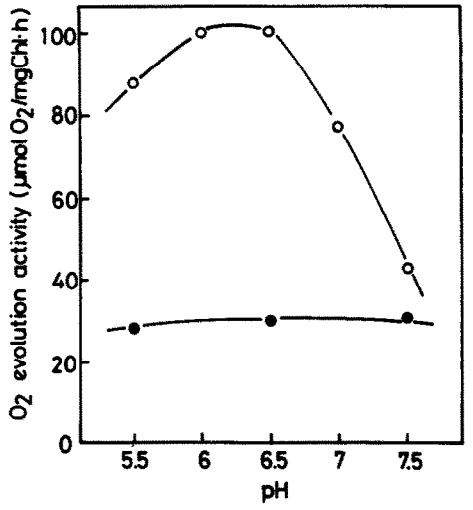

Fig.2. pH dependency of photoactivation of V_{O_2} (○). (●) V_{O_2} values of $DCIPH_2$-treated $CaCl_2$-TMF-2 similarly incubated, but in darkness. At pH 7.0 and 7.5, Mes was replaced by Hepes. See section 2 for details.

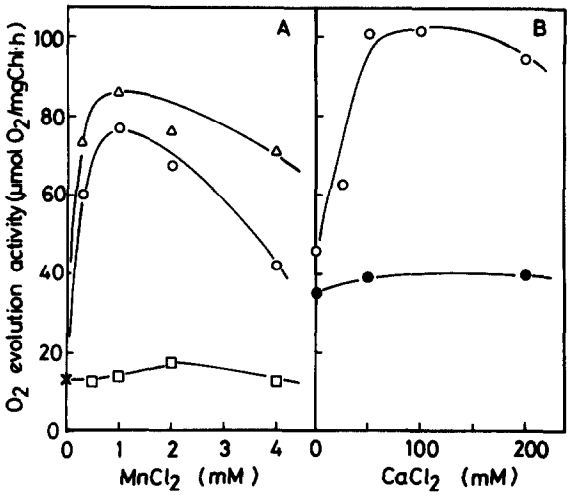

Fig.3. Effects of $MnCl_2$ (A) and $CaCl_2$ (B) concentrations on photoactivation of V_{O_2}. The concentration of $CaCl_2$ in A was 0 mM (□), 50 mM (○) and 125 mM (△). (×) Obtained in the absence of both $MnCl_2$ and $CaCl_2$. In B, incubations were in the presence of 2 mM $MnCl_2$ in either light (○) or darkness (●).

affinity constant for Mn^{2+} at its site of oxidation by PS II or differences in diffusional barriers to Mn^{2+}. The marked decreases in yield of photoactivation at 50 mM $CaCl_2$ and >1.5 mM $MnCl_2$ (fig.3A) were significantly diminished by increased $CaCl_2$ concentrations (125 mM), a result suggesting competition between these cations in processes essential for photoligation of Mn^{2+} and its stabilization in $DCIPH_2$-treated $CaCl_2$-TMF-2. Mn^{2+} and Ca^{2+} competition in photooxidation of Mn^{2+} by NH_2OH-Tris washed PS II membranes has been observed by Velthuys [21]. Additionally, Ono and Inoue [7] have implicated the existence of a similar competition in studies of photoactivation with intact chloroplasts; however, it is possible that with intact chloroplasts the observed competition could have been only remotely related to PS II events and photoactivation per se.

