Calcium Modulates the Photoassembly of Photosystem II $(Mn)_4$-Clusters by Preventing Ligation of Nonfunctional High-Valency States of Manganese†

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ABSTRACT: The requirement for Ca^{2+} in the Mn^{2+}-dependent photoactivation of oxygen evolution was re-evaluated using 17 kDa/24 kDa-less photosystem II (PSII) membranes depleted of $(Mn)_4$-clusters by NH_2OH extraction. At optimum conditions (1 mM Mn^{2+}/10 μM 2,6-dichlorphenolindophenol (DCIP)/ 20 mM Ca^{2+}), the light-induced increase of oxygen-evolution activity, the increase of membrane-bound Mn, and the B-band thermoluminescence emission intensity occurred in parallel. The extent of recovery of the oxygen-evolution activity was equivalent to 88% and 66% of the activity shown by parent NaCl-extracted PSII membranes and by PSII membranes, respectively. Neither photodamage of primary electron transport nor photoligation of nonfunctional $Mn^{\geq 3+}$ occurred. Analyses of the Ca^{2+} concentration dependence for the maximum recovery of oxygen evolution activity gave evidence for Ca^{2+}-binding site(s) having K_m values of ~38 and ~1300 μM. Illumination of membranes in the strict absence of Ca^{2+} resulted in large increases (up to 18 Mn/200 chlorophyll) of EDTA nonextractable, EPR silent, nonfunctional membrane-bound $Mn^{\geq 3+}$ and small increases of oxygen-evolution capability, dependent on pH and concentrations of Mn^{2+} and DCIP. No photodamage of primary electron transport and only ~17% decrease of A_T-band thermoluminescence occurred during the photoligation of the $Mn^{\geq 3+}$. In the strict absence of Ca^{2+}, significant recovery of oxygen-evolution activity was obtained under a limited set of conditions permitting photoligation of a limited abundance of the nonfunctional $Mn^{\geq 3+}$. Small (NH_2-OH, H_2O_2) as well as bulky external reductants readily reduced and dissociated the $Mn^{\geq 3+}$ from the membranes. Reillumination of these membranes under optimal conditions for photoactivation (plus Ca^{2+}) gave a high yield of $(Mn)_4$-clusters and oxygen-evolution capability. Similarly, simple addition of Ca^{2+} to membranes containing nonfunctional $Mn^{\geq 3+}$ followed by reillumination resulted in the conversion of $Mn^{\geq 3+}$ to $(Mn)_4$-clusters. It is argued that Ca^{2+} promotes the conformational change involved in the conversion of the Mn^{2+} mononuclear intermediate to the $Mn^{3+}-Mn^{2+}$ binuclear intermediate in the photoactivation mechanism, thereby permitting photoassembly of $(Mn)_4$-clusters and preventing photoinactivation by $Mn^{\geq 3+}$ ions.

Photosystem II (PSII)[1] couples the oxidation of water to molecular oxygen with the reduction of plastoquinone. The oxygen-evolving reaction occurs at an $(Mn)_4$-cluster located near the lumenal site of the thylakoid membrane. In close proximity to the $(Mn)_4$-cluster are one or more Cl^- ions and 1−2 Ca^{2+} ions which are essential for optimum cycling of redox states of the $(Mn)_4$-cluster during water oxidation. At the heart of PSII is the reaction center (RC) consisting of a heterodimer of homologous polypeptides, D_1 and D_2, which together bind the primary electron donor chlorophyll(s), P680, the symmetry-related secondary electron donors $TyrY_Z$ and $TyrY_D$ of D_1 and D_2, respectively, the immediate electron acceptor to P680, pheophytin (Pheo), and the primary (Q_A) and the secondary (Q_B) plastoquinone electron acceptors along with a nonheme Fe. Light absorption causes a charge separation event between P680 and a nearby Pheo. This charge separation is stabilized on the acceptor side by the oxidation of $Pheo^-$ by Q_A and on the donor side by the reduction of $P680^+$ by $TyrY_Z$. Further stabilization is accomplished by the subsequent oxidation of Q_A^- by Q_B and the reduction of $TyrY_Z^+$ by the $(Mn)_4$-cluster on the acceptor and donor sides of the RC, respectively. Following the accumulation of four oxidizing equivalents by the $(Mn)_4$-cluster, two molecules of water are oxidized to yield one molecule of oxygen [for reviews see Debus (1992), Rutherford et al. (1992), and Babcock (1993)].

X-ray absorption and EPR studies have given evidence that the $(Mn)_4$-cluster exists as a pair of di-μ-oxo-bridged Mn binuclear clusters in which the four Mn atoms exist as two Mn(III) and two Mn(IV) in the S_1 state. Reduction of the III and IV valencies to the II valency state causes dissociation of the $(Mn)_4$-cluster and concomitant inactivation of oxygen evolution [for reviews, see Debus (1992), Ruth-

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[1] Abbreviations: Aces, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; Chl, chlorophyll; D_1 and D_2, homologous 32 kDa polypeptides which, as a dimer, form the PSII core; DCIP and $DCIPH_2$, the oxidized and reduced forms of 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; EPR, electron paramagnetic resonance; HQ, hydroquinone; kDa, kilodalton; LHCP, light-harvesting chlorophyll protein complex; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NaCl/NH_2OH-PSII, PSII membranes extracted with NaCl and then NH_2OH; P680, PSII primary electron donor chlorophyll; PBQ, phenyl-*p*-benzoquinone; Pheo, pheophytin; PSII, photosystem II; Q_A and Q_B, primary and secondary plastoquinone electron acceptors of PSII, respectively; RC, reaction center; S_n (state), oxidation state of the water-oxidizing complex; TL, thermoluminescence; $TyrY_Z$, redox-active tyrosine residue acting as an electron donor to $P680^+$; V_{O_2}, rate of oxygen evolution.

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erford et al. (1992), and Babcock (1993)]. The assembly of Mn^{2+} ions as an $(Mn)_4$-cluster has long been known to be dependent on a process termed "photoactivation". Early kinetic studies using Mn-deficient, NH_2OH-extracted or dark-grown algae or wheat leaves greened under intermittent illumination established that this process occurs via a series mechanism in which two light-dependent reactions separated by a light-independent reaction are required to produce a semistable intermediate. The two light-dependent reactions were postulated to involve photooxidation of Mn^{2+} by the PSII RC, with the Mn^{3+} product of the first light-dependent reaction being unstable (Radmer & Cheniae, 1977). More recent kinetic analyses made with Tris-extracted chloroplasts (Yamashita et al., 1978), NH_2OH-PSII membranes (Tamura & Cheniae, 1987a; Miller & Brudvig, 1989, 1990; Miyao & Inoue, 1991a,b), PSII membranes from dark-grown spruce seedlings (Kamachi et al., 1994), and a wheat PSII core complex depleted of its $(Mn)_4$-cluster, LHCP, and extrinsic proteins (Tamura et al., 1991) generally corroborate the two-quantum mechanism deduced using algae and wheat leaves; however, the rate constants for the different reactions of photoactivation proved different depending on the type of preparation and the experimental conditions.

These kinetics of photoactivation are thought to reflect a reaction sequence of (a) photooxidation of an Mn^{2+} ion ligated to a high-affinity site (Blubaugh & Cheniae, 1992), possibly to D-170 of D_1 (Boerner et al., 1992; Diner & Nixon, 1992; Nixon & Diner, 1992; Chu et al., 1995a,b) to form an unstable mononuclear Mn^{3+} complex; (b) the conversion of the mononuclear Mn^{3+} complex to a more stable binuclear $Mn^{2+}-Mn^{3+}$ complex via a dark reaction involving a conformational rearrangement which may limit the overall rate of photoactivation; (c) the photooxidation of the $Mn^{2+}-Mn^{3+}$ complex to form an $Mn^{3+}-Mn^{3+}$ complex; and (d) a spontaneous light-independent ligation of two Mn^{2+} ions to form a $(Mn^{3+})_2(Mn^{2+})_2$ tetramanganese complex. This $(Mn)_4$ complex is possibly equivalent to the rather stable S_{-1} state formed by reaction of dark-adapted OEC with micromolar concentrations of NH_2OH (Bouges, 1971). EPR measurements (Tamura & Cheniae, 1988; Miller & Brudvig, 1990) as well as indirect evidence (Cheniae & Martin, 1972; Ono & Inoue, 1987; Tamura & Cheniae, 1987b) support the idea that Mn^{2+} photooxidation is involved in photoactivation. Also, direct evidence supporting the two-quantum mechanism recently has been obtained with NaCl/NH_2OH-PSII membranes (Miyao-Tokutomi & Inoue, 1992). [For partial reviews on photoactivation, see Tamura and-Cheniae (1988) and Debus (1992).]

Efforts to define the optimum conditions for photoactivation have led to the general realization that Cl^- ions and PSII electron acceptors such as DCIP (Tamura & Cheniae, 1987a) and specific substituted 1,4-benzoquinones (e.g., PBQ) (Miyao-Tokutomi & Inoue, 1992) improve the yield of the process by poorly understood mechanisms; however, the electron acceptors are not strictly essential for photoactivation. Apparently, the electron acceptors most effective in photoactivation are those that support a high rate of photoactivation while preventing possible irreversible photodamages to RC components and light-dependent inactivation of photoactivation (Miller & Brudvig, 1989; Miyao & Inoue, 1991a).

A requirement for Ca^{2+} in photoactivation was first noted using cyanobacterial preparations (Pistorius & Schmid, 1984) and intact chloroplasts (Ono & Inoue, 1983). The Ca^{2+} requirement was subsequently confirmed using PSII membranes depleted of their $(Mn)_4$-cluster by various procedures (Klimov et al., 1987; Tamura & Cheniae, 1987a; Miller & Brudvig, 1989). All of these studies indicate occurrence of competition between Mn^{2+} and Ca^{2+} for the Ca^{2+}-binding site(s) and between Ca^{2+} and Mn^{2+} for the Mn^{2+}-binding site(s). According to Ono and Inoue (1983), both Ca^{2+} and Mn^{2+} must be bound to their specific sites for assembly of the $(Mn)_4$-cluster via photoactivation. In this case, photoactivation would occur in one stage, dependent on both Mn^{2+} and Ca^{2+}. However, some data indicate that PSII can assemble some $(Mn)_4$-clusters without recovery of O_2-evolution activity in the absence of Ca^{2+} and then acquire O_2-evolution activity following dark incubation with Ca^{2+} (Tamura & Cheniae, 1987a; Tamura et al., 1989), suggesting that photoactivation may proceed in two stages: photoassembly of Mn^{2+} into an inactive $(Mn)_4$-cluster and then binding of Ca^{2+}, and the conversion of the inactive cluster to an active O_2-evolving cluster. According to Tamura and Cheniae (1988), one Ca^{2+}-binding site is "created" during the photoassembly of the $(Mn)_4$-cluster [see, however, Shenohara et al. (1992)]. Support of the two-stage hypothesis for photoactivation has appeared (Miller & Brudvig, 1989; Miyao & Inoue, 1991b).

Though the studies of photoactivation with PSII membranes/thylakoids have yielded some insight into the overall process, definitive chemical results explaining the kinetics of the process are still lacking. At least two factors have contributed to this problem: (1) the estimated quantum yield of photoactivation is low, ranging from only ~0.01 (Tamura & Cheniae, 1987a) to ~0.10 (Miyao-Tokutomi & Inoue, 1992); and (2) the final extent of recovery of O_2-evolution capability by various types of isolated $(Mn)_4$-depleted PSII preparations generally is appreciably less than the O_2-evolution capability of the parent PSII preparations. According to Miller and Brudvig (1989), this less than complete photoactivation is not a consequence of the $(Mn)_4$-less PSII centers being incompetent to undergo photoactivation at the outset of the photoactivation procedure but rather is due to photodamages affecting primary electron transport ("photoinhibition") and/or to a light-induced loss of photoactivation capability without loss of primary electron transport ("photoinactivation"). Conceivably, the photoinactivation might be due to photodamage of a protein-based Mn ligand, to photooxidation of inappropriately bound Mn^{2+}, or to photooxidation of appropriately bound Mn^{2+} to a wrong valency state. Though the results of Miller and Brudvig (1989) gave some evidence for the occurrence of photoinactivation and "photoinhibition" during their regime for photoactivation, no specific lesion was identified.

In the experiments reported here, we re-evaluated the function(s) of Ca^{2+} and DCIP in the photoassembly of the $(Mn)_4$-cluster by 17 kDa/24 kDa-less NH_2OH-PSII membranes (NaCl/NH_2OH-PSII). The totality of the results indicates that Ca^{2+} and DCIP affect the yield of photoactivation by modulating the extent of photoinactivation caused by inappropriately bound $Mn^{\geq 3+}$. Reduction and dissociation of the inappropriately bound $Mn^{\geq 3+}$ from the membranes followed by reillumination of the membranes under optimum conditions for photoactivation permits the assembly of catalytically active O_2-evolving $(Mn)_4$-clusters. Nevertheless, under optimum conditions for photoactivation, 100% yield

was not obtained even when "photoinhibition" and photoinactivation were nondetectable.

