

Probing the Functional Role of Ca^{2+} in the Oxygen-Evolving Complex of Photosystem II by Metal Ion Inhibition[†]

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ABSTRACT: Photosynthetic oxygen evolution in photosystem II (PSII) takes place in the oxygen-evolving complex (OEC) that is comprised of a tetranuclear manganese cluster (Mn_4), a redox-active tyrosine residue (Y_Z), and Ca^{2+} and Cl^- cofactors. The OEC is successively oxidized by the absorption of 4 quanta of light that results in the oxidation of water and the release of O_2 . Ca^{2+} is an essential cofactor in the water-oxidation reaction, as its depletion causes the loss of the oxygen-evolution activity in PSII. In recent X-ray crystal structures, Ca^{2+} has been revealed to be associated with the Mn_4 cluster of PSII. Although several mechanisms have been proposed for the water-oxidation reaction of PSII, the role of Ca^{2+} in oxygen evolution remains unclear. In this study, we probe the role of Ca^{2+} in oxygen evolution by monitoring the S_1 to S_2 state transition in PSII membranes and PSII core complexes upon inhibition of oxygen evolution by Dy^{3+} , Cu^{2+} , and Cd^{2+} ions. By using a cation-exchange procedure in which Ca^{2+} is not removed prior to addition of the studied cations, we achieve a high degree of reversible inhibition of PSII membranes and PSII core complexes by Dy^{3+} , Cu^{2+} , and Cd^{2+} ions. EPR spectroscopy is used to quantitate the number of bound Dy^{3+} and Cu^{2+} ions per PSII center and to determine the proximity of Dy^{3+} to other paramagnetic centers in PSII. We observe, for the first time, the S_2 state multiline electron paramagnetic resonance (EPR) signal in Dy^{3+} - and Cd^{2+} -inhibited PSII and conclude that the Ca^{2+} cofactor is not specifically required for the S_1 to S_2 state transition of PSII. This observation provides direct support for the proposal that Ca^{2+} plays a structural role in the early S-state transitions, which can be fulfilled by other cations of similar ionic radius, and that the functional role of Ca^{2+} to activate water in the O–O bond-forming reaction that occurs in the final step of the S state cycle can only be fulfilled by Ca^{2+} and Sr^{2+} , which have similar Lewis acidities.

The photooxidation of water to dioxygen in plants, algae, and cyanobacteria is catalyzed by the oxygen-evolving complex (OEC)¹ of photosystem II (PSII). The photochemistry of PSII is initiated by photoinduced charge separation from a primary chlorophyll, P_{680} , to a pheophytin (Pheo) and subsequently to the membrane-bound quinone cofactors, Q_A

and Q_B , which results in stabilization of the charge-separated states. The OEC of PSII is comprised of a tetranuclear manganese cluster (Mn_4), a redox-active tyrosine residue (Y_Z), and calcium ion (Ca^{2+}) and chloride ion (Cl^-) cofactors (Figure 1). To further stabilize the charge-separated state, P_{680}^{+} is reduced by a tyrosine residue, Y_Z , in the OEC, which in turn is reduced by the Mn_4 cluster. During the oxygen-evolution reaction, the Mn_4 cluster is sequentially oxidized in four light-driven steps. The successive formation of the higher oxidation states of the Mn_4 cluster, called S_n states ($n = 0-4$), leads to the oxidation of two water molecules and the release of one molecule of dioxygen (1, 2).

Ca^{2+} has been identified as an essential cofactor in the oxygen-evolution reaction, as the depletion of Ca^{2+} ions from the OEC causes the loss of oxygen-evolving activity in PSII. The Ca^{2+} -binding sites in PSII have been previously studied by several methods (3–6). Although a varying number of Ca^{2+} -binding site(s) have been reported in the literature, it is generally accepted that one Ca^{2+} ion per PSII center is required for oxygen evolution and that this Ca^{2+} ion is tightly bound ($K_\text{D} \sim \mu\text{M}$) in the OEC. Besides the direct functional role of Ca^{2+} ions in oxygen evolution, it has been suggested that a Ca^{2+} site in the 33 kDa polypeptide on the donor side of PSII could regulate H^+ transfer pathways through the

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¹ Abbreviations: bRC: bacterial reaction center; Chl: chlorophyll; DCBQ: 2,5-dichloro-*p*-benzoquinone; EDTA: ethylenediaminetetraacetic acid; EPR: electron paramagnetic resonance; EXAFS: extended X-ray absorption fine structure; FTIR: Fourier transform infrared; kDa: kilodalton; MES: 2-(*N*-morpholino)ethanesulfonic acid; Mn_4 : tetranuclear manganese; OEC: oxygen-evolving complex; OGP: *n*-octyl- β -D-glucopyranoside; $\text{P}_{1/2}$: microwave power at half saturation; P_{680} : primary chlorophyll electron donor of PSII; Pheo: pheophytin; PPBQ: para-phenyl benzoquinone; PSII: photosystem II; Q_A : primary plastoquinone electron acceptor of PSII; Q_B : secondary plastoquinone electron acceptor of PSII; S_n states: S_0 to S_4 oxidation states of the Mn_4 cluster; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Y_D : redox-active tyrosine-160 residue of the D2 protein; Y_Z : redox-active tyrosine-161 residue of the D1 protein.

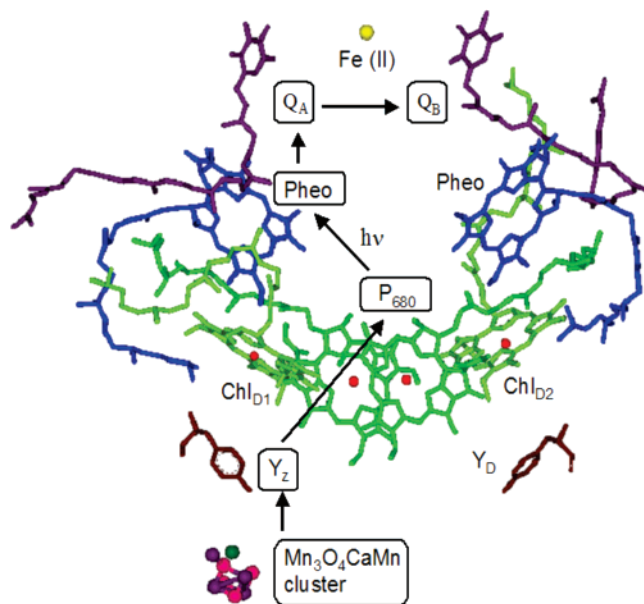


FIGURE 1: Relative locations of important redox components in PSII based on the 3.5 Å resolution crystal structure (PDB code: 1S5L) (23). Indicated with black arrows is the primary electron-transfer pathway of PSII.

water channel of the OEC (7, 8) and a Ca^{2+} site on the acceptor side of PSII could regulate proton-coupled electron-transfer pathways at the secondary quinone site of PSII (9). In addition, Shen, Ono, and co-workers have suggested the presence of additional Ca^{2+} sites that could also indirectly influence O_2 evolution (10, 11), and a weaker binding Ca^{2+} site has been identified in the CP29 polypeptide of the light harvesting complex II (LHCII) (12) of PSII. However, in the absence of direct experimental evidence, the role(s) of these additional Ca^{2+} -binding site(s) in PSII are currently under debate.

Sr^{2+} is the only cation that can functionally substitute for Ca^{2+} in the OEC. The biosynthetic incorporation of Sr^{2+} in the cyanobacterium *Thermosynechococcus elongatus* has recently been reported by Sugiura and co-workers (13). This study demonstrates that Ca^{2+} can be biosynthetically replaced by Sr^{2+} while retaining photosynthetic growth. Moreover in the thermophile, any Sr^{2+} bound outside the OEC can be exchanged with Ca^{2+} after isolating PSII, while the Sr^{2+} ion bound in the OEC is nonexchangeable (13). The Ca^{2+} -binding properties of PSII from this thermophile differ from nonthermophilic PSII, for which Ca^{2+} can be reversibly exchanged with di- and trivalent ions (1, 11, 14–20).

Previous results from extended X-ray absorption fine structure (EXAFS) measurements have indicated that the Ca^{2+} ion in the OEC is located very close to the Mn_4 cluster with corresponding Mn–Ca distances of 3.3 Å (17–19) and 4.2 Å (20). More recently, the X-ray crystal structure of PSII has been solved at 3.8–3.0 Å resolution (21–24). The structure of the OEC cannot be discerned in atomic-level detail in these X-ray crystal structures. However, using the 3.5 Å resolution X-ray crystallography maps in conjunction with anomalous diffraction and molecular modeling, Barber, Iwata, and co-workers have identified a Ca^{2+} ion as part of the OEC, which is modeled as a manganese-oxo cubane structure (Mn_3CaO_4) with a fourth manganese attached as a dangling atom (Figure 1). Additionally, Saenger, Zouni and

co-workers solved the X-ray crystal structure of PSII at 3.0 Å resolution (24). The 3.0 Å resolution X-ray crystal structure identifies a Mn_4Ca cluster, although the ligand assignment of the Mn_4Ca cluster is different from the Mn_3CaO_4 cubane proposed by Barber, Iwata, and co-workers (23). The crystallographic results showing that calcium is part of the OEC emphasize the important role of a Ca^{2+} ion in oxygen evolution.

Early proposals for the mechanism of oxygen evolution in PSII did not explain the role of Ca^{2+} (25), which is known to be an essential cofactor for oxygen evolution. More recently, studies have suggested that Ca^{2+} is directly involved in oxygen evolution (26–29). On the basis of density functional theory calculations, which showed that Ca^{2+} might be directly bridged to the Mn_4 cluster by $\mu\text{-OH}$ or $\mu\text{-O}$ ligands, it has been suggested that Ca^{2+} is required to form an oxygen radical in the S_3 state of the OEC (27). In a separate proposal, Pecoraro and co-workers have suggested that a terminal Mn(V)=O undergoes a nucleophilic attack by a Ca^{2+} -bound hydroxide ligand to form a Mn-bound hydroperoxide (28). Brudvig and co-workers have also proposed a mechanism in which a Ca^{2+} ion plays a role as a weak Lewis acid beyond the S_3 state of the OEC whereby a Ca^{2+} -bound water reacts with a Mn(V)=O species to form the O–O bond through a nucleophilic attack (26).