Fig.3B shows $CaCl_2$ concentration dependency for photoactivation of V_{O_2} at 2 mM $MnCl_2$. No increase in V_{O_2} occurred in darkness irrespective of the Ca^{2+} concentration. The photoactivation process required very high Ca^{2+} concentrations (half-maximal and maximal of about 20 and 50 mM $CaCl_2$, respectively) compared to the Ca^{2+} concentrations required for photoreactivation [8] or photoactivation [7] of chloroplasts. Increasing $CaCl_2$ concentrations did not diminish the yield of photoactivation (cf fig.3A). Apparently, Mn^{2+} can compete well with Ca^{2+} for essential Ca^{2+} binding sites but Ca^{2+} competes poorly with Mn^{2+} for the binding sites for Mn^{2+} which are essential for the photoactivation of V_{O_2} in $DCIPH_2$-treated $CaCl_2$-TMF-2. Similarly [7,8], neither 50 mM $MgCl_2$ nor 100 mM KCl effectively replaced the Ca^{2+} requirement for photoactivation. This remarkably high Ca^{2+} requirement was also observed when Ca^{2+} (acetate) and Cl^- (100 mM NaCl) were used in the incubations.

Most data indicate that a tetra-Mn complex of the water-oxidizing enzyme constitutes the major fraction of the total non-adventitiously PS II Mn (~4–5 Mn/200 Chl) [2,9,13,15]. Any PS II Mn (≤1 Mn/200 Chl) not directly correlating with O_2 evolution is a somewhat variable quantity [2,9,13,22]. Data shown in table 2 reveal the effects of $DCIPH_2$ treatment of $CaCl_2$-TMF-2 and subsequent dark or light incubation in the presence of $MnCl_2$ and 33 kDa protein on PS II Mn abundance. First, the data show that $DCIPH_2$ treatment decreased the ~4 Mn/200 Chl in parent

Table 2

Effects of $MnCl_2$, $CaCl_2$ and the 33 kDa extrinsic protein on photoligation of Mn^{2+}

Additions	Mn per 200 Chl			
	Expt 1		Expt 2	
	Dark	Light	Dark	Light
None	0.48	–	0.30	0.21
+ $MnCl_2$	1.05	1.42	–	–
+ $MnCl_2$, $CaCl_2$	–	4.13	1.28	1.92
+ $MnCl_2$, $CaCl_2$, and 33 kDa protein	1.56	3.83	1.03	2.04

$DCIPH_2$-treated $CaCl_2$-TMF-2 (350 μg Chl/ml) was incubated for 20 min in light or dark in buffer A containing where noted 2 mM $MnCl_2$, 50 mM $CaCl_2$ and 400 μg 33 kDa protein/ml. Following incubation, the membranes were pelleted then twice washed (27 μg Chl/ml with buffer A containing 10 μM A23187 and 1 mM EDTA) before Mn analyses [13]. Addition of 2 mM NH_2OH to the wash (30 min incubation), decreased the Mn/200 Chl values shown to 1.10 ± 0.21 Mn/200 Chl

$CaCl_2$-TMF-2 [4,13] to only <1 Mn/200 Chl. Second, the data indicate that incubation of $DCIPH_2$-treated $CaCl_2$-TMF-2 with $MnCl_2$ under any of the conditions shown resulted in some increase of total PS II Mn abundance above a value of 1.10 ± 0.21 Mn/200 Chl, the value obtained by NH_2OH, A23187 and EDTA washing, but without increase of V_{O_2}. We therefore assume any increase of PS II Mn abundance greater than this value reflects Mn^{2+} ligated into the tetra-Mn complex of the S-state enzyme.

Accordingly, the data of table 2 show that ligation of Mn^{2+} into the tetra-Mn complex of PS II membranes with everted orientation is not only light-dependent [5–11] but also dependent on Ca^{2+} [7,8]. We thus reject the supposition [11,12] that photoactivation merely reflects light-dependent transport of Mn^{2+} across thylakoids. The data also show that photoligation of Mn^{2+} with intrinsic membrane components is independent of the 33 kDa extrinsic protein even though the expression of catalytic activity of the photoligated Mn is dependent on this protein ([4]; table 1).