MATERIALS AND METHODS

Preparation of PSII-Membranes and NH_2OH-Extracted PSII Membranes. PSII-membranes (Berthold et al., 1981) were prepared in dim light from chloroplasts isolated from 7−9 day old wheat seedlings as previously described (Radmer et al., 1986) and then resuspended (>3 mg of Chl/mL) in 0.4 M sucrose/20 mM Mes−NaOH/15 mM NaCl/5 mM $MgCl_2$, pH 6.2, and stored at −80 °C until use. These membranes have abundances of ~1 P_{680}, 4 Mn, and 2.5 equiv of Q_A/Q_B per 200 Chl and yield maximum rates of O_2 evolution of ≥700 μmol of O_2 (mg of Chl·h)$^{-1}$.

NaCl/NH_2OH-PSII membranes were prepared as described by Miyao and Inoue (1991a) from PSII membranes that had been extracted with 1.5 M NaCl to remove the 17 and 24 kDa extrinsic proteins. Depletion of these proteins increases the rate of NH_2OH-induced dissociation of the Mn-cluster relative to parent PSII membranes (Tamura & Cheniae, 1985) and permits dissociation of the $(Mn)_4$-cluster at lower NH_2OH concentrations than required with the parent membranes. The NaCl/NH_2OH-PSII membranes were resuspended (≥2 mg of Chl/mL) in buffer A (0.4 M sucrose/25 mM Mes−NaOH/100 mM NaCl, pH 6.5) and used directly or stored at −80 °C until use. The preparation of NaCl/NH_2OH-PSII was done in near darkness to avoid photodamages to PSII donor-side redox components adversely affecting photoactivation capability (Blubaugh & Cheniae, 1990; Ono & Inoue, 1991a; Chen et al., 1992, 1995). Equivalent rates and extents of photoactivation were obtained using freshly prepared or stored NaCl/NH_2OH-PSII membranes. When "Ca^{2+}-free" conditions were used during illumination of NaCl/NH_2OH-PSII, the membranes were washed once at ~250 μg of Chl/mL with Chelex-100 resin (Bio-Rad Laboratories, Inc.)-treated buffer A before resuspension in this buffer. In this case, all the labware used for washing and for illumination of the membranes was decontaminated of Ca^{2+} by washing in ~2 N HNO_3 and rinsing with Milli-Q water the day before use. The Ca^{2+} abundance of such washed NaCl/NH_2OH-PSII was 1 Ca^{2+}/200 Chl.

Illumination Conditions. NaCl/NH_2OH-PSII or NH_2OH-PSII membranes were diluted to 250 μg of Chl/mL in buffer A supplemented with the $MnCl_2$, $CaCl_2$, and DCIP concentrations noted in the figure legends. Aliquots of 500 μL were distributed to 15 mm diameter glass vials in a rack to give a sample thickness of ~2 mm. Following 10 min of dark preincubation at room temperature, the membranes were illuminated from above with fluorescent white light (30 μE m^{-2} s^{-1}) for various durations while being shaken continuously. The light intensity varied less than 1% from the mean over the area occupied by the samples. In any given experiment, all samples were kept at room temperature for the same length of time regardless of the duration of illumination after which all were placed at 4 °C in darkness. Subsequently, the capability of the membranes to evolve oxygen was determined directly.

Activity Assays. O_2 evolution of membranes was measured polarographically in saturating light at 23 °C. The assay mixture contained 0.8 M sucrose/50 mM Mes−NaOH/15 mM $CaCl_2$, pH 6.2/1 mM FeCN/300 μM PBQ and membranes equivalent to 10 μg of Chl/mL. Light from a Dolan−Jenner high-intensity illuminator (Series 180) was filtered by two Schott KG-1 filters, one Corion FR-400-S filter, and one Corning 2-63 filter and focused on the polarograph vessel. Rates of PSII exogenous electron donor photooxidations were made essentially as described by Blubaugh and Cheniae (1990). The assay mixture contained 0.8 M sucrose/50 mM Mes−NaOH/15 mM NaCl, pH 6.5, 50 μM DCIP, membranes equivalent to 20 μg of Chl/mL, and either 1 mM DPC or 100 μM Mn^{2+} or 5 μM Mn^{2+}/3 mM H_2O_2 as exogenous electron donors.

Sonication and Density Gradient Centrifugation. NaCl/NH_2OH-PSII membranes photoactivated in buffer A containing 10 μM DCIP and either 1 mM Mn^{2+} or 1 mM Mn^{2+}/20 mM Ca^{2+} were pelleted, washed three times at 250 μg of Chl/mL with buffer A containing 2 mM EDTA, and then washed twice (500 μg of Chl/mL) and resuspended in Chelex-100 resin-treated buffer A to give ~1.5 mg of Chl/mL. The membranes (200 μL in 1.5 mL microfuge tubes at 4 °C) were then sonicated for 5 s using a Tekmar sonic disrupter Model ASI. After ~5 min incubation on ice, the sonication/cooling was repeated until 30 s total time of sonication had accrued. Following dilution with 0.2 mL of buffer A, the membranes were subjected to discontinuous sucrose density gradient centrifugation (0.80−1.6 M sucrose in 50 mM Mes/15 mM NaCl, pH 6.5) at 16 320*g* for 30 min in an HB-4 rotor. After recovery of the membranes from the interface of the 1.2 and 1.6 M sucrose buffers, they were diluted with ~3 volumes of cold Milli-Q water per volume of membranes and then pelleted (17 000*g* for 20 min). Subsequently, the membranes were washed twice (~200 μg of Chl/mL) with Chelex-100 resin-treated buffer A containing 2 mM EDTA and once (~400 μg of Chl/mL) with Chelex-100 resin-treated buffer A before resuspension in this same buffer to give 200−400 μg of Chl/mL. Prior to Chl and Mn determinations, the membranes were sonicated briefly to give a homogeneous suspension.

Mn Determinations. Mn determinations were made at 279.5 nm using flameless atomic absorption (Perkin-Elmer Zeeman 5000 spectrometer equipped with an HGA-500 system). Membranes (equivalent to 25 μg of Chl/mL) were incubated in buffer A containing 2 mM EDTA for 10 min, washed two times in this same buffer (25 μg of Chl/mL), and then pelleted. Subsequently, they were washed once with Chelex-100 resin-treated buffer A (50 μg of Chl/mL) and then resuspended in Chelex-100 resin-treated buffer A (200 μg of Chl/mL) before dilution to 10−100 μg of Chl/mL with Milli-Q water. The sample (10 μL) plus 5 μL of a chemical modifier (Welz et al., 1988) were then pipetted into the graphite furnace. This sequence of incubation and washes effectively removed any adventitious Mn from oxygen-evolving PSII membranes or photoactivated membranes without causing more than 10% loss of their oxygen-evolving activity.

Other Methods. A_T- and B-band thermoluminescence was measured with a setup similar to the one described by Ichikawa et al. (1975) following the procedures described by Koike et al. (1986) and Rutherford et al. (1982). After illumination, the photoactivated membranes were washed three times in buffer A containing 2 mM EDTA (250 μg of Chl/mL) and then washed once and resuspended in Chelex-100 resin-treated buffer A before the measurements of the B-band emission were made, since trace amounts of DCIP severely suppressed the emission. Before measurements of

the A_T-band emission were made, the membranes were extracted with 1 mM NH_2OH for 5 min and then washed and resuspended as described above.

Determinations of chlorophyll concentration were made as described by MacKinney (1941). The concentration of DCIP was calculated on the basis of the extinction coefficient of 18.1 mM^{-1} cm^{-1} of DCIP at 600 nm at pH 6.5 (Armstrong, 1964). All buffers were treated with Chelex-100 resin, Na^+ form (Bio-Rad), by direct addition of the resin (30 g/L) to the buffer followed by readjustment of pH to 6.5. Before each use, the suspension was stirred for ~15 min, and then the resin settled before any remaining resin was filtered off using a chromatography column.

For reconstitution experiments, a 17 kDa/24 kDa protein fraction was obtained by extraction of wheat PSII membranes with 1.5 M NaCl as described by Miyao and Inoue (1991b). Following concentration and desalting (Amicon PM 10 membrane), the protein fraction was dialyzed overnight against 2 L of 0.4 M sucrose/5 mM Mes−NaOH/30 mM NaCl, pH 6.2, containing 60 g of Chelex-100 resin to remove Ca^{2+}. The protein fraction was lyophilized in a 2 N HNO_3/Milli-Q water-cleaned flask, solubilized in Chelex-100 resin-treated buffer A, and stored at −80 °C until use. Reconstitution of NaCl/NH_2OH-PSII was carried out by preincubation (30 min/4 °C) of membranes (2.2 mg of Chl at 0.5 mg of Chl/mL) with 0.8 mg of the 17 kDa/24 kDa proteins either before or after illumination. Assuming 50% purity of the 17 kDa/24 kDa protein fraction, this reconstitution mixture gives approximately two copies of these proteins per reaction center.

RESULTS

Effects of Ca^{2+} on Photoactivation versus Photoligation of Mn. According to the one-stage model of photoactivation of Ono and Inoue (1983), the first-order rate constant for the assembly of O_2-evolving centers is dependent on the extent of occupancy of both Mn^{2+} and Ca^{2+} at their specific binding sites during illumination of photosystem II centers lacking the $(Mn)_4$-cluster. On the other hand, the two-stage model of photoactivation predicts that the rate of assembly of $(Mn)_4$-clusters will be dependent only on Mn^{2+} and is not expected to be enhanced by the presence of Ca^{2+} during illumination (Tamura & Cheniae, 1987a; Tamura et al., 1989; Miller & Brudvig, 1989). In this case, however, subsequent dark incubation of the assembled $(Mn)_4$-clusters with Ca^{2+} is required to confer O_2-evolution activity. This simplistic comparison of the two models assumes that no inactivating photochemical process occurs in parallel during the photoactivation regime.

Figure 1A shows the time-course of photoactivation of O_2 evolution by NaCl/NH_2OH-PSII membranes in the presence and absence of Ca^{2+}. In these experiments, the membranes were illuminated for times shown in buffer A containing either 1 mM Mn^{2+}/10 μM DCIP ("minus Ca^{2+}") or 1 mM Mn^{2+}/10 μM DCIP/20 mM Ca^{2+} ("plus Ca^{2+}"), the latter condition permitting maximal rate and yield of photoactivation (Miyao & Inoue, 1991b). As shown, illumination of NaCl/NH_2OH-PSII membranes in the absence of Ca^{2+} gave essentially no increase of rate of O_2 evolution, even though they were incubated following illumination with 50 mM Ca^{2+} for 60 min prior to assay of O_2-evolution capability. In contrast, membranes illuminated in the presence of Mn^{2+} and Ca^{2+} exhibited rapid ($t_{1/2} \approx 3$ min) and significant yield of photoactivation, measured either as an increase in O_2-evolution capability [465 μmol of O_2 (mg of Chl·h)$^{-1}$] or as an increase in abundance of the $(Mn)_4$-cluster (2.33 Mn/200 Chl).

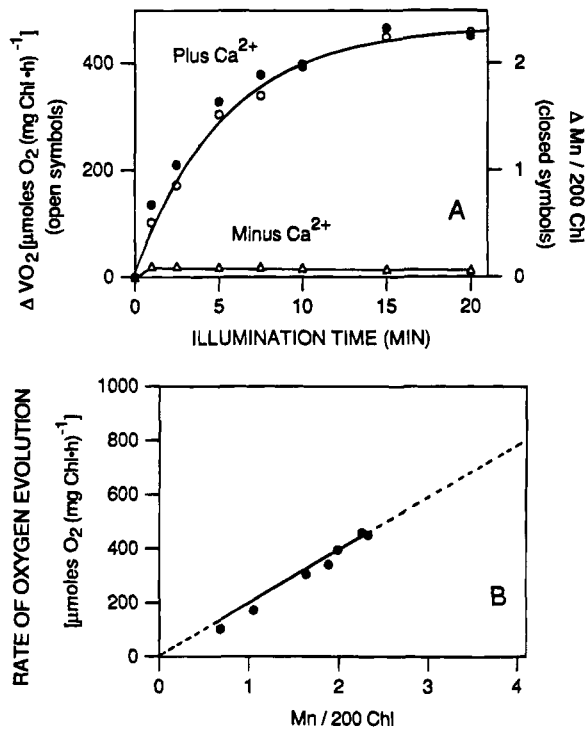


FIGURE 1: Illumination of NaCl/NH_2OH-PSII in the presence of Ca^{2+}/Mn^{2+}/DCIP results in parallel increases of rate of O_2-evolution and Mn abundance. (A) Time-course of increase of rate of O_2-evolution for membranes illuminated in buffer A containing 10 μM DCIP and 1 mM $MnCl_2$/20 mM Ca^{2+} (plus Ca^{2+}) or 1 mM $MnCl_2$ (minus Ca^{2+}). The Mn abundances are those for membranes illuminated in the plus Ca^{2+} mixture. Membranes illuminated in the minus Ca^{2+} mixture were postincubated with Ca^{2+} (50 mM/60 min/4 °C) before assaying. (B) Replot of the plus Ca^{2+} data of panel A. Extrapolation of the solid line to a 4 Mn/200 Chl value yielded a rate of O_2-evolution of 750 μmol of O_2 (mg of Chl·h)$^{-1}$, which was essentially equivalent to the rate of O_2-evolution by parent PSII membranes.