In previous metal-ion inhibition studies, Brudvig and co-workers investigated the binding of mono-, di-, and trivalent cations to the Ca^{2+} -binding site in the OEC (29). These studies have suggested that the Ca^{2+} -binding site in the OEC of PSII is size-selective, similar to the binding of Ca^{2+} ions in EF-hand proteins (29). Among the di- and trivalent cations that have been used to replace Ca^{2+} in PSII, Sr^{2+} is the only cation that maintains the O_2 -evolving activity of PSII. This may be because Sr^{2+} has a similar Lewis acidity to Ca^{2+} (29, 30). Sr^{2+} ion substitution has been shown to alter the S_2 state multiline EPR signal (31). It has previously been demonstrated that the depletion of Ca^{2+} ions in PSII does not inhibit the formation of the S_2 state (32, 33). This result suggests that Ca^{2+} may play a functional role beyond the S_2 state of the Mn_4 cluster in the OEC. It is thought that the incubation of PSII with Dy^{3+} (34, 35), La^{3+} (35), and Cd^{2+} (11, 36, 37) leads to loss of the ability to advance to the S_2 state in PSII as the S_2 state EPR signal has not been observed in these samples. The failure to observe the S_2 state EPR signal in these samples is inconsistent with the previous proposal that Ca^{2+} is not required in the S_1 to S_2 state transition, but this could be ascribed to several other possibilities. First, the Dy^{3+} , La^{3+} , and Cd^{2+} treatments may disrupt the calcium site or even disassemble the Mn_4 cluster and thus interrupt the S_1 to S_2 state transition. Second, the substitution of Ca^{2+} by Dy^{3+} , La^{3+} , and Cd^{2+} may decrease the rate of electron transfer from the Mn_4 cluster such that the conditions necessary to monitor the Dy^{3+} -, La^{3+} -, and Cd^{2+} -treated PSII samples are different from the conditions previously used for untreated PSII samples.

In this study, we examine the inhibition of oxygen evolution by divalent and trivalent metal ions in both BBY-type PSII-enriched membranes and PSII core complexes. By using a cation-exchange procedure in which Ca^{2+} is not removed prior to addition of the studied cations (29), we achieve a high degree of reversible inhibition of PSII membranes and PSII core complexes by Dy^{3+} , Cu^{2+} , and

Cd^{2+} ions. A single tightly bound Dy^{3+} gives large and reversible inhibition of O_2 evolution and a significant enhancement of the $P_{1/2}$ of the S_2 state multiline signal, showing that this Dy^{3+} binds close to Mn_4 cluster. We observe, for the first time, that both Dy^{3+} - and Cd^{2+} -inhibited PSII core complexes advance to the S_2 state and conclude that the Ca^{2+} cofactor is not specifically required for the S_1 to S_2 state transition of PSII.

MATERIALS AND METHODS

BBY and Core Preparations of PSII. BBY-type PSII-enriched membranes were isolated from market spinach according to the procedure of Berthold et al. (38) with the modifications of Beck et al. (39). The PSII samples were stored until use at 77 K in buffer containing 20 mM MES (pH 6.0), 15 mM NaCl, and 30% (v/v) ethylene glycol. PSII core complexes were prepared by the method of Ghanotakis et al. (40) with modifications. The BBY-type PSII-enriched membranes were washed three times with wash buffer (50 mM MES (pH 6), 10 mM NaCl, and 0.4 M sucrose) and resuspended in wash buffer to 2.5 mg of Chl/mL. The crucial step in the preparation of PSII core complexes is the dropwise addition of an equal volume of solubilization buffer (50 mM MES (pH 6), 5 mM CaCl_2 , 10 mM NaCl, 1 M sucrose, and 70 mM *n*-octyl- β -D-glucopyranoside (OGP)), which dilutes the samples to 1.25 mg of Chl/mL. The 35 mM OGP extraction lasted for 10 min. Subsequently, the concentration of OGP was diluted from 35 mM to 15 mM by the addition of dilution buffer (50 mM MES (pH 6), 5 mM CaCl_2 , 10 mM NaCl, 1 M sucrose), and the mixture was incubated at 0 °C for 5 min. PSII core complexes were soluble after a 20 min centrifuge spin at 34000g, while LHCII complexes precipitated as a pellet. The suspension was subsequently dialyzed against buffer containing 50 mM MES (pH 6), 10 mM NaCl, 5 mM CaCl_2 , and 30% (v/v) ethylene glycol. Typically, repeated 30 min dialyses (2–3 times) will cause the aggregation of PSII core complexes. Two-fold dilution of the core complexes with dialysis buffer was followed by a centrifuge spin at 34000g for 30 min, which sediments the PSII core complexes. Collection of the PSII core complexes was achieved by resuspending PSII with a minimum amount of dialysis buffer. Complete removal of the light harvesting complexes and the 17 and 23 kDa extrinsic proteins was confirmed by SDS–PAGE using a 12% (w/v) acrylamide resolving gel (data not shown).

O_2 -Evolution Activity Measurements. O_2 -evolving activity measurements of PSII samples were performed with a Clark-type electrode at 25 °C (39). An Oriel 1000 W tungsten lamp, fitted with a liquid filter (filled with distilled water), a 610 nm cutoff filter (LP 610), and a heat filter, was used to initiate the photochemistry. 250 μM DCBQ and 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ were added as an artificial electron acceptor and oxidant, respectively. Typically, O_2 -evolution rates of 400–600 and 1000–1500 μmol of O_2 (mg of Chl) $^{-1}$ h $^{-1}$ were observed for PSII membranes and PSII core complexes, respectively, in buffer containing 20 mM MES (pH 6.0), 15 mM NaCl, and 20 mM CaCl_2 .

Time Course of Cation-Exchange Equilibrium. The time course of the exchange of Ca^{2+} in PSII with divalent and trivalent cations was investigated by monitoring the inhibition of O_2 -evolving activity of PSII during the course of the cation

treatments. The completion of the cation exchange is determined to be the point at which the O_2 -evolving activity of the PSII sample no longer decreases. The equilibrium time of cation exchange in PSII membranes and in PSII core complexes are compared in this study.

Inhibition of Oxygen Evolution by Dy^{3+} Ions. The inhibition of oxygen evolution by Dy^{3+} ions in BBY-type PSII membranes has been conducted under various conditions. The Dy^{3+} -inhibited PSII samples, Dy1–Dy3, were prepared by a 4-h incubation of PSII membranes in the presence of 20 mM MES (pH 6), 15 mM NaCl, and 20 mM DyCl_3 (Dy1), subsequent incubation with 50 mM MES (pH 6), 15 mM OGP, 5 mM CaCl_2 , 0.4 M NaCl, and 1 M sucrose (Dy2), and further treatment with 1 mM EDTA (Dy3). PSII samples, Dy4 and Dy5, were both prepared directly from PSII membranes by 15 min incubation with 50 mM MES (pH 6), 15 mM OGP, 0.4 M NaCl, 1 M sucrose, and 0.5 mM DyCl_3 (Dy4) or 0.1 mM DyCl_3 (Dy5), respectively. PSII samples, Dy6 and Dy7, were obtained by a method similar to the preparation of Dy5 except that the buffer was pretreated with Chelex 100 for one and two times, respectively, to remove residual Ca^{2+} ions. All of the Dy^{3+} -inhibited PSII samples (Dy1–Dy7, respectively) were washed or dialyzed against Dy^{3+} -free buffer to remove excess unbound Dy^{3+} ions in solution. The inhibition of oxygen evolution by Dy^{3+} was confirmed by the significant decrease of the oxygen-evolving activity upon treatment with Dy^{3+} ions. Further, the reversibility of the Dy^{3+} treatment was examined by monitoring the oxygen-evolving activity after reconstitution of PSII with 20 mM CaCl_2 . The inhibition of oxygen evolution by Dy^{3+} in PSII core complexes was examined in Chelex 100 treated buffer (20 mM MES (pH 6) and 15 mM NaCl) by the addition of 0.001 to 0.3 mM DyCl_3 followed by a 5 min incubation period. Similarly, the Dy^{3+} inhibition and Ca^{2+} reconstitution were examined by conducting oxygen-evolving activity assays after the sample treatments. The correlation of the EPR signal intensity of DyCl_3 EPR spin standards was established as shown in Figure 2. The concentration of Dy^{3+} ions in each of the Dy^{3+} -inhibited PSII samples was quantitated by comparison of the EPR signal intensity of Dy^{3+} in PSII with that of the DyCl_3 EPR spin standards. The number of metal ions per PSII center was estimated based on the calculation that one BBY-type PSII center has 200 chlorophylls and one PSII core complex has 38 chlorophylls.

Inhibition of Oxygen Evolution by Cu^{2+} and Cd^{2+} Ions. The inhibition of oxygen evolution by Cu^{2+} was achieved by methods similar to those used for Dy^{3+} inhibition of PSII. The PSII samples, Cu1, Cu2, and Cu3, were prepared from BBY-type PSII membranes. PSII samples, Cu1 and Cu2, were incubated at pH 6 with CuCl_2 (1.5 and 0.5 mM for Cu1 and Cu2, respectively), 15 mM OGP, 0.4 M NaCl, 1 M sucrose, and 50 mM MES for 15 min, followed by 3 washes with an excess of solution containing 20 mM MES, 15 mM NaCl, and 0.1 mM EDTA (pH 6). For preparation of the PSII sample, Cu3, all of the buffers were pretreated with Chelex 100 twice; otherwise, the procedure was the same as that used to prepare the PSII sample, Cu2. The Cu^{2+} inhibition of PSII core complexes was investigated over the range from 0.001 to 2 mM CuCl_2 obtained by addition of a stock solution to Chelex 100 treated buffer (20 mM MES (pH 6) and 15 mM NaCl). After incubation with CuCl_2 -

containing buffers, all the samples were washed with Cu^{2+} -free buffers several times to remove unbound Cu^{2+} ions. The number of Cu^{2+} ions was determined by comparison of the EPR signal intensity of the $g = 2.6$ to $g = 1.8$ signals in Cu^{2+} -inhibited PSII samples with those of Cu^{2+} -EDTA EPR spin standards.

The inhibition of oxygen evolution by Cd^{2+} was conducted only in PSII core complexes. The inhibition was achieved by incubating the PSII core complexes in buffers at pH 6 containing 20 mM MES and 15 mM NaCl, and CdCl_2 at concentrations ranging from 0.5 to 6 mM.