Reassembly of the 17 and 23 kDa polypeptides with thylakoids of NH_2OH extracted leaves occurs during photoactivation but not during even prolonged (≥6 h) dark incubations [9,10]. Fig.4 shows SDS-PAGE analysis of the capacity of variously treated and incubated $CaCl_2$-TMF-2 to rebind the 33 kDa protein. Examination of lane 1 ($CaCl_2$-TMF-2) and lane 2 ($CaCl_2$-TMF-2 plus 33 kDa) reveals rebinding of the 33 kDa. However, inspection of lanes 3–5 (variously incubated $DCIPH_2$-treated $CaCl_2$-TMF-2) and lanes 6–7 (NH_2OH or Tris-extracted $DCIPH_2$-treated $CaCl_2$-TMF-2 to further deplete PS II Mn to ≤0.4 Mn/200 Chl) shows that equivalent rebinding of the 33 kDa occurred in all cases. Additionally, the rebinding of the 33 kDa with $DCIPH_2$-treated $CaCl_2$-TMF-2 and $CaCl_2$-TMF-2 [23] showed entirely similar dependency on 33 kDa concentration over the range of 0.03–0.5 μg 33 kDa/μg Chl (not shown). Clearly, rebinding per se of the 33 kDa protein to the membranes, in contrast to the 17 and 23 kDa proteins [9,10] is independent of Mn ligated into the S-state complex and photoactivation events.

Nevertheless, ligation of Mn into the tetra-Mn complex of the water oxidizing enzyme occurs only in light, is independent of the 17, 23 and 33 kDa

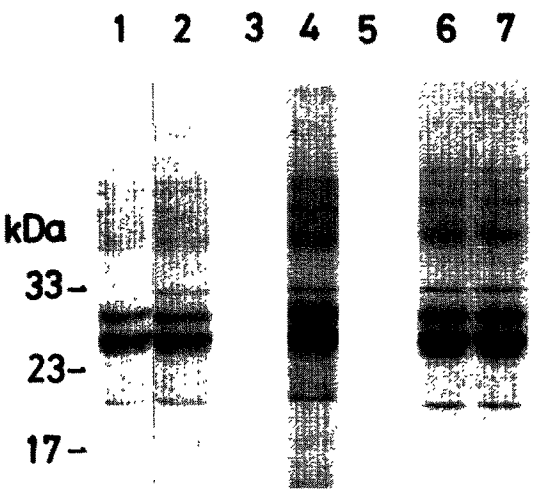

Fig.4. Rebinding of 33 kDa protein with PS II membranes is independent of the Mn-S-state complex. $CaCl_2$-TMF-2 (lanes 1,2) with and without 33 kDa addition, respectively; $DCIPH_2$-treated $CaCl_2$-TMF-2 (lanes 3,4) incubated in light in the absence or presence of $MnCl_2$, $CaCl_2$, 33 kDa protein, respectively; lane 5, as 4, but dark incubated; lanes 6 and 7, extracted with 2 mM NH_2OH and 1.0 M Tris, pH 8.0, respectively, for 40 min before incubation in darkness with 33 kDa. Following incubations (250 μg Chl/ml and 125 μg 33 kDa/ml for 30 min at 4°C in buffer A), the PS II membranes were washed twice at 25 μg Chl/ml with buffer A, before collection and SDS-PAGE analyses.

extrinsic proteins, but requires the reassembly of minimally the 33 kDa protein for expression of water oxidizing activity of the photoligated Mn. Data in fig.4 do not permit distinction between non-specific rebinding vs specific reassembly. Some of these same conclusions reached here also are observed in the photoactivation of NH_2OH-treated TMF-2. In this case photoactivation specifically causes an increase ($t_{1/2}$ ~5 min) of O_2 evolution (≥300 μmol O_2/mg Chl per h), is essentially independent of 33 kDa protein additions but is dependent on Mn^{2+}, Ca^{2+} and Cl^- additions; however, the Ca^{2+} concentration dependency is more complex than the enhancement of V_{O_2} by Ca^{2+} in 17 and 23 kDa depleted PS II membranes containing the tetra-Mn complex [24].

These conclusions seemingly contrast to those in [25] showing appreciable enhancement of V_{O_2} by dark incubation of EDTA washed *Synechococcus* PS II membranes with Mn^{2+} and Ca^{2+}.

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