The NaCl-PSII membranes used for preparation of the NaCl/NH_2OH-PSII membranes (Miyao & Inoue, 1991b) of this experiment gave 531 μmol of O_2 (mg of Chl·h)$^{-1}$ after a 10 min room temperature dark preincubation and a 20 min illumination regime as used for the photoactivation of the NaCl/NH_2OH-PSII membranes. Accordingly, about 88% of the O_2-evolving activity of the NaCl-PSII membranes was obtained. However, on the basis of the activity of parent PSII membrane preparations of 750−800 μmol of O_2 (mg Chl·h)$^{-1}$, only about 66% of the maximal activity could be recovered during photoactivation in the presence of Mn^{2+} and Ca^{2+}. A replot (Figure 1B) of the rates of O_2 evolution versus the Mn abundances resulting from photoactivation in the presence of Mn^{2+}/Ca^{2+} from data of Figure 1A yields a straight line, which, when extrapolated to the typical 4 Mn/200 Chl abundance of our wheat PSII membranes, indicates an expected rate of ~750 μmol of O_2 (mg Chl·h)$^{-1}$, a value consistent with the rate we commonly observe with PSII membranes from wheat.

These data therefore indicate that all of the Mn that becomes ligated during illumination of the NaCl/NH_2OH-PSII membranes in the presence of Mn^{2+}/Ca^{2+} is assembled

as $(Mn)_4$-clusters. They also support the generally accepted belief [see, however, Pauly and Witt (1992)] that the catalytic site for O_2 evolution is an $(Mn)_4$ complex. Additionally, the rather high and the very low yields of photoactivation observed in the presence of Mn^{2+}/Ca^{2+} and Mn^{2+} only, respectively, confirm and extend previous observations made by Miyao and Inoue (1991b), also using NaCl/NH_2OH-PSII membranes. The combined data lend no support to the two-stage model of photoactivation advanced by Tamura et al. (1989) on the basis of results obtained under different experimental conditions and with NH_2OH-PSII membranes less depleted of the 17 kDa/24 kDa extrinsic PSII polypeptides than the NaCl/NH_2OH-PSII membranes used here.

The less than complete photoactivation in the presence of 1 mM Mn^{2+}/20 mM Ca^{2+} and its near absence without addition of Ca^{2+} is not due to photodamages affecting photoactivation and donor side redox active components as seen with weak or strong light illumination of NH_2OH-PSII in the absence of PSII exogenous electron donors (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991; Ono & Inoue, 1991a; Chen et al., 1992, 1995). Both the 1 mM Mn^{2+}/20 mM Ca^{2+}- and 1 mM Mn^{2+}-illuminated (20 min) membranes gave rates of DCIP photoreduction by 5 μM Mn^{2+}/3 mM H_2O_2, 100 μM Mn^{2+}, or 1 mM DPC equivalent to dark incubated membranes after extraction with 0.8 M Tris, pH 8.3 (data not shown). These results contrast to a report (Miller & Brudvig, 1989) indicating 25% irreversible loss of DPC photooxidation capability from weak light illumination of NH_2OH-PSII membranes in the presence of Mn^{2+}/Ca^{2+}. They agree, however, with the observations showing that 1 mM Mn^{2+} gives virtually complete protection of NH_2-OH-PSII from weak light induced photodamages (Blubaugh & Cheniae, 1990; Chen et al., 1992, 1995).

In the experiments of Figure 2, we asked if the failure to observe photoactivation with membranes illuminated with Mn^{2+} in the absence of Ca^{2+} was a consequence of failure to photoligate Mn. Figure 2A shows the increase in abundance of membrane-bound, EDTA nonextractable Mn during illumination of NaCl/NH_2OH-PSII in the presence of 10 μM DCIP and either 1 mM Mn^{2+}/20 mM Ca^{2+} or 1 mM Mn^{2+} only. In the absence of Ca^{2+}, the increase of abundance of photoligated Mn was even more rapid and greater than in the presence of Ca^{2+}. As shown, the increase of Mn abundance reached a maximum (2.33 ΔMn/200 Chl) after ~15 min of illumination of the membranes in the presence of Mn^{2+}/Ca^{2+}. In the absence of Ca^{2+}, however, the abundance of photoligated Mn continued to increase slowly even after 15 min of illumination and reached a value of ~8 ΔMn/200 Chl after 20 min of illumination. From experiment to experiment with different preparations of NaCl/NH_2OH-PSII, illumination of the membranes with 1 mM Mn^{2+}/10 μM DCIP for 20 min resulted in variable increases (~8−18 ΔMn/200 Chl) of Mn abundance. Such values are 3−4 times greater than those observed by Tamura and Cheniae (1987a) and Tamura et al. (1989) following illumination of wheat NH_2OH-PSII for 40 min in the absence of Ca^{2+} and in the presence of 100 μM DCIP and either 100 μM Mn^{2+} or 1 mM Mn^{2+}. In those experiments, postincubation of the membranes with 50 mM $CaCl_2$ resulted in partial decreases of the abundance of photoligated Mn and ~3-fold increases of rates of O_2 evolution, presumably as a result of dissociation and replacement by Ca^{2+} of some Mn occupying the Ca^{2-} site essential for O_2 evolution.

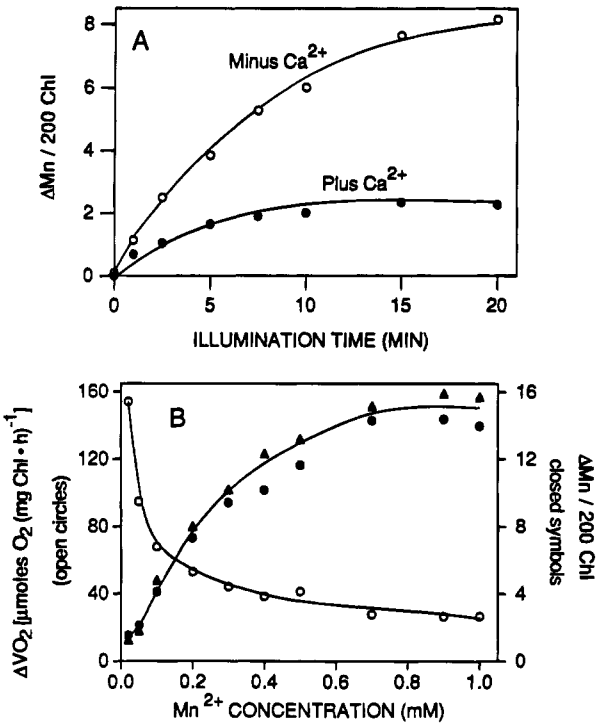


FIGURE 2: (A) Time-course of photoligation of Mn by NaCl/NH_2-OH-PSII in the presence and absence of Ca^{2+}. Membranes were illuminated as indicated in the legend of Figure 1 with the exception of omission of Ca^{2+}, as noted. (B) Mn^{2+} concentration dependence of photoligation of Mn and increase of rate of O_2-evolution with illumination of NaCl/NH_2OH-PSII in the absence of Ca^{2+}. Membranes were illuminated as above for 20 min in the Mn^{2+} concentrations indicated in the absence of Ca^{2+}. One set of samples was incubated with 50 mM $CaCl_2$ for 60 min at 4 °C in darkness before assay of O_2-evolution capability (open circles). No additions were made to the other set. Subsequently, the membranes were repetitively washed with EDTA and then analyzed for EDTA nonextractable photoligated Mn. The closed circles and triangles record the abundance of photoligated Mn of membranes receiving no addition and 50 mM $CaCl_2$ addition/postincubation, respectively.

Figure 2B summarizes typical results from experiments in which NaCl/NH_2OH-PSII membranes were illuminated in the absence of Ca^{2+} for 20 min in the presence of 10 μM DCIP and the various indicated concentrations of Mn^{2+}. After illumination, one set of membranes received ~150 min of dark incubation at 4 °C with 50 mM Ca^{2+} (closed and open circles) while the other set (closed triangles) received no Ca^{2+} before assays of O_2-evolution capability. The membranes receiving no postaddition of Ca^{2+} and incubation following illumination with any Mn^{2+} concentration evolved essentially no O_2 when assayed directly in the absence of Ca^{2+}. On the other hand, as shown by the open circles, the membranes illuminated with ≤0.1 mM Mn^{2+} exhibited significant rates of O_2 evolution after addition and incubation with Ca^{2+}. The highest rate was observed with membranes containing the least abundance of EDTA nonextractable photoligated Mn, identified by either the closed circles or triangles. As shown, increasing Mn^{2+} concentrations during illumination led to decreasing O_2-evolution capability and increasing abundance of photoligated Mn. This antiparallel response contrasts sharply to that seen in Figure 1. At any level of membrane-bound Mn abundance, prolonged postincubation with Ca^{2+} (closed circles) did not significantly change the Mn abundance compared to membranes receiving no Ca^{2+} treatment.

We estimate from the data of Figure 2B that half-maximal and maximal membrane-bound Mn was obtained from

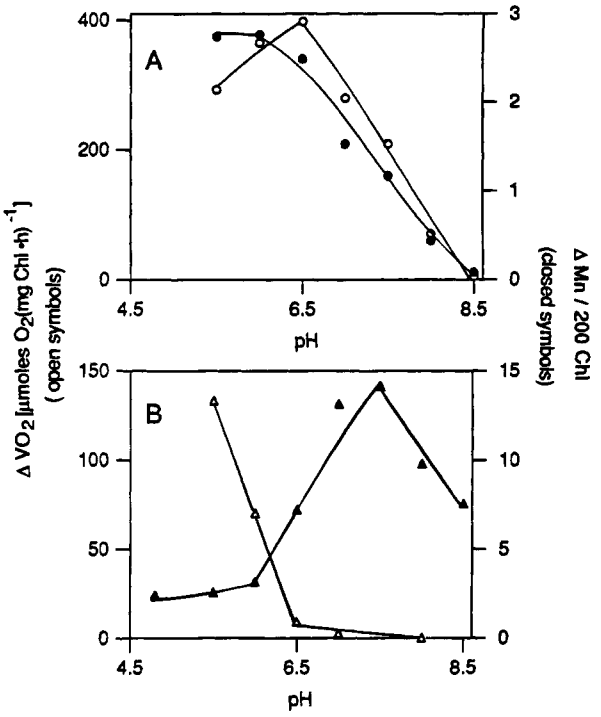


FIGURE 3: pH dependence of increase in rate of O_2-evolution and abundance of membrane-bound Mn. (A) Membranes were illuminated for 20 min in 0.4 M sucrose/100 mM NaCl/25 mM buffer containing 1 mM Mn^{2+}/20 mM Ca^{2+}/10 μM DCIP. (B) Membranes were illuminated as above except for omission of Ca^{2+}. The buffers were acetate (pH 4.8), Mes (pH 5.5−6.5), Aces (pH 7.0−7.5), and Tris (pH 8.0−8.5). After assay of O_2-evolution capability, the membranes were repetitively washed with EDTA to remove adventitious Mn^{2+} before determinations of Mn abundance.

illumination of NaCl/NH_2OH-PSII in the presence of 200 and 800 μM Mn^{2+}, respectively. This estimated 200 μM K_m Mn^{2+} value is much greater than the 4 μM K_m Mn^{2+} value for photoactivation using wheat NH_2OH-PSII (Blubaugh & Cheniae, 1990) and the 50 μM dissociation constant for Mn^{2+} at the Mn site essential for photoactivation as estimated by Miller and Brudvig (1989). Additionally, it does not compare favorably with the ≤1−10 μM K_m Mn^{2+} values for reduction of $TyrY_Z^+$ found by different workers using differing experimental conditions [see Chen et al. (1995) and references therein] and the 17 μM K_m Mn^{2+} value for Mn^{2+} suppression of the A_T-band thermoluminescence (One & Inoue, 1991b). However, the 200 μM K_m Mn^{2+} value extracted from the data of Figure 2B equates with the K_m value for Mn^{2+} photooxidation via the chemically unidentified site 2 locus of electron donation to the PSII RC (Blubaugh & Cheniae, 1990).

Effects of pH and DCIP Concentration. The contrasting relationship seen in Figures 1 and 2 between increase in O_2-evolution capability and the photoligation of functional and apparently nonfunctional Mn, depending on the presence of Ca^{2+} during illumination, prompted us to examine if this occurred at conditions different from those used in the experiments of Figures 1 and 2. Figure 3 compares the effects of pH on the light-dependent increase of O_2-evolution capability and membrane-bound Mn resulting from 20 min of illumination of NaCl/NH_2OH-PSII in the presence (A) and absence (B) of Ca^{2+}. With NH_2OH-PSII membranes in the presence of Mn^{2+}/Ca^{2+} and 100 μM DCIP, past studies have shown that the pH dependence of the light-induced increase of O_2-evolution capability exhibits a rather sharp optimum at pH 6.5, declining sharply with increasing pH values and becoming zero at ~pH 8.5 (Tamura & Cheniae, 1987a). In contrast, PSII membranes isolated from dark-grown spruce seedlings and NH_2OH-treated PSII membranes from light-grown spruce seedlings show an optimum of pH 5.3−5.5 for photoactivation in the presence of Mn^{2+}/Ca^{2+} and DCIP (Kamachi et al., 1994). Similarly, an NH_2OH-treated PSII core complex preparation containing primarily only the D_1/D_2/47 kDa/43 kDa/Cyt b_{559} proteins exhibits a weak pH optimum at pH 5.3−5.5 for photoactivation when illuminated in Mn^{2+}/Ca^{2+} and DCIP (Tamura et al., 1991). In the above referenced studies, O_2-evolution capability but not Mn abundance was assayed to determine the extent of photoactivation.