EPR Measurements. (a) *EPR Spectroscopy of the Light-Induced Signals of PSII.* All the PSII samples were dark adapted after the addition of 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 0.5 mM PPBQ to keep the electron acceptors in PSII completely oxidized prior to conducting EPR measurements. The S_2 state multiline EPR signal was obtained by 1–5 min illumination at 200 K (in a dry ice and acetone bath) followed by rapid freezing of the PSII samples at 77 K. The dark-stable Y_D^\bullet EPR signal was induced by illumination at 0 °C for 2 to 5 min. All the EPR scans were acquired on a Varian E-line EPR spectrometer equipped with a TE_{102} cavity or a Bruker ELEXYS E500 EPR spectrometer equipped with a SHQ resonator and an Oxford ESR-900 helium-flow cryostat. The S_2 state multiline EPR signal was recorded at 6 K with the following instrumental parameters: microwave frequency = 9.28 GHz (Varian) or 9.38 GHz (Bruker), modulation frequency = 100 kHz, modulation amplitude = 20 G. The experimental parameters for the observation of the Y_D^\bullet EPR signal were as follows: temperature = 30 K, microwave frequency = 9.28 GHz (Varian) or 9.38 GHz (Bruker), modulation frequency = 100 kHz, modulation amplitude = 2 G. The microwave power was varied as indicated in the figure captions of the EPR spectra.

(b) *Simulation of EPR Progressive Power Saturation Data.* The EPR progressive power saturation curves were simulated using the equation:

$$A = \frac{K\sqrt{P}}{(1 + P/P_{1/2})^{b/2}} \quad (1)$$

where A is the EPR signal amplitude, K is a constant, P is the microwave power, $P_{1/2}$ is the microwave power at half-saturation, and b is the inhomogeneity parameter. For a first-derivative EPR spectrum, $b = 1$ in the inhomogeneous limit and $b = 3$ in the homogeneous limit. In this work, the inhomogeneous limit was used, and the value of b was set to 1. The progressive power-saturation curves were simulated using Origin 6.0 (Microcal Software, Inc.).

(c) *Dy^{3+} EPR Signals.* The EPR signal of Dy^{3+} has a broad peak located at $g \sim 16$ (Figure 2). The EPR signal of Dy^{3+} ions in the DyCl_3 EPR spin standards and Dy^{3+} ions in PSII are compared for the quantitation of bound Dy^{3+} in PSII. The instrumental parameters used to observe the Dy^{3+} EPR signals were as follows: microwave frequency = 9.28 GHz, modulation frequency = 100 kHz, modulation amplitude = 20 G, microwave power = 5 mW and the EPR spectra were recorded at 6 K on a Varian EPR spectrometer.

(d) *Cu^{2+} EPR Signals.* The EPR signal of Cu^{2+} consists of hyperfine splittings between $g = 1.8$ and $g = 2.6$ that are sensitive to the binding environment of the Cu^{2+} ions. The

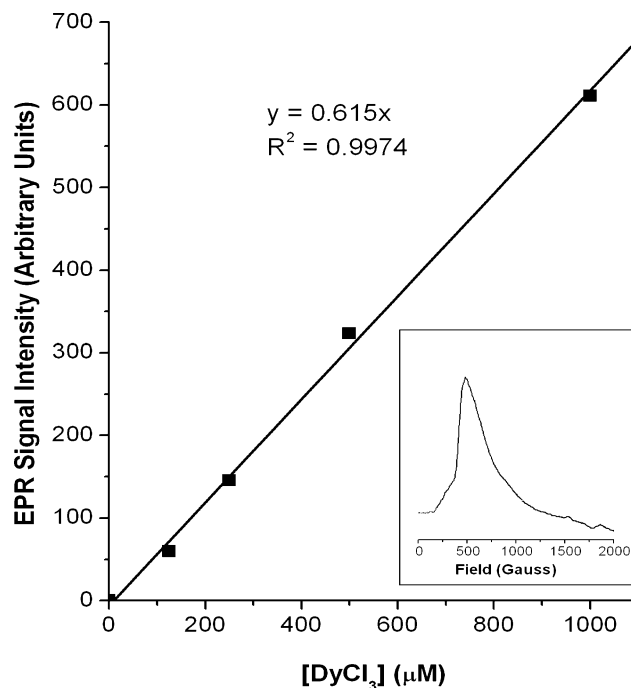


FIGURE 2: Correlation of the EPR signal intensity with the concentration of DyCl_3 spin standards. The Dy^{3+} EPR signal is shown in the inset.

instrumental parameters for observation of the Cu^{2+} ions are same as those used for recording Dy^{3+} EPR signals. The quantitation of bound Cu^{2+} ions in PSII was achieved by double integration of the Cu^{2+} hyperfine peaks using the method of Aasa and Vänngård (41).

RESULTS

Inhibition by Dy^{3+} Ions. (a) *Oxygen-Evolution Inhibition Data.* In the present study, the inhibition of oxygen evolution of PSII by Dy^{3+} has been conducted in both PSII membranes and PSII core complexes. The inhibition of oxygen evolution by Dy^{3+} in PSII membranes requires a 4 h incubation period to achieve equilibrium, whereas the inhibition of oxygen evolution with Dy^{3+} in PSII core complexes is rapid and complete within few minutes (Figure 3). Several conditions have been examined for the preparation of Dy^{3+} -inhibited PSII membranes. Table 1 summarizes the characterization of Dy^{3+} -inhibited PSII membranes and Dy^{3+} -inhibited PSII core complexes. Most notably, the addition of 20 mM CaCl_2 to the Dy^{3+} -treated PSII samples (Dy4–Dy7) restores 60–71% of the original oxygen-evolving activity of untreated PSII.

The incubation of PSII membranes with excess DyCl_3 (sample Dy1) for 4 h achieves complete Dy^{3+} inhibition, and the number of bound Dy^{3+} /PSII center is determined to be 24 (Table 1). Further treatment with EDTA lowers the number of bound Dy^{3+} /PSII to 19 and 6 for samples Dy2 and Dy3, respectively. Incubation of PSII membranes with lower concentrations of DyCl_3 gives 2 to 6 Dy^{3+} /PSII as characterized in samples Dy4–Dy7. As shown in Figure 4 and Table 1, the incubation of PSII core complexes with 0.3 mM DyCl_3 results in significant inhibition (93%) of O_2 -evolving activity. It is determined that there is 1 bound Dy^{3+} /PSII present in this sample and there is high reversibility of inhibition (~62%) upon reconstitution with Ca^{2+} . In samples

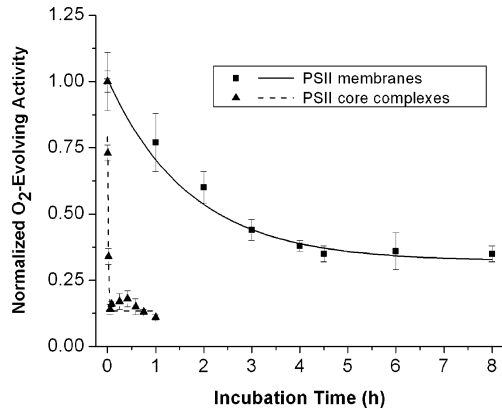


FIGURE 3: The time course of the exchange of Ca^{2+} with Dy^{3+} in BBY-type PSII membranes and PSII core complexes. Inhibition of oxygen evolution is achieved by the addition and incubation of Dy^{3+} with PSII membranes in buffer containing 20 mM MES (pH 6), 15 mM NaCl, 5 mM DyCl_3 , and 5 mM CaCl_2 and with PSII core complexes in buffer containing 20 mM MES (pH 6), 15 mM NaCl, and 5 mM DyCl_3 . The curve fits of the experimental data indicate that equilibrium is reached in 4 h with the Dy^{3+} incubation of PSII membranes and ~ 3 min with the Dy^{3+} incubation of PSII core complexes.

Table 1: Summary of the Characterization of Dy^{3+} -Inhibited PSII Samples^a

Dy^{3+} -inhibited sample	% activity after Dy^{3+} -inhibition	$\text{Dy}^{3+}/\text{PSII}$	% activity after Ca^{2+} -reconstitution
Dy1 ^b	0	24	0
Dy2 ^b	0	19	0
Dy3 ^b	0	6	0
Dy4 ^b	20 ± 1^c	6	65 ± 3^c
Dy5 ^b	45 ± 2^d	2	60 ± 5^d
Dy6 ^b	14 ± 0^e	3	71 ± 2^e
Dy7 ^b	9 ± 3^f	2	70 ± 2^f
Dy^{3+} -PSII core complex	7 ± 1^g	1	62 ± 5^g

^a See sample preparation section for details. ^b Prepared from BBY-type PSII membranes: samples Dy1–Dy3 were prepared from incubation with 20 mM DyCl_3 (Dy1), a subsequent OGP-treatment (Dy2) and a further EDTA-treatment (Dy3); samples Dy4 and Dy5 were prepared from incubation with OGP, NaCl, EDTA and 0.5 or 0.1 mM DyCl_3 , respectively; samples Dy6 and Dy7 were prepared with the same method of Dy5, but the buffer was pretreated with Chelex 100 once or twice, respectively. ^c Relative to activity of untreated PSII membranes at $630 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$. ^d Relative to activity of untreated PSII membranes at $647 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$. ^e Relative to activity of untreated PSII membranes at $460 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$. ^f Relative to activity of untreated PSII membranes at $432 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$. ^g Relative to activity of untreated PSII core complexes at $1228 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$.

Dy1–3, the O_2 -evolving activity is inhibited completely and cannot be recovered by reconstitution with CaCl_2 . However, Dy^{3+} inhibition in samples Dy4–7 is reversible. Reconstitution with Ca^{2+} restores $>60\%$ of the original O_2 -evolving activity, as shown in Table 1. Similarly, the incubation of PSII core complexes with CuCl_2 and CdCl_2 was investigated (Figure 4).