The data of Figure 3A corroborate and extend the conclusions reached by Tamura and Cheniae (1987a) in their studies of the effects of pH on photoactivation of NH_2OH-PSII membranes illuminated in a Mn^{2+}/Ca^{2+} mixture containing 100 μM DCIP. As shown, both the increase in O_2-evolution capability and ΔMn/200 Chl are affected in parallel by the pH of the suspension during illumination of NaCl/NH_2OH-PSII in a Mn^{2+}/Ca^{2+}/DCIP mixture, both parameters declining rather sharply with pH values greater than the pH 6.5 optimum. Figure 3B records similar measurements, but, in these experiments, Ca^{2+} was omitted during illumination of the membranes. At this condition, the increases of O_2-evolution capability and Mn abundance occur in an antiparallel manner over the pH range shown. Significant but small increases of O_2-evolution capability were observed only at pH < 6.5, a condition resulting in photoligation of ~2.5 Mn/200 Chl. The maximum increase of O_2-evolution capability [~135 μmol of O_2 (mg of Chl•h)$^{-1}$ at pH 5.5] in the absence of Ca^{2+} was only about one-third of the maximum increase of O_2-evolution capability seen in Figure 3A with addition of Ca^{2+} during illumination, even though similar increases of Mn abundance occurred under both conditions. According to this comparison, only a small fraction of the total Mn photoligated at pH 5.5 in the absence of Ca^{2+} is assembled as $(Mn)_4$-clusters. At pH ≥ 6.5, in the absence of Ca^{2+}, increasing photoligation of Mn occurred with increasing pH, reaching a maximum of 14.1 Mn/200 Chl at pH 7.5 before decreasing at the more alkaline pH values. However, virtually no increase of O_2-evolution capability was obtained at pH > 6.5 even after prolonged (≥120 min) incubation of the membranes with 50 mM Ca^{2+} before O_2-evolution rate assays. Apparently, illumination of NaCl/NH_2OH-PSII at pH ≥ 6.5 with 1 mM Mn^{2+}/10 μM DCIP and no Ca^{2+} yields no $(Mn)_4$-clusters having latent O_2-evolution activity. These results contrast to those previously described by several different workers using experimental conditions and membranes different than those employed in the experiments of Figure 3. This same general tentative conclusion was also reached from an analysis of the Cl^- concentration dependence for photoactivation and photoligation of Mn by NaCl/NH_2OH-PSII at pH 6.5, 1 mM Mn^{2+}/10 μM DCIP, in the absence or presence of 20 mM Ca^{2+}. In the presence of Ca^{2+}, O_2-evolution capability [520 μmol of O_2 (mg of Chl•h)$^{-1}$] and ΔMn/200 Chl (3.2 Mn/200 Chl) increased in parallel and hyperbolically over the range 0−600 mM Cl^-, supplied as tetramethylammonium chloride. In contrast, 12−15 Mn/200 Chl were photoligated with no significant increase of V_{O_2}, irrespective of Cl^- concentration in the absence of Ca^{2+}. Thus, over a large range of Cl^- concentrations at pH

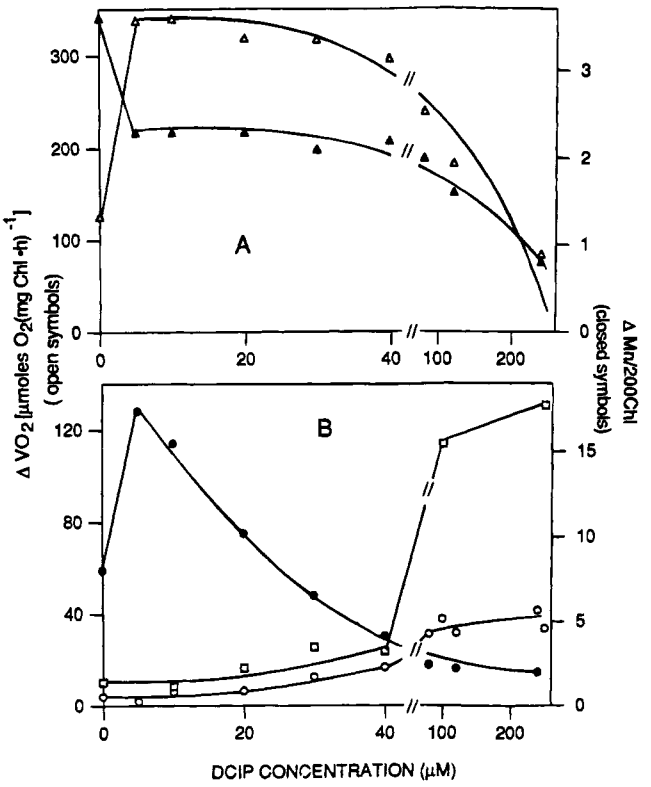


FIGURE 4: Effects of DCIP concentration on the increase in rate of O_2-evolution capability and Mn/200 Chl. (A) Membranes were illuminated for 20 min in buffer A containing 1 mM Mn^{2+}/20 mM Ca^{2+} and the indicated concentrations of DCIP. (B) Membranes were illuminated as above except for omission of Ca^{2+}. In the experiments in panel A, the rate of O_2-evolution was determined directly following the 20 min of illumination. The open circles and open squares in panel B record the increase in rate of O_2-evolution by membranes receiving no postincubation with Ca^{2+} and those postincubated with 50 mM Ca^{2+}, respectively, before assay.

6.5 and over a broad range of pH values, almost all of our data thus far suggest that both Mn^{2+} and Ca^{2+} are required for the assembly of $(Mn)_4$-clusters in the photoactivation process.

However, in the experiments of Tamura and Cheniae (1987a) and Tamura et al. (1989), illumination of NH_2OH-PSII membranes in the presence of 1 mM Mn^{2+} but no Ca^{2+} led to formation of some $(Mn)_4$-clusters which were inactive but could be converted to active O_2-evolving clusters by dark incubation with Ca^{2+}. In those experiments, a 10-fold higher concentration of DCIP was used during illumination than in the experiments summarized in Figures 1−3 showing no apparent photoassembly of $(Mn)_4$-clusters unless Ca^{2+} was present.

Figure 4 shows results from experiments in which we compared the DCIP concentration dependence for photoactivation of the O_2-evolving complex and photoligation of Mn by NaCl/NH_2OH-PSII illuminated in the presence and absence of Ca^{2+}. In the presence of Ca^{2+} (Figure 4A), a significant increase of O_2-evolution activity was observed even in the absence of DCIP addition. As shown by the open triangles, addition of DCIP over the range ~5−40 μM DCIP led to a ~3-fold greater increase of O_2-evolution activity; however, ≥80 μM DCIP concentrations caused diminishing extents of photoactivation as measured by rates of O_2 evolution (open triangles). This DCIP concentration dependence for increase of O_2-evolution activity is entirely similar to that reported by Miyao and Inoue (1991b), also using NaCl/NH_2OH-PSII. The curve described by the closed triangles of Figure 4A records the abundances of Mn that were photoligated at DCIP concentrations ranging from 5 to 240 μM DCIP. A comparison of this curve with the curve defined by the open triangles indicates that ΔMn/200 Chl and increase of O_2-evolution capability are affected by DCIP concentration in parallel during illumination of NaCl/NH_2-OH-PSII in the presence of Ca^{2+}. On the other hand, an antiparallel increase of O_2-evolution capability and decrease of ΔMn/200 Chl, relative to membranes receiving no DCIP were observed with addition of only 5 μM DCIP to NaCl/NH_2OH-PSII membranes in the presence of Mn^{2+}/Ca^{2+}. Apparently in the absence of DCIP, some of the total Mn/200 Chl was ligated as inactive $Mn^{\geq 3+}$, and the addition of only 5 μM DCIP prevented this while simultaneously promoting the assembly of active $(Mn)_4$-clusters.

In the absence of Ca^{2+} (Figure 4B), the DCIP concentration dependence for photoligation of Mn versus assembly of $(Mn)_4$-clusters having latent O_2-evolving capability was quite different than in the presence of Ca^{2+} (Figure 4A). Inspection of the data of Figure 4B reveals the following: (1) NaCl/NH_2OH-PSII membranes illuminated in the presence of any DCIP concentration in the absence of Ca^{2+} and assayed for O_2-evolution capability without dark postincubation with Ca^{2+} had extremely low capability to evolve O_2, as shown by the open circles; (2) dark postincubation of these membranes with Ca^{2+} before O_2-evolution assays (open squares) led to 3−4 fold increases in capability to evolve O_2 but only with those membranes illuminated in the presence of ≥100 μM DCIP; and (3) in the absence of DCIP addition, appreciable Mn (7.8 Mn/200 Chl) was photoligated as shown by the closed circles, and this Mn abundance was further increased ~2.3-fold by addition of 5−10 μM DCIP. With increasing DCIP concentrations, however, the abundance of photoligated Mn decreased until ultimately only ~2.1 Mn/200 Chl was photoligated in the presence of ≥100 μM DCIP. Clearly, only the membranes having such low Mn abundances showed significant increase of O_2-evolution capability following dark postincubation with Ca^{2+}. Qualitatively, the increase of O_2-evolution capability and the Mn abundance data of Figure 4B obtained from illumination of NaCl/NH_2OH-PSII in the presence of ≥100 μM DCIP concentrations and no Ca^{2+} are quite similar to those obtained from illumination of NH_2OH-PSII in a mixture of 1 mM Mn^{2-}, no Ca^{2+} and DCIP at an invariant 100 μM concentration followed by dark postincubation with Ca^{2+} (Tamura & Cheniae, 1987a; Tamura et al., 1989). Such data led these workers to conclude that Ca^{2+} is not strictly essential for the photoassembly of $(Mn)_4$-clusters possessing latent O_2-evolving activity.

Effects of Divalent Cations on Photoactivation Measured by DCIP Photoreduction. In the experiments of Figure 5, we measured the photoreduction of DCIP by Mn^{2+} in the absence and presence of Ca^{2+} and other divalent cations in efforts to gain some understanding of the basis for apparent complete dependence of photoactivation on Ca^{2+} when NaCl/NH_2OH-PSII membranes are illuminated in the presence of 1 mM Mn^{2+} and low concentrations of DCIP. Preliminary experiments established that the light intensity used in these experiments was sufficient to promote photoactivation, measured as an increase in O_2-evolution activity, at a rate comparable to the maximum rate shown in Figure 1A.

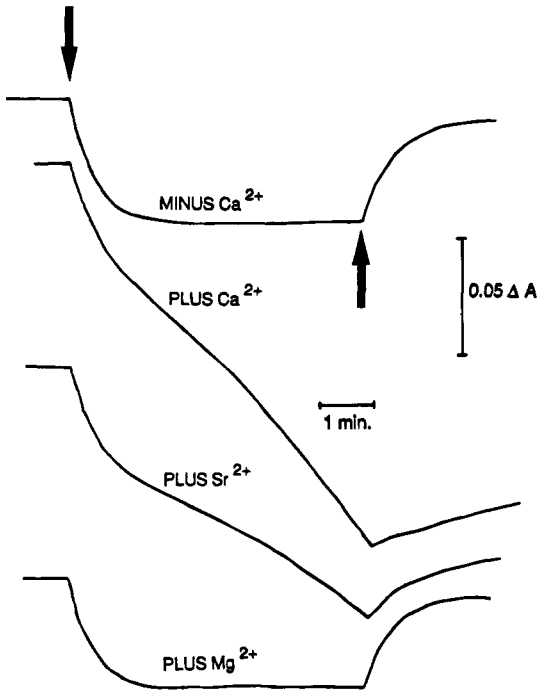


FIGURE 5: Comparisons of the rate of DCIP photoreduction under conditions nonsupportive versus supportive of the photoassembly of $(Mn)_4$-clusters. NaCl/NH_2OH-PSII membranes (40 μg of Chl/mL) were illuminated in buffer A containing 10 μM DCIP/1 mM Mn^{2+} in the absence or presence of the indicated 20 mM divalent cation. Preliminary experiments established that the light intensity gave the optimal rate of photoactivation. The rates of DCIP photoreduction were recorded at 600 nm using an Aminco spectrophotometer equipped with side-window illumination. The down- and up-pointing arrows indicate when the light was turned on and off, respectively. (See text for details.)

Inspection of the traces reveals that all show an initial relatively rapid rate of Mn^{2+}-dependent DCIP photoreduction followed by a gradual exponential rate decline of DCIP photoreduction in $\leq$15 s. The initial rates [18 μequiv of electrons (mg of Chl·h)$^{-1}$] in the absence of Ca^{2+} or in the presence of Mg^{2+} were equivalent and ~42% greater than those seen in the presence of Ca^{2+} or Sr^{2+}, presumably as a result of some competition by Ca^{2+} or Sr^{2+} with Mn^{2+} at its site of oxidation by $TyrY_Z^+$. This magnitude of difference between rates of Mn^{2+}-dependent DCIP photoreduction is entirely comparable to the difference in the initial rates of photoligation of Mn in the absence and presence of Ca^{2+} (Figure 2A). After this rapid initial exponentially decaying phase of Mn^{2+}-dependent DCIP photoreduction, the membranes receiving no Ca^{2+} or an addition of Mg^{2+} gave extremely low rates of DCIP photoreduction over the next ~4 min of illumination, even though both Mn^{2+} and DCIP were still present in excess concentrations. On cessation of illumination of the minus Ca^{2+} and the plus Mg^{2+} samples, most (84%) of the photoaccumulated $DCIPH_2$ was oxidized rapidly presumably by back-reaction with some of the $Mn^{\geq 3+}$ generated during illumination (Velthuys, 1983). This result explains the absence of accumulation of $DCIPH_2$ in supernatants from NaCl/NH_2OH-PSII illuminated in the presence of Mn^{2+} without Ca^{2+} (Miyao & Inoue, 1991b).

With addition of Ca^{2+}, the comparatively rapid initial rate of Mn^{2+}-dependent photoreduction of DCIP was followed by a transient slower rate which then increased ~1.5-fold. On cessation of illumination, essentially no oxidation of $DCIPH_2$ was observed. Parallel measurements of O_2-evolution capability showed that the time (~3.2 min) for onset of this increased rate of DCIP photoreduction correlated well with the half-time of photoactivation. Similar kinetic behavior was observed when Sr^{2+} was used; however, in this case, the rate measured just before cessation of illumination was only ~52% of that seen with Ca^{2+}. This diminished effectiveness of Sr^{2+} relative to Ca^{2+} is similar to what is observed in the reconstitution of O_2 evolution in Ca^{2+}-depleted PSII membranes (Yocum, 1991) and for photoactivation of NH_2OH-PSII using 1 mM Mn^{2+}/100 μM DCIP (Tamura et al., 1989). The diminished effectiveness of Sr^{2+} for promoting photoactivation in the experiments of Figure 5 is seen also in the rate of oxidation of the photoaccumulated $DCIPH_2$ after illumination. Although the total amount of DCIP photoreduced in the presence of Sr^{2+} was only 65% of that seen with Ca^{2+}, the initial rate of $DCIPH_2$ oxidation in darkness was ~3.5-fold greater than with Ca^{2+}. Apparently, virtually all the Mn that is photoligated when Ca^{2+} is present is assembled as O_2-evolving $(Mn)_4$-clusters, while with Sr^{2+} both $(Mn)_4$-clusters and $Mn^{\geq 3+}$ are products and with Mg^{2+} or without Ca^{2+} only $Mn^{\geq 3+}$ becomes ligated. This supposition finds support from measurements of the Mn abundance in membranes illuminated for 20 min in the presence of 1 mM Mn^{2+} or 1 mM Mn^{2+}/20 mM Mg^{2+}. Equal and high Mn abundances (~11 Mn/200 Chl) with no increase of rate of O_2 evolution were found.

Ca^{2+} Concentration Dependence. Much of the data in preceding sections shows that Ca^{2-} promoted the photoassembly of $(Mn)_4$-clusters as shown previously [for reviews, see Tamura and Cheniae (1988) and Debus (1992)]. Additionally, the data show that Ca^{2+} inhibited ligation of a rather large amount of an Mn species assumed to be $Mn^{\geq 3+}$. Whether the binding site(s) of the Ca^{2+} involved in these processes can be identified with the binding site(s) of the Ca^{2+} required in O_2 evolution *per se* is unknown. One of us (G.M.C.) previously reported results suggesting that the binding site for the Ca^{2+} involved in O_2 evolution was created during photoactivation (Tamura & Cheniae, 1988). In the experiments of Figure 6, we measured the Ca^{2+} concentration dependence for promoting photoactivation in order to compare it with results reported for reconstitution of O_2 evolution by Ca^{2+} with Ca^{2+}-depleted PSII. Because of the competition between Mn^{2+} and Ca^{2+} at Ca^{2+}-binding sites (Yocum, 1991), only 100 μM Mn^{2+} was used rather than the usual 1 mM Mn^{2+}. Nevertheless, at optimum Ca^{2+} levels, both the half-time and extent of photoactivation were similar to that shown in Figure 1A using 1 mM Mn^{2+}.

The data points of Figure 6A show the effect of increasing Ca^{2+} concentration on the extent of photoactivation induced by a 20 min illumination. Analysis by an Eadie–Scatchard plot (Figure B) gave two distinct linear regions, indicating two independent sites of Ca^{2+} binding. For each site, K_m and V_{max} values were calculated (Spears et al., 1971), and then the dashed and dot-dashed curves shown in Figure 6A were calculated, assuming Michaelis–Menton kinetic behavior. Summation of the dashed and dot-dashed curves yielded the solid curve through the data points. The K_m and V_{max} values yielding the dot-dashed curve were 37.7 μM and 235 μmol of O_2 (mg of Chl·h)$^{-1}$, respectively. For the dashed curve, the K_m and V_{max} values were 1311 μM and 183 μmol of O_2 (mg of Chl·h)$^{-1}$, respectively. These Ca^{2+} K_m values for promoting photoactivation differ significantly from the 300 μM dissociation constant for Ca^{2+} at the Ca^{2+} site as

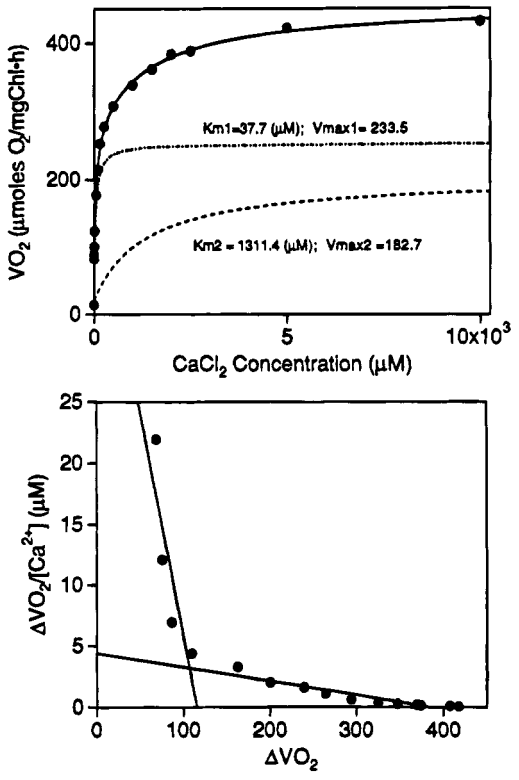


FIGURE 6: (A) Velocity curve showing the dependence of photoactivation on Ca^{2+} concentration. NaCl/NH_2OH-PSII membranes were photoactivated for 20 min in the presence of 100 μM $MnCl_2$/10 μM DCIP and the indicated concentrations of $CaCl_2$, and then rates were determined directly in the Ca^{2+}-containing assay mixture. (B) Eadie–Scatchard plot of the data of A. The dashed and dot-dashed curves of panel A are velocity curves calculated from K_m and V_{max} values determined from the Eadie–Scatchard plot by successive approximations (Spears et al., 1971; Segel, 1975, pp 64–71) for two independent sites. The dashed and dot-dashed curves of panel A were summed to produce the velocity curve fitting the experimental data. The K_m and V_{max} values for the dot-dashed velocity curve were 37.3 μM and 235 μmol of O_2 (mg of Chl•h)$^{-1}$, and the K_m and V_{max} values for the dashed curve were 1311.4 μM and 182.7 μmol of O_2 (mg of Chl•h)$^{-1}$, respectively.

deduced by Miller and Brudvig (1989) in their kinetic model treatment of photoactivation. On the other hand, they approximate the two Ca^{2+} K_m values of 50–100 μM and 1–7 mM found by different laboratories in studies of the Ca^{2+} concentration dependence for reconstitution of O_2 evolution by Ca^{2+}-depleted PSII membranes [see Debus (1992) and Yocum (1991) for reviews]. The difference in concentration of Na^+ used here versus those used in various reconstitution analyses, as well as the presence of 100 μM Mn^{2+} in our measurements, precludes a more critical comparison of the Ca^{2+} K_m values in the two processes, since both Na^+ and Mn^{2+} are weak antagonists of Ca^{2+} binding (Yocum, 1991). At this time, we are unsure whether the data of Figure 6 infer two distinct Ca^{2+}-binding sites or only one Ca^{2+}-binding site that has been partially perturbed.

Effect of Reconstitution with the 17 kDa/24 kDa Extrinsic Proteins on Photoactivation in the Absence of Ca^{2+}. Analyses of the redox properties of the S_2 state of Ca^{2+}-depleted PSII membranes containing or lacking the 24 kDa extrinsic protein have revealed that this protein markedly affects the temperature dependence of the TL emission arising from charge recombination between S_2/Q_A^- as well as the dependence on temperature for the advancement of S_1 to S_2 (Ono et al., 1992). These results, showing that the redox

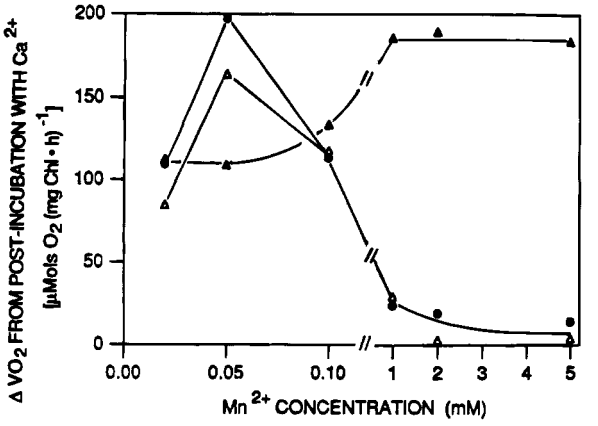


FIGURE 7: The effect of reconstitution with 17 kDa/24 kDa polypeptides on the capability of NaCl/NH_2OH-PSII to assemble $(Mn)_4$-clusters in the absence of Ca^{2+}. Membranes were preincubated in darkness (10 min) in buffer A containing 10 μM DCIP and the $MnCl_2$ concentrations shown and were then illuminated (20 min). Subsequently, rates of O_2-evolution were determined in a mixture containing 0.8 M sucrose/50 mM Mes–NaOH/2 mM EGTA/1 mM FeCN/300 μM PBQ, pH 6.2, and then, after addition of $CaCl_2$ (final concentration of 50 mM Ca^{2+}) and a 60 min dark incubation, rates were determined in a 15 mM Ca^{2+}-containing assay mixture (Materials and Methods). The difference between the two assay determinations is plotted on the ordinate. The closed circles represent NaCl/NH_2OH-PSII membranes which were not reconstituted. The open triangle data were obtained with membranes which were reconstituted after illumination and prior to postincubation with Ca^{2+}. The closed triangle data were obtained with membranes that were reconstituted prior to photoinactivation.

properties of the $(Mn)_4$-cluster depend on the 24 kDa protein, led the authors to suggest that this protein regulates the structure/function of the $(Mn)_4$-cluster in the absence of Ca^{2+} by modulating the conformation(s) of the intrinsic protein(s) ligating both the Mn and Ca^{2+} of the oxygen-evolving complex. According to this view, this protein closely interacts with both Ca^{2+} and the $(Mn)_4$-cluster. Supportive evidence for this idea come from reports showing (a) the 17 kDa/24 kDa proteins protect the $(Mn)_4$-cluster from attack by exogenous reductants (Ghanotakis et al., 1984; Tamura & Cheniae, 1985; Tamura et al., 1986; Mei & Yocum, 1990), (b) the $(Mn)_4$-cluster is required for binding of the 24 kDa protein (Becker et al., 1985; Ono et al., 1986; Kavelaki & Ghanotakis, 1991), and (c) inhibition of O_2 evolution caused by extraction of the 17 kDa/24 kDa proteins without solubilization of any bound Ca^{2+} (Shen et al., 1988; Enami et al., 1989).

In the experiments of Figure 7, we asked if reconstitution of the 17 kDa/24 kDa-less NaCl/NH_2OH-PSII membranes with these extrinsic proteins prior to a photoactivation regime in the absence of Ca^{2+} would permit the assembly of $(Mn)_4$-clusters having O_2-evolution capability after postincubation of the membranes with Ca^{2+}. These experiments were done intentionally at pH 6.5 and at 10 μM DCIP, since (a) at this condition and 1 mM Mn^{2+}, no increase of O_2-evolution capability is seen from illumination of NaCl/NH_2OH-PSII in the absence of Ca^{2+} (Figure 3B), and (b) in the presence of Ca^{2+}, maximum rate and extent of photoactivation are still observed (Figures 1A, 3A).

The closed circles of Figure 7 show the Ca^{2+}-dependent postincubation increase of O_2-evolution resulting from illumination of NaCl/NH_2OH-PSII membranes at the indicated concentrations of Mn^{2+}. The open triangle data were obtained similarly except that after illumination and before

postincubation with Ca^{2+} the membranes were reconstituted with the 17 kDa/24 kDa proteins before assays of O_2-evolution capability. Without postincubation with Ca^{2+}, all of the differently treated NaCl/NH_2OH-PSII membranes gave very low rates of O_2 evolution when assayed directly in the minus Ca^{2+} assay mixture. The highest [50−60 μmol of O_2 (mg of Chl·h)$^{-1}$], and the lowest [13−16 μmol of O_2 (mg of Chl·h)$^{-1}$] rates were recorded with membranes illuminated in 50 μM Mn^{2+} and in ≥1 mM Mn^{2+}, respectively. As shown, both the NaCl/NH_2OH-PSII membranes and those reconstituted after illumination had a similar Mn^{2+} concentration dependence for formation of $(Mn)_4$-clusters displaying O_2-evolution activity after dark incubation with Ca^{2+}. Only at <1 mM Mn^{2+}, with a maximum at 50 μM Mn^{2+}, was there apparent formation of clusters.