(b) *EPR Spectroscopic Properties of Dy^{3+} -Inhibited PSII Core Complexes.* Shown in Figure 5 is a comparison of the light-induced S_2 state multiline EPR signal in (a) untreated PSII core complexes and (b) Dy^{3+} -inhibited PSII core complexes with a single Dy^{3+} per PSII. After illumination of the Dy^{3+} -inhibited PSII core complexes at 200 K for 2 min, the S_2 state multiline and QA^- EPR signals are observed at $g \sim 2$ and $g \sim 1.86$, respectively. The S_2 state multiline EPR signal in Dy^{3+} -inhibited PSII core complexes is very

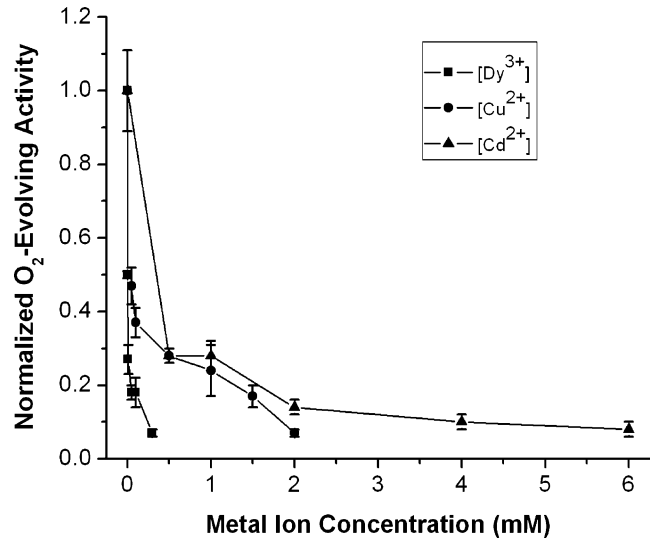


FIGURE 4: Dy^{3+} , Cu^{2+} , and Cd^{2+} inhibition of PSII core complexes. The oxygen-evolving activity of the inhibited PSII samples was normalized to the activity of untreated PSII at $1228 \mu\text{mol of O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$. See text for sample preparation.

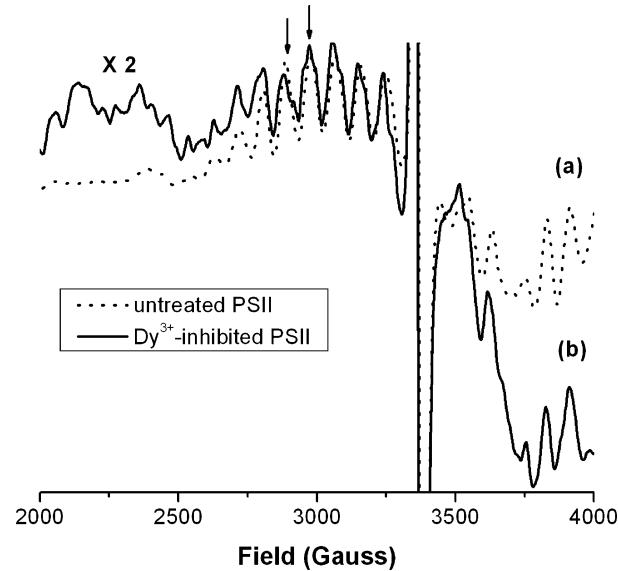


FIGURE 5: The S_2 state multiline EPR signal obtained from light-minus-dark spectra after illumination for 2 min at 200 K of (a) untreated PSII core complexes and (b) Dy^{3+} -inhibited PSII core complexes with 1 Dy^{3+} bound per PSII. The EPR spectra were acquired with 1 and 10 mW on a Bruker EPR spectrometer for untreated and Dy^{3+} -inhibited PSII core complexes, respectively. The detection powers are close to the $P_{1/2}$ values of 0.92 and 9.9 mW for untreated and Dy^{3+} -inhibited PSII core complexes, respectively. The arrows indicate the ^{55}Mn hyperfine peaks associated with the S_2 state multiline signal that were used for quantitation of the EPR signal intensity.

similar to the S_2 state multiline EPR signal in untreated PSII core complexes. However, the hyperfine peaks of the S_2 state multiline EPR signal in Dy^{3+} -inhibited PSII cores are slightly broader, and additional weak peaks are observed in the $g \sim 3$ region of the EPR spectrum. The yield of the S_2 state multiline EPR signal in Dy^{3+} -inhibited PSII core complexes and untreated PSII core complexes was investigated as a function of the illumination time at 200 K for (a) 1 min, (b) 2 min, (c) 3 min, (d) 4 min, and (e) 5 min (data not shown). It is observed that the spectral intensity of the S_2 state multiline EPR signal in untreated PSII core complexes and

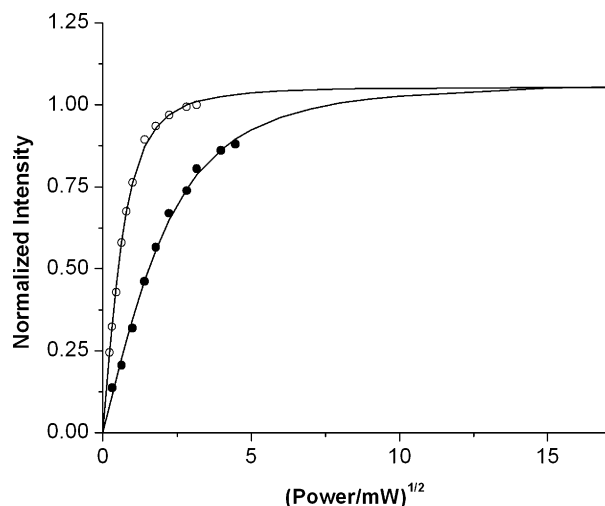


FIGURE 6: Progressive power saturation of the S_2 state multiline signal in untreated PSII core complexes (open circles) and Dy^{3+} -inhibited PSII core complexes (filled circles) after illumination at 200 K for 2 min. The $P_{1/2}$ is determined to be 0.92 mW ($R^2 = 0.9988$) and 9.9 mW ($R^2 = 0.9876$) for untreated PSII and Dy^{3+} -inhibited PSII core complexes, respectively. The spectra were collected on a Bruker EPR spectrometer.

Dy^{3+} -inhibited PSII core complexes remains unchanged with the increase in illumination time of the samples. The average yield of the S_2 state multiline EPR signal collected after 1–5 min illumination in Dy^{3+} -inhibited PSII core complexes is 24% of the average yield of the S_2 state multiline EPR signal in untreated PSII core complexes when the EPR signal intensity under non-saturating conditions is compared and corrections are made for sample concentration and microwave power.

(c) *Progressive Power Saturation of EPR Signals in Dy^{3+} -Inhibited PSII.* Figure 6 shows the progressive power saturation curves of the S_2 state multiline EPR signal in untreated PSII and Dy^{3+} -inhibited PSII core complexes. According to eq (1), the $P_{1/2}$ of the S_2 state multiline EPR signal in untreated PSII and Dy^{3+} -inhibited PSII core complexes is determined to be 0.92 and 9.9 mW, respectively. The power saturation of the Y_D^\bullet EPR signal is investigated in untreated PSII, Dy^{3+} -inhibited PSII membranes (samples Dy1, Dy4, and Dy7) and Dy^{3+} -inhibited PSII core complexes as shown in Figure 7 a,b and Table 2. The intensity of the Y_D^\bullet EPR signal is determined at the magnetic field position indicated by an arrow in the insets of Figure 7 a,b. The inhibition of oxygen evolution by Dy^{3+} in PSII membranes with 2, 6, and 24 Dy^{3+} /PSII enhances the $P_{1/2}$ of Y_D^\bullet from 0.27 mW to 0.5, 1.2, and 3.5 mW, respectively (measured on a Varian EPR spectrometer). In PSII core complexes, the inhibition of oxygen evolution by Dy^{3+} enhances the $P_{1/2}$ of Y_D^\bullet from 0.034 to 0.066 mW (measured on a Bruker EPR spectrometer). As different resonators are used on the Varian and Bruker EPR spectrometers, a normalization factor, determined by the ratio of the $P_{1/2}$ values of the S_2 state multiline EPR signal in untreated PSII membranes on the two spectrometers, was applied to the power saturation data. The raw and normalized $P_{1/2}$ values of Y_D^\bullet are listed in Table 2.

Inhibition by Cu^{2+} Ions. (a) *Oxygen-Evolution Inhibition Data.* The inhibition of oxygen evolution by Cu^{2+} has been conducted in both BBY-type PSII membranes and PSII core

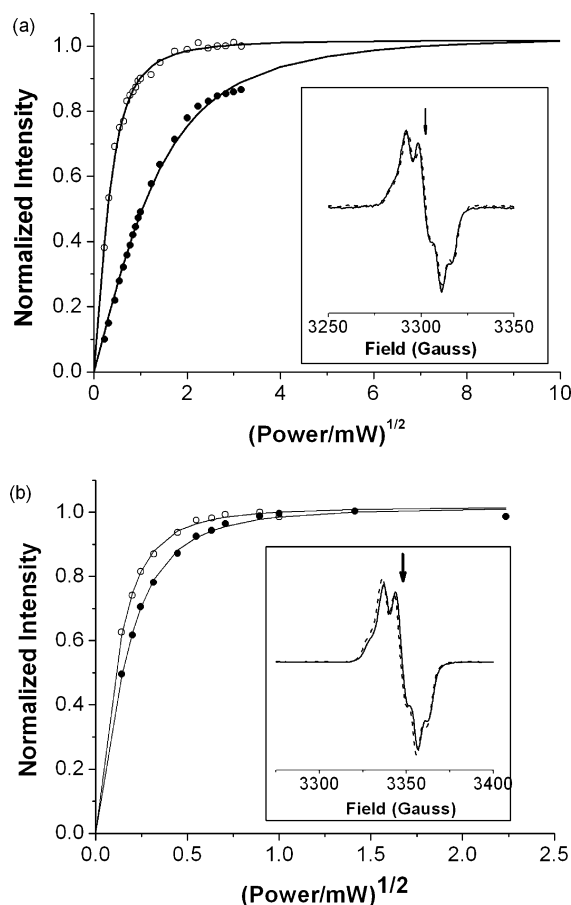


FIGURE 7: (a) Power saturation of the Y_D^\bullet EPR signal in excess Dy^{3+} -inhibited PSII membranes (sample Dy1, filled circles) and untreated PSII membranes (open circles) at 30 K, measured on a Varian EPR spectrometer. The data yield $P_{1/2}$ of 3.5 mW ($R^2 = 0.9967$) and $P_{1/2}$ of 0.27 mW ($R^2 = 0.9944$) for Dy^{3+} -inhibited PSII membranes and untreated PSII membranes, respectively. The inset shows the Y_D^\bullet EPR signal in Dy^{3+} -inhibited PSII membranes (sample Dy1, solid line) and untreated PSII membranes (dashed line) recorded at a microwave power of 0.3 mW. The arrow indicates the hyperfine peak of Y_D^\bullet EPR signal that was used for quantitation. (b) Progressive power saturation of the Y_D^\bullet signal in Dy^{3+} -inhibited PSII core complexes with 1 Dy^{3+} per PSII (filled circles) and untreated PSII core complexes (open circles) at 30 K, measured on a Bruker EPR spectrometer. The data yield $P_{1/2}$ of 0.066 mW ($R^2 = 0.9964$) and $P_{1/2}$ of 0.034 mW ($R^2 = 0.9954$) for Dy^{3+} -inhibited PSII membranes and untreated PSII membranes, respectively. The inset shows the Y_D^\bullet signal in Dy^{3+} -inhibited PSII core complexes (solid line) and untreated PSII core complexes (dashed line) recorded at a microwave power of 0.04 mW. The arrow indicates the hyperfine peak of the Y_D^\bullet EPR signal that was used for quantitation.