On the other hand, NaCl/NH_2OH-PSII membranes which had been reconstituted with the 17 kDa/24 kDa proteins prior to illumination (closed triangles) gave rather high rates of O_2 evolution after dark incubation with Ca^{2+}, even those illuminated at ≥1 mM Mn^{2+}. This effect of the 17 kDa/24 kDa proteins cannot be attributed to Ca^{2+} carry-over with the proteins, since the proteins had been dialyzed against buffer containing 30 g of Chelex-100 resin (30 g/L) and their addition had no effect on the rates of O_2 evolution measured directly in the minus Ca^{2+} assay mixture. This effect also cannot be attributed to a nonspecific protein effect, since bovine serum albumin at equivalent or 2-fold higher concentration than that used for reconstitution with the 17 kDa/24 kDa had no effect. NH_2OH-PSII membranes which lack the 17 kDa protein but contain considerable 24 kDa protein (Tamura & Cheniae, 1987a) gave results similar to NaCl/NH_2OH-PSII membranes which had been reconstituted with a 17 kDa/24 kDa fraction prior to illumination. We, therefore, attribute the reconstitution effect shown at ≥1 mM Mn^{2+} directly to the effects the 24 kDa polypeptide has on the conformations of the intrinsic protein(s) involved in the ligation of the Mn and Ca^{2+} of the water-oxidizing complex.

Thermoluminescence Analyses of NaCl/NH_2OH-PSII Photoactivated in the Absence and Presence of Ca^{2+}. The extent of restoration of O_2 evolution from photoactivation of NH_2-OH-PSII in a Mn^{2+}/Ca^{2+} mixture has been shown to correlate well with increase of Mn content and with the magnitude of the S_2 multiline EPR signal (Miller & Brudvig, 1989) or with the intensity of B-band thermoluminescence emission (Tamura et al., 1989) reflecting recombination of S_2 Q_B^- and S_3 Q_B^- charge pairs. However, the correlation between Ca^{2+}-activated O_2 evolution, Mn abundance, and B-band emission intensity after a single flash was only roughly approximate with membranes photoactivated in 1 mM Mn^{2+}/100 μM DCIP and no Ca^{2+} (Tamura et al., 1989). In particular, the Mn abundances, even after postincubation with Ca^{2+} causing release of 1−2 membrane-bound Mn per reaction center, were disproportionately high relative to the rates of O_2 evolution. Though the NaCl/NH_2OH-PSII membranes used here gave no O_2 evolution after illumination in 1 mM Mn^{2+}/10 μM DCIP and no Ca^{2+}, even following postincubation with Ca^{2+} (Figures 1A and 2B), these results do not exclude the possibility that $(Mn)_4$-clusters were formed, but their water-oxidizing activity could not be expressed because light-generated $Mn^{\geq 3+}$ was very tightly bound to the Ca^{2+} site essential for O_2 evolution and could not be displaced by Ca^{2+} in postincubations. Since Ca^{2+} is thought to be required only for the $S_3 \rightarrow S_0 + O_2$ transition (Debus, 1992; Rutherford et al., 1992), such membranes would display the S_2 multiline EPR signal and B-band emission if $(Mn)_4$-clusters were formed.

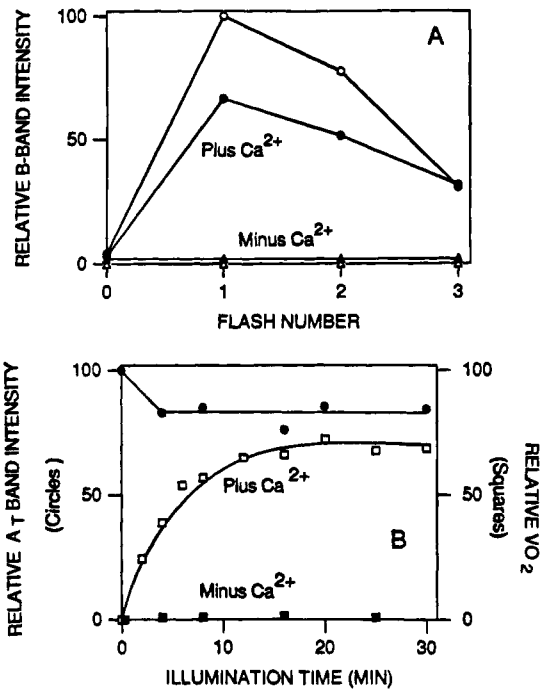


FIGURE 8: (A) Effect of illumination of NaCl/NH_2OH-PSII in the presence and absence of Ca^{2+} on the B-band TL emission in a sequence of flashes. The open and closed circle data were recorded with parent PSII-membranes and with NaCl/NH_2OH-PSII membranes that had been illuminated (20 min) in buffer A containing 1 mM Mn^{2+}/20 mM Ca^{2+}/10 μM DCIP. The open and closed triangle data were obtained with membranes illuminated as above except for the omission of Ca^{2+}. Following illumination, a portion of the membranes were incubated (60 min dark/4 °C) with 15 mM Ca^{2+} and then TL measurements were made (closed triangles). (B) Effects of illumination of NaCl/NH_2OH-PSII in the presence and absence of Ca^{2+} on the increase of rate of O_2-evolution and the intensity of the A_T-band emission. The open and closed squares record the rates of O_2-evolution by membranes illuminated in the presence and absence of Ca^{2+}, respectively, relative to the rate [694 μmol of O_2 (mg of Chl·h)$^{-1}$] by parent PSII membranes. These membranes (20 min of illumination) were used in the assays of B-band emission above. The closed circles show the relative A_T-band TL intensity of membranes that had been illuminated in the absence of Ca^{2+}, extracted (~250 μg of Chl/mL) with 1 mM NH_2-OH for 5 min, and then washed twice with buffer A before measurements of A_T-band emission (Materials and Methods).

Figure 8A shows a comparison of the B-band intensity emissions following a sequence of flashes given to parent PSII membranes (open circles) and to NaCl/NH_2OH-PSII membranes photoactivated either in the presence (closed circles) or absence (closed triangles) of Ca^{2+}. In all of these measurements, equal chlorophyll concentrations were used, thus direct comparisons can be made between the different samples. As shown, both the PSII membranes and the Mn^{2+}/Ca^{2+}-photoactivated NaCl/NH_2OH-PSII membranes gave high B-band emissions on the first two flashes before damping and becoming equivalent on the third flash. The relative intensity of the emission on the first two flashes and the rate of O_2 evolution (Figure 8B) produced by the Mn^{2+}/Ca^{2+}-photoactivated membranes were both ~66% of the values obtained with parent PSII membranes. In contrast, neither the membranes photoactivated in Mn^{2+} only (closed triangles) and analyzed directly nor those given subsequent postincubation with Ca^{2+} (open triangles) showed any B-band

emission throughout the flash regime. Moreover, such membranes, in contrast to those photoactivated in Mn^{2+}/Ca^{2+}, produced no S_2 multiline signal, nor did they exhibit a significant aquo–Mn^{2+} six-line EPR signal. Clearly, none of the rather large number of Mn that becomes ligated on illumination of NaCl/NH_2OH-PSII in 1 mM Mn^{2+}/10 μM DCIP is assembled as $(Mn)_4$-clusters. At least at this condition, both Ca^{2+} and Mn^{2+} appear to be strict requirements in the photoactivation process.

In the experiments of Figure 8B, we asked if such failure to assemble $(Mn)_4$-clusters in the absence of Ca^{2+} possibly was a consequence of photodamage of the putative His residue, possibly His-190 of D_1 (Kramer et al., 1994), contributing to the A_T thermoluminescence emission arising from His^+/Q_A^- charge recombination (Koike et al., 1986; Ono & Inoue, 1990, 1991b). Both the capabilities of A_T-band emission and photoactivation become rapidly and simultaneously inhibited by either weak or strong light treatment of NH_2OH-PSII, at least in the absence of an exogenous electron donor to PSII (Ono & Inoue, 1991a; Chen et al., 1995). These observations led Ono and Inoue to suggest that the putative His residue functions in photoactivation to mediate electron transfers between $TyrY_Z^+$ and Mn^{2+}.

The NaCl/NH_2OH-PSII membranes used in the experiments of Figure 8 exhibited a time-course of photoactivation in the presence and absence of Ca^{2+} as shown in Figure 8B by the open and closed squares, respectively. Though the membranes illuminated in the absence of Ca^{2+} were incapable of O_2 evolution and B-band generation, they were extracted with NH_2OH to remove any photoligated Mn before measurements of their capability to generate the A_T-band emission. The closed circles record the intensity of the A_T-band emission from membranes illuminated for times shown in the absence of Ca^{2+} relative to those maintained in darkness. As shown, about 17% of the capability to generate the A_T-band was lost during the first 4 min of illumination but then no more. Under similar weak light experimental conditions, but in the absence of Mn^{2+}, 50% and total suppression of the A_T-band capability of NH_2OH-PSII occur in only ~1.3 and 15 min, respectively (Ono & Inoue, 1991a). Thus, the failure to assemble $(Mn)_4$-clusters in the absence of Ca^{2+} cannot be ascribed to photodamage of the putative His residue contributing to the A_T-band and to photoactivation. Presumably, electron donation from Mn^{2+} to $TyrY_Z^+$ and/or the putative His^+ residue protects against donor-side photodamages.

General Chemical Properties of the Mn Photoligated in the Presence of Mn^{2+}/Ca^{2+} versus Mn^{2+}. Chemical reduction of the Mn ions within the $(Mn)_4$-cluster to Mn^{2+} results in destruction of the cluster, dissociation of the Mn^{2+}, and loss of O_2-evolution capability. The rate of reactivity of the cluster with reductants of different molecular size is modulated, in part, by the PSII extrinsic proteins as well as PSII Cl^- and Ca^{2+}, which together tend to provide a barrier thereby protecting the cluster (Debus, 1992).

In the experiment summarized in Table 1, the effectiveness was measured of several reductants to dissociate the Mn ligated as $(Mn)_4$-clusters during photoactivation in Mn^{2+}/Ca^{2+} versus the Mn ligated during illumination in Mn^{2+} without Ca^{2+}. The data of rows 2 and 3 show the effect of extraction with 0.5–5 mM NH_2OH and 0.8 M Tris, pH 8.3, respectively, on rates of O_2-evolution and Mn abundances

Table 1: Comparison of the Susceptibility of the Mn Photoligated in the Presence and Absence of Ca^{2+} to Dissociation by Chemical Treatments

chemical treatment[a]	plus Mn^{2+}/Ca^{2+} illumination[b] ΔV_{O_2}	plus Mn^{2+}/Ca^{2+} illumination[b] ΔMn/200 Chl	plus Mn^{2+} minus Ca^{2-} illumination,[b] ΔMn/200 Chl
1. none	406	2.24	12.1
2. 0.5–5 mM NH_2OH	0	0.43	0.53
3. 0.8 M Tris, pH 8.3	0	0.32	1.06
4. 3 mM H_2O_2	158	1.29	0.58
5. 1 mM HQ	324	1.78	0.57
6. 100 μM $DCIPH_2$[c]	388	2.21	1.21

[a] The presence of 20 mM $CaCl_2$ during chemical treatment did not significantly alter either the ΔV_{O_2} or the ΔMn/200 Chl values shown. [b] NaCl/NH_2OH-PSII membranes were illuminated for 20 min in buffer A containing 10 μM DCIP and either 1 mM Mn^{2+}/20 mM Ca^{2+} or 1 mM Mn^{2+} minus Ca^{2+}. After removal of adventitious Mn^{2+}, the membranes were incubated (200 μg of Chl/mL) in darkness for 30 min at 4 °C with the indicated chemicals and then washed with buffer A containing 2 mM EDTA before assay of rates of O_2-evolution and abundances of bound Mn. V_{O_2} assays were made directly in assay buffer containing 15 mM Ca^{2+} after ≤2 min preincubation. See Materials and Methods for details. [c] Present as 100 μM DCIP and 500 μM sodium ascorbate.

of NaCl/NH_2OH-PSII illuminated in Mn^{2+}/Ca^{2+} and in Mn^{2+} without Ca^{2+}. These two chemical treatments are frequently used diagnostically to indirectly assay for $(Mn)_4$-clusters. With membranes illuminated in the presence of Mn^{2+}/Ca^{2+}, both treatments yielded the expected results, namely, elimination of O_2-evolution capability and depletion of the Mn abundance from 2.24 to <0.5 Mn/200 Chl. As shown, the same two chemical treatments effectively dissociated most of the large amounts of Mn that were ligated during illumination in the absence of Ca^{2+} but which had essentially no O_2-evolution capability.

Rows 4–6 give results from treatment with 3 mM H_2O_2, 1 mM HQ, and 100 μM $DCIPH_2$, respectively. Previous work has revealed that 3–9 mM H_2O_2 sluggishly dissociates the $(Mn)_4$-cluster in membranes lacking the 17 kDa/24 kDa extrinsic polypeptides or the 33 kDa extrinsic polypeptide or in membranes which have been depleted of Cl^- (Debus, 1992). This small molecule caused only partial losses, relative to NH_2OH or Tris, of the O_2-evolution capability and the Mn abundance of the 17 kDa/24 kDa-less NaCl/NH_2OH-PSII membranes photoactivated in Mn^{2+}/Ca^{2+}. However, with membranes illuminated in Mn^{2+} without Ca^{2+}, 3 mM H_2O_2 was equally as effective as 0.5–5 mM NH_2OH for dissociation of the photoligated Mn as shown by rows 5 and 6.