complexes. Incubation of PSII membranes with 0.5–1.5 mM $CuCl_2$ in a buffer containing 15 mM OGP, 0.4 M NaCl, 1 M sucrose, and 50 mM MES (pH 6) for 15 min leads to inhibition of the O_2 -evolving activities to <10% of the original activity as shown in Table 3 and Figure 4. The reversibility of the treatment is examined by the addition of 20 mM $CaCl_2$. The deactivation of the PSII sample Cu1 is largely irreversible as only 18% of O_2 -evolving activity is restored upon reconstitution of the Cu1 PSII sample with 20 mM $CaCl_2$. For the PSII samples Cu2 and Cu3, the O_2 -evolving activity is partially restored to 58 and 67% of the original activity, respectively. The inhibitory effect of $CuCl_2$ on the O_2 -evolving activity of PSII core complexes is

Table 2: Progressive Power Saturation of the Y_D^\bullet Signal in Untreated PSII, Dy^{3+} -Inhibited PSII Core Complexes and Dy^{3+} -Inhibited PSII Membranes Measured on Varian EPR and Bruker EPR Spectrometers

sample	untreated PSII	Dy^{3+} -PSII core complex	Dy7	Dy4	Dy1
Dy^{3+} /PSII	0	1	2	6	24
$P_{1/2}$ measured on a Varian EPR spectrometer (raw data)	0.27 mW ($R^2 = 0.9944$)		0.5 mW ($R^2 = 0.9986$)	1.2 mW ($R^2 = 0.9921$)	3.5 mW ($R^2 = 0.9967$)
$P_{1/2}$ measured on a Varian EPR spectrometer (normalized data) ^a	0.034 mW		0.063 mW	0.152 mW	0.438 mW
$P_{1/2}$ measured on a Bruker EPR spectrometer	0.034 mW ($R^2 = 0.9954$)	0.066 mW ($R^2 = 0.9964$)			
$\Delta P_{1/2}$		0.032 mW	0	0.118 mW	0.404 mW
distance (Dy^{3+} - Y_D^\bullet)		30 Å ^b	≥ 40 Å ^c	25 Å ^c	20 Å ^c

^a The normalization factor is determined from the $P_{1/2}$ of the S_2 state multiline EPR signal of untreated PSII measured on both the Varian and Bruker EPR spectrometers. ^b Obtained from the X-ray crystal structure (23) assuming that Dy^{3+} occupies the Ca^{2+} site in the OEC. ^c Estimated by using $\Delta P_{1/2} \propto 1/r^6$ and assuming additive effects of one Dy^{3+} bound to the Ca^{2+} site in the OEC and a second Dy^{3+} bound at a distance r from Y_D^\bullet .

Table 3: Summary of the Characterization of Cu^{2+} -Inhibited PSII Samples

Cu^{2+} -inhibited sample	% activity after Cu^{2+} inhibition	Cu^{2+} ions per PSII center	% activity after Ca^{2+} reconstitution
Cu1 ^a	9 ± 1^d	N/A	18 ± 3^d
Cu2 ^b	10 ± 2^e	11	58 ± 1^e
Cu3 ^c	4 ± 0^f	8	67 ± 4^f
Cu^{2+} -PSII core complex	7 ± 1^g	N/A	17 ± 1^g

^a Prepared from PSII membranes incubated with 1.5 mM $CuCl_2$.

^b Prepared from PSII membranes incubated with 0.5 mM $CuCl_2$.

^c Prepared from PSII membranes incubated with 0.5 mM $CuCl_2$ and Chelex 100 treated buffer.

^d Relative to activity of untreated PSII membranes at $488 \mu\text{mol of O}_2$ (mg of Chl)⁻¹ h⁻¹.

^e Relative to activity of untreated PSII membranes at $403 \mu\text{mol of O}_2$ (mg of Chl)⁻¹ h⁻¹.

^f Relative to activity of untreated PSII membranes at $362 \mu\text{mol of O}_2$ (mg of Chl)⁻¹ h⁻¹.

^g Relative to activity of untreated PSII core complexes at $1228 \mu\text{mol of O}_2$ (mg of Chl)⁻¹ h⁻¹.

surveyed in the range of 0.05–2 mM $CuCl_2$. The addition of 2 mM Cu^{2+} to PSII core complexes inhibits the O_2 -evolving activity to 7% of the original activity, but reconstitution with excess $CaCl_2$ does not significantly restore the O_2 -evolving activity, as shown in Table 3.

(b) *EPR Properties of Cu^{2+} -Inhibited PSII Membranes.* The EPR spectrum of Cu^{2+} -EDTA exhibits four hyperfine peaks at $g = 2.46$, $g = 2.34$, $g = 2.22$, and $g = 2.07$, with an average hyperfine splitting of ~ 171 G. The hyperfine peaks of the Cu^{2+} ions ($g = 2.46$, 2.30, 2.17, and 2.08) in the PSII sample Cu2 and Cu3 are different from the EPR signals obtained from Cu^{2+} -EDTA. The average hyperfine coupling observed in the Cu3 PSII sample is ~ 163 G. The quantitation of Cu^{2+} in PSII membranes was performed by comparison of the double integral of the Cu^{2+} EPR signals at $g = 2.6$ to $g = 1.8$ in Cu^{2+} -inhibited PSII membranes to the Cu^{2+} EPR signals in standard Cu^{2+} -EDTA samples. The results indicate that Cu^{2+} -inhibited PSII membrane samples have a large number of bound Cu^{2+} ions; PSII samples Cu2 and Cu3 have 11 and 8 Cu^{2+} ions per PSII center, respectively. Cu^{2+} -inhibited PSII membrane sample Cu3 was illuminated at 200 K for 5 min. The light-minus-dark spectrum of this sample displays an EPR signal growing in at $g = 2$, corresponding to the Y_D^\bullet/Y_Z^\bullet EPR signals. However, the line shape of this light-induced EPR signal is quite different from the signal of Y_D^\bullet in untreated PSII membranes (42), indicating that other radicals, such as chlorophyll and/or carotenoid radicals, are also formed. The S_2 state multiline

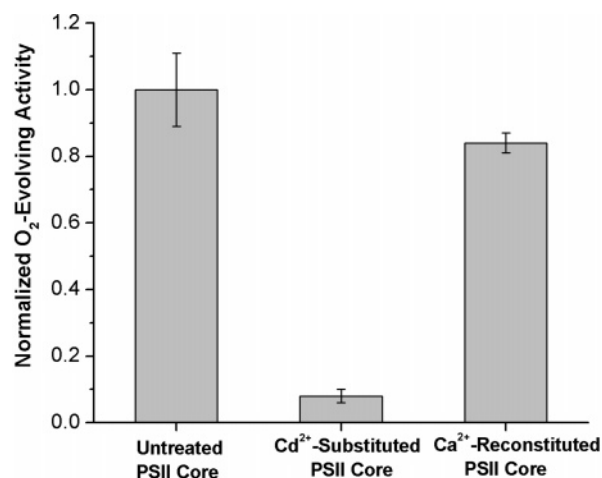


FIGURE 8: Comparison of the normalized O_2 -evolving activity of untreated PSII core complexes, Cd^{2+} -inhibited PSII core complexes, and Ca^{2+} reconstitution of Cd^{2+} -inhibited PSII core complexes. The oxygen-evolving activity of the treated PSII samples was normalized to the activity of untreated PSII at $1228 \mu\text{mol of O}_2$ (mg of Chl)⁻¹ h⁻¹.

EPR signal is not induced upon 200 K illumination for 5 min in Cu^{2+} -inhibited PSII membrane sample Cu3. Further, the $Q_A^-Fe^{2+}$ signal ($g = 1.82$ and 1.7 (43)) is also not observed in the Cu3 PSII sample.

Inhibition by Cd^{2+} Ions. (a) *Oxygen-Evolution Inhibition Data.* The inhibition of oxygen evolution by Cd^{2+} was conducted in PSII core complexes. As shown in Figure 4, the effects of incubation with 0.5 to 6 mM $CdCl_2$ were examined in this study. The treatment of PSII core complexes with 6 mM $CdCl_2$ yields 8% of the original O_2 -evolving activity. Subsequent incubation of the PSII core samples with $CaCl_2$ restores the O_2 -evolving activity to 84% (Figure 8) of the original activity of untreated PSII core complexes.