Treatment of membranes photoactivated in Mn/Ca^{2+} with bulky reductants such as 1 mM HQ or 100 μM $DCIPH_2$ caused minimal losses of O_2-evolution activity and Mn abundance, even when these treatments were done without addition of Ca^{2+} to the incubations. According to Mei and Yocum (1992), Ca^{2+} or Cl^- addition to their Cl^-/Ca^{2+} and 17 kDa/24 kDa-depleted PSII membranes is required to slow/inhibit HQ-induced loss of O_2-evolution and dissociation of Mn. Apparently, photoactivation of the 17 kDa/24 kDa-depleted NaCl/NH_2OH-PSII membranes in Mn/Ca^{2+} results in ligation of sufficient Ca^{2+} such that the addition of 20 mM Ca^{2+} to treatment with HQ or the other reductants had essentially no effect. Despite the bulkiness of HQ and $DCIPH_2$, these molecules were, nevertheless, equally as effective as the smaller sized reductants for dissociating/

solubilizing the $Mn^{\geq 3+}$ species photoligated in the absence of Ca^{2+}. Presumably, the conformational rearrangements of the PSII core polypeptides, which are believed to occur during photoactivation and to diminish the susceptibility of the ligated Mn^{3+} intermediate to exogenous reductants (Debus, 1992), do not occur in the absence of Ca^{2+}.

We sought means to evaluate whether different conformations of core polypeptides existed and contributed differently to the stability of Mn ligated during illumination of NaCl/NH_2OH-PSII in Mn^{2+}/Ca^{2+} and in Mn^{2+}. In data not shown, we found that brief sonication at 5 s intervals (over the range 0−20 s) of Mn^{2+}/Ca^{2+} photoactivated membranes followed by density gradient centrifugation and EDTA washing (see Materials and Methods) did not diminish the Mn abundance. In contrast, this same procedure caused rapid decrease of the $Mn^{\geq 3+}$ that was ligated during illumination in the absence of Ca^{2+}. After 20 s of sonication, only 20% of the original Mn abundance remained. Such results tend to reinforce the idea that photoactivation in Mn^{2+}/Ca^{2+} leads to $(Mn)_4$-clusters which are more buried than the $Mn^{\geq 3+}$ ligated in the absence of Ca^{2+}.

Reversibility of Photoinactivation. Photoinactivation has been defined as the light-induced loss of photoactivation capability without loss of primary electron transport (Miller & Brudvig, 1989). By this definition, illumination of NaCl/NH_2OH-PSII membranes in 1 mM Mn^{2+}/10 μM DCIP without Ca^{2+} results in photoinactivation on the basis of the following: (1) though the capacity of such membranes to photoreduce DCIP by 100 μM Mn^{2+}, 1 mM DPC or by a mixture of 5 μM Mn^{2+}/3 mM H_2O at light-limiting conditions is diminished by 30%−40%, full capacity is restored by NH_2-OH treatment causing dissociation of the ~12−16 $Mn^{\geq 3+}$/200 Chl; (2) the yield of flash-induced variable Chl *a* fluorescence from the membranes illuminated in 1 mM Mn^{2+}/10 μM DCIP is equivalent to that from unilluminated membranes; and (3) the intensity of the A_T-band emission from such illuminated membranes is maximally diminished by only 17% relative to those maintained in darkness (Figure 8B). These observations indicate that the primary electron transport capability was not significantly affected, even though the capability to carry out photoactivation was essentially eliminated.

Figures 9 and 10 summarize experiments testing whether photoinactivation was reversible. Bars A and B of Figure 9 show the small increase in rate of O_2 evolution resulting from 20 min of illumination of NaCl/NH_2OH-PSII in 10 μM DCIP and 50 μM and 1 mM Mn^{2+}, respectively. Bars C and D illustrate the yields of photoactivation resulting from additions of 5 and 20 mM Ca^{2+} to the 50 μM and 1 mM Mn^{2+} solutions, respectively. Subsequently, the membranes of samples A to D were briefly (5 min) extracted with 1 mM NH_2OH, washed with EDTA to remove the $Mn^{\geq 3+}$ from samples A and B and the $(Mn)_4$-cluster from samples C and D, and then all samples were reilluminated (20 min) in 10 μM DCIP/1 mM Mn^{2+}/20 mM Ca^{2+}. Bars A′ to D′ show the rates of O_2 evolution resulting from reillumination of the NH_2OH/EDTA-treated membranes of A to D in the Mn^{2+}/Ca^{2+}/DCIP mixture. Comparison of A′ with C′ and B′ with D′ reveals that the extent of photoactivation by the previously photoinactivated A and B membranes is nearly equivalent to the extent obtained with membranes experiencing initial photoactivation, NH_2OH extraction, and rephotoactivation.

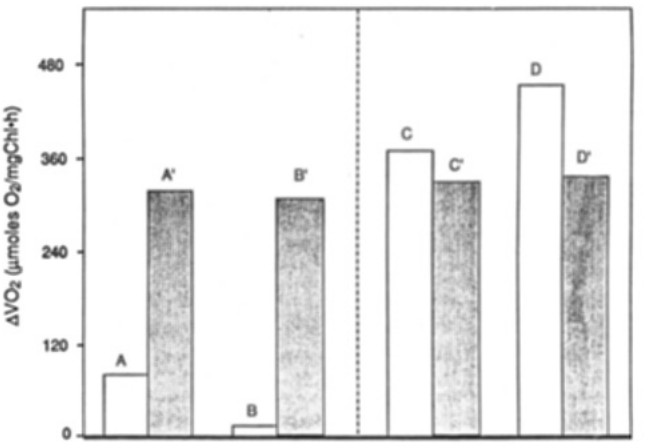


FIGURE 9: NH_2OH extraction of photoinactivated membranes followed by reillumination in the presence of Mn^{2+}/Ca^{2+}/DCIP permits assembly of $(Mn)_4$-clusters. Samples A and B were illuminated for 20 min in buffer A containing 10 μM DCIP and 50 μM and 1 mM Mn^{2+}, respectively. Samples C and D were illuminated similarly except that buffer A contained 50 μM Mn^{2+}/5 mM Ca^{2+} and 1 mM Mn^{2+}/20 mM Ca^{2+}, respectively. After rates of O_2-evolution were determined, samples A−D were extracted (1 mM NH_2OH/5 min/4 °C in the dark), washed, and then reilluminated for 20 min in buffer A containing 1 mM Mn^{2+}/10 μM DCIP/20 mM Ca^{2+} to give the corresponding samples indicated by the prime notation.

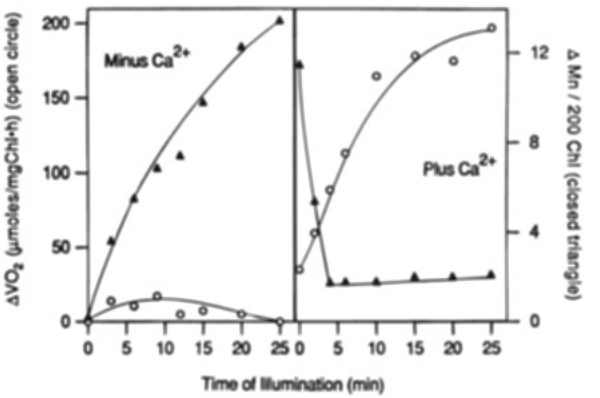


FIGURE 10: The photoinactivated state is reversed by Ca^{2+} addition and reillumination. The left panel records the increase of EDTA nonextractable Mn (closed triangles) and rates of O_2-evolution (open circles) from illumination of membranes in buffer A containing 1 mM Mn^{2+}/10 μM DCIP. Right panel: Membranes were illuminated as above for 25 min, and then $CaCl_2$ and DCIP were added in darkness to give 20 mM and 20 μM final concentrations, respectively. Subsequently, the membranes were reilluminated for times shown before determinations of rates of O_2 evolution and Mn abundances. (See text for details.)

Such reversal of photoactivation can be observed more facilely. In the experiment summarized in Figure 10, NaCl/NH_2OH-PSII membranes were illuminated in 1 mM Mn^{2+}/10 μM DCIP for the times shown on the abscissa of the left panel. The expected results, based on data of Figure 2, were obtained, namely, there was no significant increase in rate of O_2 evolution (open circles); however, a large amount of $Mn^{\geq 3+}$ species was ligated. Another set of membrane samples was illuminated under the exact same conditions noted above for 25 min. Then $CaCl_2$ and DCIP were added to each sample to give final concentrations of 20 mM and 20 μM, respectively. After dark incubation for 10 min, the samples were reilluminated for times shown and then assayed for O_2-evolution capability and Mn abundance. The right panel of Figure 10 records the results. As shown by the

closed triangles, a precipitous decrease in the abundance of $Mn^{\geq 3+}$ occurred during the first 2 min of illumination, reached a minimum value (1.6 Mn/200 Chl) after 4 min, and then slowly increased slightly with continued illumination. Accompanying this change was a large increase in O_2-evolution capability as shown by the open circles of the right panel. No parallel behavior between the increase of rate of O_2 evolution and the increase of Mn abundance was observed, in contrast to the results shown in Figure 1A. We interpret the results shown in the right panel as follows: (1) Ca^{2+} addition promotes and stabilizes the light-induced conformational change within the intrinsic protein(s) that is necessary for photoactivation; (2) during the early illumination period, sufficient $DCIPH_2$ is formed to dissociate as Mn^{2+} a large fraction of the $Mn^{\geq 3+}$ ligated during photoinactivation, and (3) this Mn^{2+} and perhaps some remaining nonfunctional $Mn^{\geq 3+}$ is then assembled as O_2-evolving $(Mn)_4$-clusters during continued illumination. The suggestion that sufficient $DCIPH_2$ is formed to dissociate the $Mn^{\geq 3+}$ that is inhibitory to photoactivation finds support from experiments in which photoinactivated membranes containing high abundances of the $Mn^{\geq 3+}$ were illuminated with 10 μM DCIP in the absence and presence of 100 μM atrazine, an inhibitor of Q_A^- to Q_B electron transfer. Membranes illuminated in the presence of atrazine lost <10% of the $Mn^{\geq 3+}$ over a period of 20 min illumination while those illuminated in the absence of atrazine lost ~75% within 5 min.

DISCUSSION

This work was directed toward evaluating two issues pertaining to the mechanism of photoactivation of O_2 evolution: (1) the relative merits of the one-stage model versus the two-stage model proposed originally by Ono and Inoue (1983) and Tamura and Cheniae (1987a), respectively; and (2) an assessment of contributions of photoinhibition, photoinactivation, and inherent membrane/protein structural deformation(s) to limitations of the extent of photoactivation seen often with PSII membranes depleted of their $(Mn)_4$-complexes and one or more of the PSII extrinsic proteins.

The one-stage model was advanced on the basis of studies with intact chloroplasts from leaves grown under intermittent flash illumination (Ono & Inoue, 1983). In these studies, the divalent cation ionophore, A23187, was used in conjunction with EGTA and additions of various concentrations of Mn^{2+} and Ca^{2+} or other divalent cations to analyze their effects on the increase of rate of DCIP photoreduction capability following illumination for a time (8 min) insufficient to give maximum increase of photoactivation. It was tacitly assumed that the rate of photoactivation was strictly proportional to the single measurement of DCIP photoreduction activity induced by the brief illumination of the chloroplasts in the presence of various Mn^{2+}/Ca^{2+} concentrations. Additionally, in these experiments, the specific individual effects of Ca^{2+} and/or Mn^{2+} ions on the final extent of photoactivation measured either by increase of DCIP photoreduction activity or by increase of membrane bound Mn were not shown. Thus, these experiments did not distinguish between promotion of the initial rate of photoassembly of $(Mn)_4$-clusters by Mn^{2+} and Ca^{2+} versus activation of latent water-oxidizing activity by Ca^{2+} of any $(Mn)_4$-clusters photoassembled in the absence of Ca^{2+}.

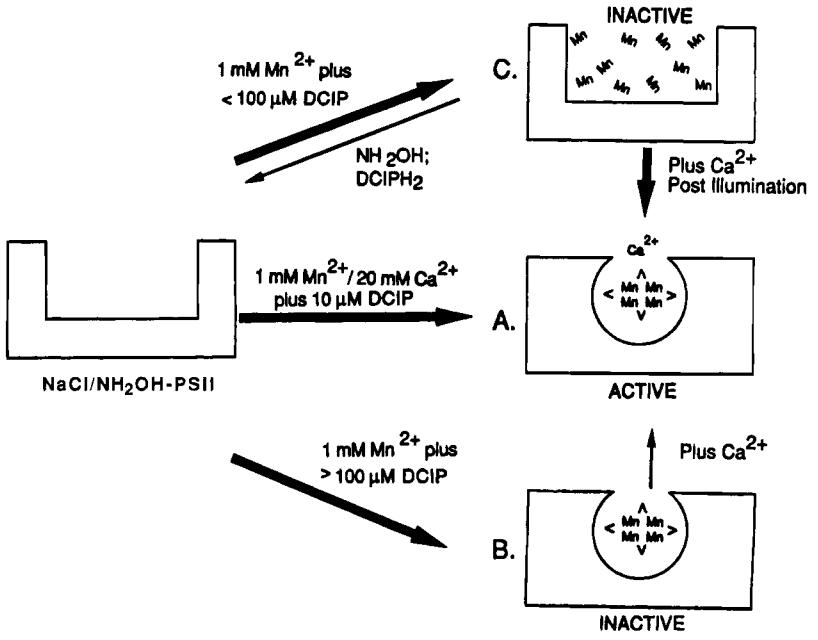
The recognition of conditions necessary for obtaining significant photoactivation of O_2 evolution and photoligation of Mn by NH_2OH- or Tris-PSII membranes permitted testing of the effects of illumination in the presence of DCIP containing Mn^{2+}/Ca^{2+} mixtures, or Mn^{2+} only, on the increases of O_2-evolution capability and membrane-bound Mn (Tamura & Cheniae, 1987a; Tamura et al., 1989; Miller & Brudvig, 1989). Illumination of membranes in a mixture of 100 μM DCIP and 1 mM Mn^{2+} at an optimal pH, without rigorous attempts to exclude contaminating Ca^{2+}, resulted in photoligation of ~3−4 Mn/RC which were EDTA nonextractable and which expressed O_2-evolution capability and B-band oscillations after postincubation with Ca^{2+} (Tamura & Cheniae, 1987a; Tamura et al., 1989). The authors noted, however, that the O_2-evolution capability of such membranes was low relative to the abundance of photoligated Mn even after postincubation with Ca^{2+}. Moreover, the yield of photoactivation under optimal conditions in the presence of both Mn^{2+}/Ca^{2+} was low which may have compromised some of the thinking leading to the two-stage model proposal.