(b) *S_1 to S_2 State Transition in Cd^{2+} -Inhibited PSII.* The S_1 to S_2 state transition in Cd^{2+} -inhibited PSII is examined by illumination of the Cd^{2+} -inhibited PSII sample for 3 min under the same conditions used to induce the S_2 state multiline EPR signal in untreated PSII. As can be seen in Figure 9, Cd^{2+} -inhibited PSII exhibits an S_2 state multiline EPR signal upon illumination at 200 K that is very similar in both yield and line shape to the signal observed in untreated PSII core complexes. On the basis of the intensity of the ^{55}Mn hyperfine peaks at $g = 2.33$ and $g = 2.25$ as marked in Figure 9, the yield of the S_1 to S_2 state transition

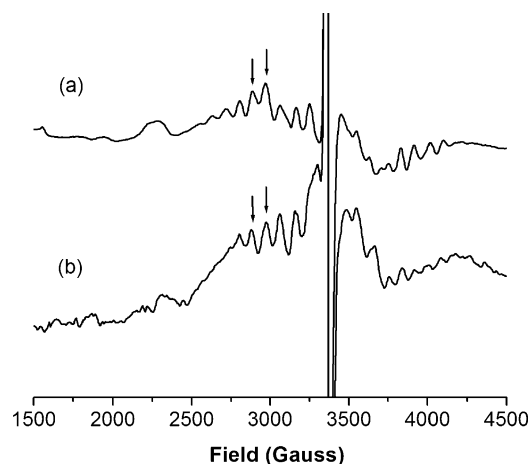


FIGURE 9: Comparison of the S_2 state multiline EPR signal induced by 200 K illumination for 3 min of (a) untreated PSII core complexes (3.9 mg of Chl/mL) and (b) Cd^{2+} -inhibited PSII core complexes (3.8 mg of Chl/mL). The EPR spectra were obtained from the light-minus dark difference of the individual spectra measured at 1 mW microwave power on a Bruker EPR spectrometer. The arrows indicate the ^{55}Mn hyperfine peaks associated with the S_2 multiline EPR signal, which were used for quantitation.

in Cd^{2+} -inhibited PSII core complexes is estimated to be 84% of the S_1 to S_2 state transition in untreated PSII core complexes.

DISCUSSION

Biochemistry and Quantitation of Metal–Ion Inhibition. Cation treatments cause the loss of the O_2 -evolving activity due to the replacement of Ca^{2+} , but they could also potentially disassemble the Mn_4 cluster of PSII (as is the case for the Cu^{2+} treatment of PSII reported previously by Styring and co-workers (44)). Therefore, it is essential to examine the reversibility of the inhibition caused by the cation treatments by reconstitution of Ca^{2+} . In addition, previous studies have indicated that PSII membranes have several Ca^{2+} -binding sites, in addition to the Ca^{2+} -binding site in the OEC. To determine whether metal ions are bound to additional sites besides the Ca^{2+} -binding site in the OEC, we carefully quantitate the number of Dy^{3+} and Cu^{2+} per PSII based on their EPR signal intensity.

Inhibition by Dy^{3+} . Dy^{3+} ion is a suitable EPR spin probe to replace Ca^{2+} in PSII due to the similarity of the ionic radii of Dy^{3+} and Ca^{2+} . Although Dy^{3+} is a trivalent ion, previous studies have shown that it binds as a dipositive ion (29), presumably because Dy^{3+} has a coordinated hydroxide. The EPR signal of Dy^{3+} ($g \sim 16$) is distinct and does not overlap with the EPR signals arising from PSII components (42); therefore, the stoichiometry of Dy^{3+} ions per PSII center can be quantified using the intensity of the Dy^{3+} EPR signal. In addition, the spin–lattice relaxation rate of the Dy^{3+} is rapid in comparison to the spin–lattice relaxation rate of the Mn_4 cluster and the Y_D^\bullet radical of PSII. This difference in spin–lattice relaxation rates can be exploited to determine the location of Dy^{3+} ions in PSII by monitoring the spin–lattice relaxation enhancement of the Mn_4 and Y_D^\bullet EPR species in the presence of the Dy^{3+} ions. Thus, the inhibition of oxygen evolution by Dy^{3+} ions in the OEC can be utilized to determine both the location and function of the Ca^{2+} cofactor in PSII. In contrast to previous studies (34, 45), the

Dy^{3+} -inhibited PSII samples in this study are prepared using an ion-exchange procedure in which Ca^{2+} ions are not removed prior to the Dy^{3+} -inhibition of PSII (29, 30). Thus, the PSII complexes remain structurally intact after the inhibitory treatments. This is confirmed by reconstitution of oxygen-evolution activity by addition of Ca^{2+} to the Dy^{3+} -inhibited PSII. Further, Dy^{3+} binding within the PSII core complex is confirmed by the effect of Dy^{3+} ions on the EPR progressive power saturation behavior of the S_2 multiline and Y_D^\bullet EPR signals.

As can be seen in Table 1, there is a loss of O_2 -evolving activity of PSII complexes when Dy^{3+} ions are added to PSII. The extent and reversibility of inhibition varies with the different treatments, with generally greater, and more irreversible, inhibition and more binding of Dy^{3+} when higher concentrations of DyCl_3 are added.

Other than the Ca^{2+} -binding site in the OEC, two additional weak Ca^{2+} -binding sites in PSII have previously been reported in literature. One Ca^{2+} -binding site is suggested to be present on the CP29 polypeptide (12), and the second Ca^{2+} -binding site is suggested to be on the 33 kDa polypeptide of PSII (7, 8). Dy^{3+} ions can easily replace Ca^{2+} in all three binding sites and could also bind to other metal-binding sites including nonspecific surface binding sites on the membranes. A study on the phosphorylation of the light-harvesting complexes has shown that, as the PSII membranes stack, two LHCII complexes may bind one Ca^{2+} ion after the LHCII proteins are phosphorylated (46). One PSII membrane sample has 200 chlorophylls, and approximately 150 chlorophylls are located in the LHCII. On the basis of the knowledge that each LHCII contains 15 chlorophylls (47), the number of the LHCII in PSII membranes can be estimated to be 10. After the LHCII complexes are phosphorylated, five Ca^{2+} ions could bind to the LHCII of stacked PSII membranes. Including the Ca^{2+} ion in the OEC and Ca^{2+} bound to the LHCII, six Ca^{2+} ions in total could bind to each PSII membrane complex. This prediction is consistent with the characterization of Dy3 and Dy4 PSII samples as they both contain six Dy^{3+} ions per PSII center (Table 1).

PSII samples treated with 20 mM DyCl_3 bind 24 Dy^{3+} /PSII and the number decreases to 6 Dy^{3+} /PSII upon treatment with OGP and EDTA (Dy3). When PSII membranes are treated with ≤ 0.5 mM DyCl_3 , such as samples Dy4–Dy7, the number of bound Dy^{3+} ions per PSII complex further decreases to ≤ 6 Dy^{3+} /PSII. When the light-harvesting complexes and the 17 and 23 kDa proteins are partially dissociated from the PSII core by the addition of high salt (0.4 M NaCl), most of the bound Dy^{3+} ions dissociate into the solution. When the NaCl-treated PSII membranes with partially dissociated light-harvesting complexes and 17, 23 kDa proteins (PSII samples Dy5, Dy6, and Dy7) are treated with 0.1 mM DyCl_3 , each PSII center is determined to have 2–3 Dy^{3+} /PSII. This is consistent with studies by Grove et al. (6) and Kalosaka et al. (4), which have reported that 17 and 23 kDa-depleted PSII has 2.5 high-affinity Ca^{2+} -binding sites per PSII.

The Ca^{2+} site in the OEC has been proposed to be the strongest Ca^{2+} -binding site in PSII, and it has previously been suggested that the Ca^{2+} ion located in the 33 kDa extrinsic protein of PSII membranes can be easily replaced by Dy^{3+} ions for spectroscopic studies (7). This may explain why the minimum number of Dy^{3+} ions per PSII membrane

determined in this study is two rather than one.

Although several studies have reported multiple Ca^{2+} -binding sites in PSII, in general, it is believed that the OEC has only one Ca^{2+} -binding site. However, previous Dy^{3+} -inhibition conditions in PSII membranes did not successfully yield samples with one Dy^{3+} ion bound per PSII. In the present study, for the first time, we demonstrate that it is possible to substitute one Dy^{3+} ion per PSII by the use of PSII core complexes, which are depleted of the LHCII and the 17, 23 kDa extrinsic polypeptides. In the absence of the 17 and 23 kDa extrinsic polypeptides, weakly bound ions in the Ca^{2+} -binding site in the 33 kDa extrinsic protein will dissociate with buffer washes.

EPR spectroscopic studies of PSII samples that have been treated with lanthanide ions (such as Dy^{3+} , La^{3+} , Lu^{3+} , and Pr^{3+}) have previously been reported by Bakou et al. (34, 45). On the basis of the loss of the S_2 state multiline EPR signals in lanthanide-treated PSII samples, it was suggested that the S_1 to S_2 state transition is blocked by these cation treatments (34, 45). These studies also reported that the amplitude of the light-induced EPR signal from Y_Z^{\bullet} varies with the treated lanthanide ions (45) and the line shape of the Y_D^{\bullet} EPR signal is broadened due to a magnetic interaction with the treated Dy^{3+} ions (34). However, the PSII samples used in the measurements of Y_D^{\bullet} by Bakou et al. contained 14–15 Dy^{3+} ions per PSII complex (34). PSII samples with more than one Dy^{3+} ion per PSII could lead to misinterpretation of the effects of Dy^{3+} on the OEC, especially if Dy^{3+} ions bind in sites outside of the OEC of PSII.

EPR progressive power saturation is commonly used to determine the location of paramagnetic centers in proteins by the measurement of the spin–lattice relaxation effects caused by long-range (<40 Å) spin–spin interactions (48). Measurements of $P_{1/2}$ provide information on the distance (r) between fast- and slow-relaxing spins because the change of $P_{1/2}$ caused by the spin–spin interaction is proportional to $1/r^6$. Dy^{3+} is a fast-relaxing species and, therefore, the binding of Dy^{3+} to the Ca^{2+} -site of the OEC or other Ca^{2+} sites of PSII can be easily identified by the change of the power saturation of the Y_D^{\bullet} and S_2 state multiline EPR signals.

The progressive power saturation behavior of the dark-stable Y_D^{\bullet} radical of PSII was examined in untreated and Dy^{3+} -treated PSII samples to determine the location of Dy^{3+} ions bound to PSII. As can be seen in Figure 7, binding of excess Dy^{3+} to PSII membranes (sample Dy1 with 24 Dy^{3+} /PSII) causes a 13-fold increase in the $P_{1/2}$ of Y_D^{\bullet} relative to that of untreated PSII membranes, whereas the binding of 1 Dy^{3+} to PSII core complexes causes only a 2-fold increase. These results indicate that the highest affinity site is quite distant from Y_D^{\bullet} , consistent with the highest affinity Dy^{3+} -binding site being the Ca^{2+} -binding site of the OEC, which is known to be located 30 Å from Y_D^{\bullet} based on the X-ray structures. When a larger number of Dy^{3+} ions are bound to PSII, there are additional Dy^{3+} ions bound in closer proximity to Y_D^{\bullet} in the D2 polypeptide of PSII. The 13-fold increase of $P_{1/2}$ of Y_D^{\bullet} in PSII membranes with 24 Dy^{3+} /PSII can either be due to one Dy^{3+} ion bound at a short distance from Y_D^{\bullet} or several Dy^{3+} ions bound at slightly longer distances from Y_D^{\bullet} . Assuming that Dy^{3+} in PSII core complexes (1 Dy^{3+} /PSII) is bound in the OEC, we can use the distance between the Ca^{2+} ion of the OEC and the Y_D^{\bullet} radical

determined by the X-ray crystal structure (30 Å) as a reference. With this reference, we can estimate that a second Dy^{3+} ion would have to be bound at a distance of 20 Å from the Y_D^{\bullet} radical to account for the change of $P_{1/2}$ by Dy^{3+} inhibition in sample Dy1. Notably, the 20 Å distance between Dy^{3+} and Y_D^{\bullet} is estimated with the assumption that the change of the power saturation is contributed solely by the additive effects of two Dy^{3+} ions: one bound in the OEC and the second Dy^{3+} ion closest to Y_D^{\bullet} . Similarly, the closest Dy^{3+} to the Y_D^{\bullet} radical in sample Dy4 can be estimated to be 25 Å from the Y_D^{\bullet} radical. In sample Dy7, which has 2 Dy^{3+} /PSII, $P_{1/2}$ of Y_D^{\bullet} is same as $P_{1/2}$ of Y_D^{\bullet} in PSII core complexes with 1 Dy^{3+} /PSII. The same value of $P_{1/2}$ in the two PSII samples suggests that the second Dy^{3+} in the sample with 2 Dy^{3+} /PSII is located ≥ 40 Å from Y_D^{\bullet} .

The PSII protein surfaces on the luminal and stromal sides of the membrane are at distances of ~ 27 and 26 Å, respectively, from Y_D^{\bullet} according to an EPR study of PSII membranes depleted of all three extrinsic proteins (49). This EPR study on untreated PSII membranes also indicated that the distance from Y_D^{\bullet} to the luminal surface in the presence of the extrinsic proteins is ~ 41 Å. According to these EPR distance measurements, surface bound Dy^{3+} ions, on either the luminal or the stromal side of the membrane, can affect the power saturation of Y_D^{\bullet} in the absence of the extrinsic proteins. In terms of the power saturation effect on Y_D^{\bullet} , PSII core complexes are similar to extrinsic protein-depleted PSII membranes because the 17 and 23 kDa extrinsic proteins are removed. For sample Dy1, which retains the extrinsic proteins, the surface bound Dy^{3+} ions on the luminal side would be too distant to have a significant influence on the power saturation of Y_D^{\bullet} , while Dy^{3+} ions in the stromal side could affect the power saturation of Y_D^{\bullet} . We searched for acidic residues as possible ligands of Dy^{3+} ions within a radius of 19–21 Å of Y_D^{\bullet} in the 3.5 Å X-ray crystal structure of PSII (23). No acidic residues were found at 19–21 Å from Y_D^{\bullet} in the PSII structure. Therefore, we propose that the Dy^{3+} -binding site nearest to Y_D^{\bullet} is not located in the PSII core. The structure of the PSII–LHCII supercomplex obtained by cryo-electron microscopy identified that light-harvesting proteins, the LHCII, CP29 and CP26, are attached to the CP43 and CP47 polypeptides of the PSII core (50). The distance between CP29 and Y_D^{\bullet} is not known, but the closest Dy^{3+} -binding site to Y_D^{\bullet} most likely is located on CP29 because the CP29 polypeptide is closer to Y_D^{\bullet} than the CP26 polypeptide. Furthermore, a Ca^{2+} site on CP29 has been previously reported (12).

PSII core complexes containing a single Dy^{3+} ion exhibit the S_2 state multiline EPR signal upon low-temperature illumination. As can be seen in Figure 5, the S_2 state multiline EPR signal in Dy^{3+} -inhibited PSII core complexes is very similar to the S_2 state multiline EPR signal in untreated PSII, in contrast to the altered S_2 state multiline EPR signal observed in Sr^{2+} -inhibited PSII (31). However, Dy^{3+} inhibition of PSII does lead to a slight broadening of the hyperfine peaks in comparison to those in untreated PSII. The X-ray crystal structure has characterized the distance of Ca–Mn as 3.3 and 4.0 Å (23). It might be expected that binding of Dy^{3+} to the OEC in place of Ca^{2+} would perturb the exchange interactions between the Mn ions and the electron–nuclear hyperfine interactions between the $S = 1/2$ spin of the Mn_4 cluster and the four $I = 5/2$ ^{55}Mn spins and, thereby,

alter the S_2 state multiline signal. There are two effects of Dy^{3+} binding that need to be considered: the effect of the size and charge of Dy^{3+} relative to Ca^{2+} and the effect of the paramagnetism of Dy^{3+} .

Vrettos et al. (29) have shown that the Ca^{2+} site in the OEC is a rigid, size-selective site. On the basis of this, we expect that Dy^{3+} (and also Cd^{2+}) substitute into the Ca^{2+} site better than Sr^{2+} because they have much closer ionic radii (ionic radii of aqua Ca^{2+} , Cd^{2+} , Dy^{3+} , and Sr^{2+} are 0.99, 0.97, 0.91, and 1.12 Å, respectively). Therefore, the perturbation of the Mn_4 geometry caused by Dy^{3+} (or Cd^{2+}) substitution is expected to be smaller than that caused by Sr^{2+} ; Sr^{2+} is the only cation in this group that is too big to fit into the site. Note that Vrettos et al. (29) also have shown that Ca^{2+} , Cd^{2+} , Sr^{2+} , and Dy^{3+} all bind as dipositive ions in the OEC, presumably because Dy^{3+} has a coordinated hydroxide. Thus, there are no charge effects on the structure upon substitution of Ca^{2+} by Dy^{3+} .

The spin–lattice relaxation rate of Dy^{3+} has been estimated to be 6 ns at 6 K (51), which is much faster than the spin–lattice relaxation rate of Mn_4 cluster in the S_2 state at the same temperature (8 μ s at 6 K, (52)). Thus, it is possible that the rapid spin–lattice relaxation rate of the Dy^{3+} ion could average the exchange and dipolar interactions between the Dy^{3+} spin and the S_2 state, which would render the line shape of the S_2 state multiline EPR signal in the Dy^{3+} -inhibited PSII core complexes very similar to the S_2 state multiline EPR signal in untreated PSII. For example, the broad low-temperature $S_2 Y_Z^*$ EPR signal of acetate-treated PSII converts to a narrow Y_Z^* EPR signal at room-temperature due to averaging of the exchange and dipolar interactions between Y_Z^* and the S_2 state of the Mn_4 cluster by rapid spin–lattice relaxation of the Mn_4 cluster (53).

We examine the effects of Dy^{3+} inhibition on the progressive power saturation behavior of the S_2 state of the Mn_4 cluster to study the location of Dy^{3+} in the Dy^{3+} -inhibited PSII samples. As shown in Figure 6, $P_{1/2}$ of the S_2 state multiline in Dy^{3+} -inhibited PSII core complexes containing a single Dy^{3+} is 11-fold higher than $P_{1/2}$ of the S_2 state multiline in untreated PSII (9.9 and 0.92 mW, respectively). The enhancement of the $P_{1/2}$ value of the S_2 state multiline EPR signal upon Dy^{3+} inhibition is due to the spin–spin interaction between the fast-relaxing Dy^{3+} spin and the relatively slow-relaxing Mn_4 cluster.

We also observe that Dy^{3+} -inhibited PSII cores have only 7% of the original O_2 -evolving activity of untreated PSII cores. This confirms that the Dy^{3+} inhibition is nearly complete ($\sim 93\%$) in PSII core complexes. We ascribe the residual activity to centers in which Dy^{3+} has not replaced Ca^{2+} . Further, 62% of the original O_2 -evolving activity of Dy^{3+} -inhibited PSII cores can be restored upon addition of excess $CaCl_2$. This indicates that the Dy^{3+} treatment may have denatured the OEC in 38% of the PSII centers such that only 62% reconstitution of O_2 -evolving activity is possible. The intensity of the S_2 state multiline EPR signals of Dy^{3+} -inhibited PSII is 24% of the intensity of the S_2 state multiline EPR signals in untreated PSII. This 24% yield of the S_2 state multiline signal in Dy^{3+} -inhibited PSII is low but still significantly more than the residual Ca^{2+} -bound PSII could form. The low yield of the S_2 state multiline EPR signal could be due to several reasons. First, the S_1 to S_2 state transition could be slowed down by Dy^{3+} inhibition such

that a complete reaction requires illumination at higher temperature, as has been observed in the case of low-pH treated PSII (54, 55). In low-pH-treated PSII, the S_2 state multiline EPR signal is observed after illumination at 0 °C (70 °C higher than the illumination temperature to induce the normal S_2 multiline EPR signal). Second, there are 1–2 weak Ca^{2+} -binding sites in PSII besides the Ca^{2+} in the OEC (4, 6) that may affect the OEC (10, 11). Ono and Inoue have investigated the effects of replacement of Ca^{2+} by several divalent cations (Cd^{2+} , Sr^{2+} , Mg^{2+} , and Ba^{2+}) in PSII membranes after the depletion of Ca^{2+} by a low-pH treatment (11). It was found that Sr^{2+} and Cd^{2+} ions have smaller effects on the thermoluminescence properties of the S_2QA^- state than other divalent metal ions that were tested. It was suggested that one weakly bound Ca^{2+} ion is essential for the S_2 to S_3 state transition. Kimura and Ono have studied the effect on carboxylate ligands in the OEC of depletion of weakly bound Ca^{2+} ion(s) by high salt treatment (56). The FTIR spectra of these samples suggested that one Ca^{2+} ion is close to the OEC, but not directly incorporated in the OEC. Kruk and co-workers proposed that a Ca^{2+} ion on the 33 kDa extrinsic protein regulates the H^+ transfer through the water channel of the OEC (7). Recently, Barber and co-workers reinvestigated previous anomalous diffraction data (23) and have identified a Ca^{2+} ion that binds to the 33 kDa protein at the exit of the putative proton channel (8). The distances between this bound Ca^{2+} to Y_D^* and to the OEC are ~ 60 Å and ~ 35 Å, respectively (8). It is possible that a Dy^{3+} ion binds to this Ca^{2+} site; however, a Dy^{3+} ion that binds to this Ca^{2+} site is not expected to affect the power saturation behavior of the S_2 state multiline EPR signal significantly, contrary to the 11-fold increase of $P_{1/2}$ of the S_2 state multiline EPR signal observed in this study. Moreover, the Dy^{3+} -inhibited PSII core complexes studied here have only 1 Dy^{3+} /PSII on an average, such that most PSII centers should have Dy^{3+} bound only to the high-affinity site in the OEC. It is conceivable that some PSII centers have Dy^{3+} bound to another site that affects the activity of the OEC. In this case, somewhat more than 7% of the PSII centers could still have Ca^{2+} bound in the OEC, if these centers do not evolve O_2 due to an inhibitory effect of Dy^{3+} bound to another site. It seems unlikely, however, that the Dy^{3+} -inhibited PSII core complexes could have a sufficient fraction of active centers to account for the observation that 24% of Dy^{3+} -treated PSII core complexes ($\sim 40\%$ of PSII with reversibility) advance to the S_2 state. Moreover, such a second binding site would have to be very close to the Mn_4 cluster, so that binding of Dy^{3+} to this site could significantly change the power saturation of the S_2 state multiline EPR signal.