In the evaluation of the two models reported here, NaCl/NH_2OH-PSII membranes (Miyao & Inoue, 1991a) were used, since these membranes, under optimum conditions, show a significantly greater extent and quantum efficiency of photoactivation than the NH_2OH-PSII membranes previously employed. These membranes are almost completely depleted of the 17 kDa/24 kDa extrinsic proteins as well as $(Mn)_4$-clusters; additionally, they are depleted of the 1 Ca^{2+}/RC required in O_2 evolution. Moreover, in contrast to the previous studies, stringent efforts were made to remove the ubiquitous, contaminating Ca^{2+} ions from buffers and labware.

Scheme 1 attempts to illustrate the conversions and interconversions of the differing states of NaCl/NH_2OH-PSII membranes as a result of their illumination under different conditions. In this scheme, the broad and narrow arrows represent light and dark reactions, respectively. The two differently shaped objects represent different conformations of PSII core complex polypeptides, particularly the D_1-polypeptide, since site-directed mutagenesis studies strongly suggest that this polypeptide contributes many of the amino acid residues that coordinate the $(Mn)_4$-cluster and the Ca^{2+} essential for the $S_3 \rightarrow S_0 + O_2$ transition [Chu et al., 1995a,b, and references therein; for reviews see Debus (1992) and Nixon and Diner (1994)]. The circular shaped object enclosing the $(Mn)_4$-cluster with the Ca^{2+} in close proximity to the cluster equates with the "jaws" model of the Ca^{2+}−$(Mn)_4$-cluster assemblage (Yachandra et al., 1993).

Path A depicts the photoassembly of a $(Mn)_4$-cluster/Ca^{2+} complex resulting from illumination of NaCl/NH_2OH-PSII in a 1 mM Mn^{2+}/20 mM Ca^{2+}/10 μM DCIP mixture (pH 6.5). The maximum rate ($t_{1/2} \approx 3$ min) and extent of recovery of O_2-evolution capability (~88% and 66% relative to NaCl-PSII and PSII membranes, respectively) were obtained (Figure 1), thus corroborating the results of Miyao and Inoue (199a); additionally, the maximum extent of recovery of B-band emission capability was obtained (~66% relative to PSII membranes, Figure 8A). Under this condition, the Mn that is photoligated is assembled as $(Mn)_4$-clusters based on the following: (1) The observed increase of Mn abundance and O_2-evolution capability exhibit a linear relationship that fits the relationship predicted based on O_2-

Scheme 1. Depiction of the Various States Formed from Illumination of NaCl/NH_2OH-PSII under Various Conditions[a]



[a] See Discussion for details.

evolution capability of PSII membranes (Figure 1A); and (2) the observed chemical reactivity of all the photoligated Mn to exogenous reductants (Table 1) (NH_2OH, Tris > H_2O_2 ≫ HQ, $DCIPH_2$) was typical for $(Mn)_4$-clusters of PSII membranes (Debus, 1992; Mei & Yocum, 1992, and references therein). Illumination of membranes in a mixture of 10 μM DCIP/50−100 μM Mn^{2+}/5 mM Ca^{2+} (Figures 6A and 9) also gave extents of recovery nearly equivalent to that observed with 10 μM DCIP/1 mM Mn^{2+}/20 mM Ca^{2+}. We believe this reflects the competition between Mn^{2+} and Ca^{2+} at each other's binding site, with the Mn^{2+}-binding site having a high specificity for Mn^{2+} and a low affinity for Ca^{2+} (Ono & Inoue, 1983; Tamura & Cheniae, 1987a; Miller & Brudvig, 1989).

On the other hand, with omission of Ca^{2+}, any significant increase of O_2-evolution capability following illumination and postincubation with Ca^{2+} was observed only under a limited set of conditions: (1) 1 mM Mn^{2+} and ≥100 μM DCIP (Figure 4B, path B of Scheme 1); (2) 10 μM DCIP and either ≤50 μM Mn^{2+} (Figure 2B) or 1 mM Mn^{2+} but at pH < 6.5 (Figure 3B); and (3) 10 μM DCIP and ≥1 mM Mn^{2+} with membranes that had been reconstituted with a 17 kDa/24 kDa extrinsic protein fraction prior to the photoactivation regime (Figure 7). The first condition noted above is the same condition employed by Tamura et al. (1989), and our results from this condition are qualitatively similar to theirs. However, the extent of recovery of latent O_2-evolution capability we observed in the absence of Ca^{2+} under any of the above conditions was far less than the recovery obtained in the presence of Ca^{2+} under optimal conditions (path A), even though the Mn abundances were similar. This result infers that only a fraction of the total Mn photoligated in the absence of Ca^{2+} under the above limited set of conditions was assembled as inactive O_2-evolving $(Mn)_4$-clusters that could be made active by incubation with Ca^{2+} in darkness.

Illumination of NaCl/NH_2OH-PSII in the absence of Ca^{2+} at all other conditions (pH, concentration of Mn^{2+} or DCIP) than those cited above exacerbated this relationship. As many as ~18 Mn/200 Chl were photoligated which were EDTA nonextractable, had little or no O_2-evolving activity, and produced no B-band TL emission (Figure 8A). This is illustrated in Scheme 1 by path C. Moreover, the chemical reactivity of such photoligated Mn contrasts greatly to the Mn assembled as $(Mn)_4$-clusters: (1) small-sized hydrophilic reductants (NH_2OH, H_2O_2) as well as large-sized lipophilic reductants (HQ, $DCIPH_2$) readily reduce/dissociate it (Table 1); additionally, the presence of Ca^{2+} does not inhibit the HQ-induced reduction/dissociation as seen with 17 kDa/24 kDa-depleted, $(Mn)_4$-cluster-containing PSII membranes (Debus, 1992; Mei & Yocum, 1992, and references therein); and (2) a sonication/EDTA/density gradient centrifugation treatment of membranes containing this aberrant Mn results in its dissociation. It can be inferred from such results that the conformation of the proteins ligating this $Mn^{\geq 3+}$ is greatly different than that ligating the $(Mn)_4$-cluster.

Our observations of the photoligation of $Mn^{\geq 3+}$ by NaCl/NH_2OH-PSII in the absence of Ca^{2+} are consistent with a report of Mn^{2+} photooxidation and Mn ligation without restoration of O_2-evolution capability (Klimov et al., 1982), and with the observation of silencing of the EPR signal from more than 4 Mn/PSII RC on illumination of NH_2OH-PSII in a solution containing a high ratio of Mn^{2+} to Ca^{2+} (Miller & Brudvig, 1990).

We believe that ligation of the $Mn^{\geq 3+}$ ions strongly inhibits photoassembly of $(Mn)_4$-clusters by occupying the sites providing ligands for the Mn^{3+} mononuclear complex and the Mn^{3+}−Mn^{2+} binuclear complex intermediates in the photoactivation process (Tamura & Cheniae, 1987a). While ligation of 12−16 $Mn^{\geq 3+}$ by NaCl/NH_2OH-PSII decreases the quantum efficiency of the photooxidation of 100 μM Mn^{2+} (or 1 mM DPC or 5 μM Mn^{2+}/3 mM H_2O_2) by 30%−40%, this would not explain the observed almost complete loss of photoactivation capability by membranes; thus, we equate the ligation of $Mn^{\geq 3+}$ with the photoinactivation process, namely, the loss of capability to photoassemble $(Mn)_4$-clusters without greatly affecting PSII primary electron transport (Miller & Brudvig, 1989).

Furthermore, our data indicate that the photoinactivation process is the major factor limiting the extent of recovery of O_2-evolution capability, at least with NaCl/NH_2OH-PSII membranes suffering virtually complete loss of capability to photoassemble $(Mn)_4$-clusters: (1) they were as capable as dark treated PSII membranes of photooxidation of exogenous electron donors (DPC, Mn/H_2O_2, Mn^{2+}) following extraction with NH_2OH or Tris [see, however, Miller and Brudvig (1989)]; (2) the capability to give the A_T-band emission was not seriously impaired (Figure 8B); and (3) following dissociation of the $Mn^{\geq 3+}$, recovery of O_2-evolution could be obtained by reillumination of the membranes under conditions optimum for photoactivation (Figures 9 and 10). In this case, the extent of recovery was almost equivalent to the extent observed with membranes initially photoactivated under optimum conditions, extracted with NH_2OH, then again photoactivated under optimum conditions. The recovery of O_2-evolution capability by membranes experiencing two cycles of photoactivation under optimum conditions was only ~25% less than that observed with one cycle. This relatively small decrease is attributed to a relatively small irreversible deformation/conformation of the PSII polypeptides constituting the microenvironment of the $(Mn)_4$-cluster.

We believe that the events leading to photoinactivation versus photoassembly of O_2-evolving $(Mn)_4$-clusters (photoactivation) reflect events associated with the rate-limiting step in photoactivation and that Ca^{2+} modulates these events. According to Tamura and Cheniae (1987a) [see also Miller and Brudvig (1989)], this rate-limiting step involves a conformational change which allows formation of the $Mn^{3+}-Mn^{2+}$ binuclear complex from the Mn^{3+} mononuclear complex formed in the first light reaction. Subsequently, in photoactivation, the $Mn^{3+}-Mn^{2+}$ binuclear complex is thought to be converted to the $Mn^{3+}-Mn^{3+}$ binuclear complex by the second light reaction, and then this complex spontaneously ligates two Mn^{2+} ions to yield an $(Mn^{3+})_2-(Mn^{2+})_2$-cluster whose redox state may be equivalent to the S_{-1} state (Bouges, 1971). We speculate that Ca^{2+} promotes and stabilizes the conformational change, thus permitting subsequent reactions leading to an $(Mn)_4$-cluster. In the absence of Ca^{2+} and without the conformational change under the conditions of a high Mn^{2+} concentration (1 mM) and a low DCIP concentration (10 μM), maximum rates of Mn^{2+} photooxidation with minimum back-reaction between $Mn^{3+}/DCIPH_2$ would occur leading to an accumulation of Mn^{3+} ions. This condition would result in the binding of the Mn^{3+} with high affinity without assembly of O_2-evolving $(Mn)_4$-clusters. Possibly some of the Mn^{3+} would dismutate to form Mn^{2-} and Mn^{4+}, both paramagnetic ions; however, the $Mn^{\geq 3+}$ ligated during photoinactivation is essentially EPR silent.

Earlier, we noted that a small abundance of $(Mn)_4$-clusters could be assembled in the absence of Ca^{2+} under a very limited set of conditions. With the one exception of NaCl/NH_2OH-PSII membranes reconstituted with 17 kDa/24 kDa polypeptides, all of the other conditions are ones that would result in minimal accumulation of $Mn^{\geq 3+}$ because of either limited Mn^{2+} photooxidation or back-reactions between photoaccumulated $DCIPH_2$ and the $Mn^{\geq 3+}$. This infers that the accumulation of $Mn^{\geq 3+}$ exacerbates the condition observed in the absence of Ca^{2+}. We believe that the explanations proposed above for the function of Ca^{2+} in photoactivation may be relevant to some of the observations reported by Chu et al. (1995a,b). We speculate that the Asp-59 and Asp-61 residues located near the base of the A helix of D_1 that they identified as PSII Ca^{2+}-binding sites are identifiable with the binding sites for the Ca^{2+}, causing the change of conformation that contributes to photoactivation. This postulated change of conformation also may relate to previous observations showing that Ca^{2+} specifically enhances the reaction rate between polar electron donors and $TyrY_Z^+$ (Yerkes & Babcock, 1981). We further speculate that this conformational change brings those D_1 residues within the carboxy-terminal domain that contributes ligands to the $(Mn)_4$-cluster in close proximity to D_1–Asp-170. This residue is known to ligate Mn^{2+} with high affinity for its photooxidation by $TyrY_Z^+$ (Boerner et al., 1992; Diner & Nixon, 1992; Nixon & Diner, 1992), and this photooxidation is thought to be the first step in the photoactivation process (Blubaugh & Cheniae, 1992). Finally, we believe that the site created during photoactivation for the binding of Ca^{2+} required in O_2-evolution (Tamura & Cheniae, 1988) involves a structural change associated with the final step(s) in the assembly of the $(Mn)_4$-cluster, and this structural change causes a downshift in the redox potential of Q_A during so-called photoactivation (Johnson et al., 1995).

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