Previous studies have concluded that Ca^{2+} -depleted PSII is capable of advancing to the S_2 state, but it has been reported that Dy^{3+} - (34, 35), La^{3+} - (35), or Cd^{2+} -inhibited (11, 36) PSII samples do not advance to the S_2 state upon illumination. To maintain the S_n state transitions of PSII, it is important to preserve the intact structure of the OEC during the treatments to substitute other metal ions into the Ca^{2+} site. In the study of Bakou et al. (34), the lanthanide treatments were irreversible, except for the sample of Dy^{3+} /EDTA-incubated PSII membranes. In the Dy^{3+} /EDTA-treated PSII membranes, Ca^{2+} reconstitution recovered 43% of the original activity and the Ca^{2+} -reconstituted PSII

formed the S_2 state multiline EPR signal. However, this Dy^{3+} /EDTA-treated PSII sample did not form the S_2 state multiline EPR signal prior to the addition of Ca^{2+} under the same sample illumination and spectrometer instrumental conditions used. In the present study, careful and systematic characterization of Dy^{3+} -inhibited PSII membranes (samples Dy1–Dy3) indicates that excess Dy^{3+} can easily inhibit O_2 -evolving activity but Ca^{2+} reconstitution cannot restore the oxygen-evolving activity in these samples. We propose that the lack of S_n state transitions in previous studies (34) could be due to alteration of the intact OEC structure with lanthanide treatment. If the same percentage of reconstitutable Dy^{3+} -inhibited PSII centers advance to the S_2 state as observed in the present study, then a Dy^{3+} -treated PSII membrane sample with 43% reversibility, as in the study of Bakou et al. (34), would be estimated to exhibit 17% of the S_2 state multiline EPR signal relative to untreated PSII. It is possible that it was not possible to detect such a low yield in the previous study considering that the Dy^{3+} /EDTA treated PSII membrane sample used by Bakou et al. was less concentrated (5 mg of Chl/mL) than the Dy^{3+} -inhibited PSII core complexes used in the present EPR measurements (3.1 mg of Chl/mL, equivalent to 15.5 mg of Chl/mL of PSII membranes).

Inhibition by Cu^{2+} and Cd^{2+} . A comparison of the effects of divalent cations with those of Dy^{3+} ions will help clarify the role of Ca^{2+} ions in oxygen evolution. Brudvig and co-workers previously proposed that Ca^{2+} functions in the OEC as a weak Lewis acid (26). Cd^{2+} is ideal to replace Ca^{2+} as it has a similar ionic radius (0.99 and 0.97 Å for Ca^{2+} and Cd^{2+} , respectively, (57)) but is a stronger Lewis acid than Ca^{2+} (pK_a 's of aqua Cd^{2+} and Ca^{2+} ions are 12.80 and 9.00, respectively (58)). In a prior study, Cu^{2+} was found to inhibit oxygen evolution, although reversibility was not achieved (44). Studying the inhibition of oxygen evolution by Cu^{2+} ions by using samples prepared with the same procedure used for Dy^{3+} -treated PSII provides more insight on the effect of Cu^{2+} on the electron-transfer pathway.

Cu^{2+} significantly inactivates the OEC (Table 3). For Cu^{2+} -inhibited PSII membrane samples, addition of excess $CaCl_2$ partially restores the O_2 -evolving activity. The reversibility of inhibition is as high as 67 and 58% for samples Cu2 and Cu3, respectively. In PSII core complexes, the O_2 -evolving activity is inhibited to 7% of the original activity by the addition of 2 mM $CuCl_2$, but the inhibition is not reversible. The inhibition of oxygen evolution caused by Cu^{2+} treatment is conceivably a Ca^{2+} -dependent effect, although samples Cu2 and Cu3 are characterized to have 8 and 11 Cu^{2+} ions per PSII center, respectively, which are more than the number of Ca^{2+} ions in the PSII membrane samples. The hyperfine coupling of Cu^{2+} ions in Cu^{2+} -inhibited PSII sample Cu3 is different from the hyperfine couplings in the EPR signal of aqueous Cu^{2+} -EDTA, which indicates that most of the added Cu^{2+} ions are bound to PSII. The large number of Cu^{2+} ions that bind to PSII indicates that PSII membranes have many unidentified binding sites for Cu^{2+} . The large extent of reversibility of Cu^{2+} -inhibited PSII membrane samples Cu2 and Cu3 indicates that the Cu^{2+} treatment does not cause significant damage to the Mn_4 clusters in PSII membranes. The lack of reversibility for PSII core complexes might reflect the instability of the protein or inhibition that is not related to Ca^{2+} in the OEC.

Cd^{2+} ions are almost the same size as Ca^{2+} ions and are, therefore, likely to bind well to Ca^{2+} sites that discriminate metal ions by size. With the same charge as Ca^{2+} ions, Cd^{2+} ions will largely retain the charge distribution of the Mn_4 cluster. We find reversible inhibition of oxygen evolution by Cd^{2+} . Treatment of PSII core complexes with Cd^{2+} inhibits the O_2 -evolving activity to 8% of the original activity and the addition of excess $CaCl_2$ reactivates 84% of the O_2 -evolving activity (Figure 8). The number of bound Cd^{2+} ions bound to PSII was not characterized because Cd^{2+} ions are EPR silent. Ono and Inoue (11) and Sigfridsson et al. (37) have studied the interaction of Cd^{2+} with PSII, including the effects on the electron donor and acceptor sides, and the inhibition of O_2 evolution by a Ca^{2+} -dependent reaction. The reversibility of Cd^{2+} inhibition is a key difference for the Cd^{2+} -inhibited PSII core complexes used in the present investigation and these previous studies.

As shown in Figure 9, an S_2 state multiline EPR signal is observed in Cd^{2+} -inhibited PSII core complexes, and this signal is very similar to that in untreated PSII. From the good correlation between the extent of reversibility of Cd^{2+} treatment and the yield of the S_2 state multiline EPR signal in Cd^{2+} -inhibited PSII core complexes (both 84%), it is clear that Cd^{2+} -inhibited PSII centers are capable of advancing from the S_1 to the S_2 state. Because Cd^{2+} is diamagnetic, the binding of Cd^{2+} to the OEC would only affect the line shape of the S_2 state multiline signal if Cd^{2+} ions change the geometry of the Mn_4 cluster. The observation of very similar S_2 state multiline EPR signals in both Cd^{2+} -inhibited and untreated PSII core complexes indicates that Cd^{2+} binds to the calcium site in the OEC with very little structural perturbation, consistent with the similar ionic radii of Ca^{2+} and Cd^{2+} .

Role of Ca^{2+} in the Oxygen-Evolution Reaction. In the models proposed by Brudvig, Pecoraro, and co-workers, Ca^{2+} is suggested to function as a Lewis acid that binds and activates a substrate water molecule (or hydroxide ion) for nucleophilic attack on a $Mn(V)=O$ species in the O–O bond-formation reaction (26, 28). The OEC remains active when Ca^{2+} is replaced by Sr^{2+} , although the activity is reduced to ~40% of untreated PSII. This functional replacement of Ca^{2+} by Sr^{2+} has been ascribed to the similar pK_a of Ca^{2+} and Sr^{2+} ions (26, 29). Notably, the substitution of Ca^{2+} by Sr^{2+} changes the pK_a of the protonation reaction in the OEC (30). The upshift of the pK_a by Sr^{2+} substitution supports the proposal that Ca^{2+} acts as a Lewis acid in the oxygen-evolution reaction.

Dy^{3+} and Cd^{2+} inhibition of the OEC cause the loss of O_2 -evolving activity, but this work shows that both Dy^{3+} - and Cd^{2+} -inhibited PSII core complexes are capable of advancing from the S_1 to the S_2 state. These observations are consistent with the previous observation that Ca^{2+} -depleted PSII forms the S_2 state multiline EPR signal upon illumination at 200 K and indicate that the specific functional role of Ca^{2+} in the OEC is beyond the S_2 state, as proposed by Brudvig, Pecoraro, and co-workers.

CONCLUSIONS

In conclusion, we present a new, reversible procedure to substitute metal ions into the calcium-binding site in the OEC and provide quantitation of the substituted metal ions and

distance estimates for the locations of bound metal ions. The observation that both Dy^{3+} - and Cd^{2+} -inhibited PSII core complexes that are capable of advancing from the S_1 to the S_2 state provides support for the proposal that Ca^{2+} plays a structural role in the early S state transitions, which can be fulfilled by other cations of similar ionic radius, and that the functional role of Ca^{2+} to activate water in the O–O bond-forming reaction, which occurs in the final step of the S state cycle, can only be fulfilled by Ca^{2+} and Sr^{2+} , two cations with similar Lewis acidities. As Ca^{2+} is a spectroscopically silent ion, there has long been a quest for more informative metal ions to be substituted into the Ca^{2+} -binding site of PSII. This work successfully introduces a new method for further studies to understand oxygen evolution in PSII.

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