Comparative Properties of Hydroquinone and Hydroxylamine Reduction of the Ca^{2+}-Stabilized O_2-Evolving Complex of Photosystem II: Reductant-Dependent Mn^{2+} Formation and Activity Inhibition†

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ABSTRACT: Calcium binding to photosystem II slows NH_2OH inhibition of O_2 evolution; Mn^{2+} is retained by the O_2-evolving complex [Mei, R., & Yocum, C. F. (1991) *Biochemistry 30*, 7836–7842]. This Ca^{2+}-induced stability has been further characterized using the large reductant hydroquinone. Salt-washed photosystem II membranes reduced by hydroquinone in the presence of Ca^{2+} retain 80% of steady-state O_2 evolution activity and contain about 2 Mn^{2+}/reaction center that can be detected at room temperature by electron paramagnetic resonance. This Mn^{2+} produces a weak enhancement of H_2O proton spin–lattice relaxation rates, cannot be easily extracted by a chelator, and is reincorporated into the O_2-evolving complex upon illumination. A comparison of the properties of Ca^{2+}-supplemented photosystem II samples reduced by hydroquinone or NH_2OH alone or in sequence reveals the presence of a subpopulation of manganese atoms at the active site of H_2O oxidation that is not accessible to facile hydroquinone reduction. At least one of these manganese atoms can be readily reduced by NH_2OH following a noninhibitory hydroquinone reduction step. Under these conditions, about 3 Mn^{2+}/reaction center are lost and O_2 evolution activity is irreversibly inhibited. We interpret the existence of distinct sites of reductant action on manganese as further evidence that the Ca^{2+}-binding site in photosystem II participates in regulation of the organization of manganese-binding ligands and the overall structure of the O_2-evolving complex.

Water oxidation by PSII[1] is postulated to occur by successive formation of five oxidation states, S_i (i = 0–4); S_4 decays spontaneously to produce S_0 and O_2 (Kok et al., 1970). Four atoms of Mn form the redox-active site of the O_2-evolving complex; Ca^{2+} and Cl^- are also required for activity (Amesz, 1983; Babcock, 1987; Brudvig et al., 1989; Ghanotakis & Yocum, 1990). A requirement for added Ca^{2+} to restore O_2 evolution activity in PSII preparations is observed when extrinsic 23- and 17-kDa polypeptides are removed from PSII (Ghanotakis et al., 1984a; Miyao & Murata, 1984; Nakatani, 1984). It has been proposed that these polypeptides and an extrinsic 33-kDa species provide part of a structure that concentrates inorganic ion cofactors at the site of H_2O oxidation (Ghanotakis et al., 1984b).

The observation that Ca^{2+} extraction from PSII disrupts electron transfer from Mn to Y_Z^+ suggests that the metal is required for S-state cycling (Dekker et al., 1984; Kalosaka et al., 1990). Analyses of delayed fluorescence yields from PSII indicated that Ca^{2+} extraction impedes the $S_3 \rightarrow S_4$ transition (Boussac et al., 1985), and some EPR measurements suggested that the $S_1 \rightarrow S_2$ transition is affected by Ca^{2+} removal (de Paula et al., 1986; Ghanotakis et al., 1987; Kalosaka et al., 1990). However, if Ca^{2+} extraction by EGTA, EDTA, or citrate is carried out in the light, a modified S_2 EPR multiline signal is observed (Boussac et al., 1989), and thermoluminescence measurements on samples from which Ca^{2+} has been extracted in darkness at pH 3 also show that a modified form of S_2 is formed (Ono & Inoue, 1989). The disparities surrounding Ca^{2+} involvement in S_2 formation may be due to an increased low-temperature limit for S_2 formation, induced by Ca^{2+} extraction (Ono & Inoue, 1990; Boussac et al., 1990).

The EPR-detectable S_2 multiline signal is not observed after illumination of Cl^--depleted PSII; subsequent dark addition of Cl^- produces the signal (Ono et al., 1986). Substitution of F^- for Cl^- generates the g = 4.1 precursor to the multiline signal (Casey & Sauer, 1984), and NH_3 can produce a similar result if the Cl^- concentration is suppressed (Beck & Brudvig, 1986). Chloride sensitivity of ligand binding on or near Mn in PSII defines two types of sites. Small ligands (NH_3, NH_2OH) bind at a Cl^--insensitive site (Sandusky & Yocum, 1984; Beck & Brudvig, 1986; Mei & Yocum, 1990, 1991) whereas a second, Cl^--sensitive site binds NH_3 and larger primary amines, as well as *N*-methyl derivatives of NH_2OH and large species such as PD or TMPD (Sandusky & Yocum, 1984; Tamura et al., 1986; Beck & Brudvig, 1988; Mei & Yocum, 1990; Rickert et al., 1991).

Exposure of PSII to the small reductant NH_2OH can produce two effects. Concentrations greater than 0.1–0.2 mM reduce the Mn complex with a loss of activity and of three to four EPR-detectable Mn^{2+} per reaction center (Cheniae & Martin, 1971; Yocum et al., 1981). An enhancement of H_2O proton spin–lattice relaxation rates, attributed to weak binding of Mn^{2+} to nonfunctional sites outside the O_2-evolving complex, is also observed (Sharp & Yocum, 1981). Lower NH_2OH concentrations ($\ll$100 μM) generate a S_{-1} state from which O_2 release is delayed by two flashes (Bouges, 1971; Kok & Velthuys, 1977). Steady-state illumination of thylakoids incubated with NH_2OH consumes the reductant and prevents inhibitory Mn^{2+} release (Sharp & Yocum, 1981); flash

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; H_2Q, hydroquinone; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Mn, manganese ligated to photosystem II in oxidation states higher than +2; NMR, nuclear magnetic resonance; PS, photosystem; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; XANES, X-ray absorption near-edge structure; Y_Z^+, redox-active tyrosine 161 on the D1 polypeptide of photosystem II.

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illumination of a similar system has been shown to generate N_2 (Radmer & Ollinger, 1983). The latter observation has been interpreted as indicating that NH_2OH binds to the Mn complex in the dark and reduces it by one electron to form N_2 and S_0, which binds another NH_2OH to form S_0–NH_2-OH. A single-turnover flash is proposed to oxidize S_0–NH_2-OH by a one-electron mechanism to generate S_0 and N_2 (Radmer, 1983). Messinger et al. (1991) also interpreted their data from thylakoids on reduction of S states by NH_2-OH as indicating that the reductant acts by a one-electron mechanism.

An examination of the effect of NH_2OH on multiline signal formation revealed a two-turnover delay in optimal signal yield that followed an NH_2OH concentration dependence like that for O_2 flash yields (Andreasson & Hansson, 1986; Sivaraja & Dismukes, 1988a,b). Paramagnetic one-electron oxidation products of NH_2OH, predicted as intermediates in N_2 release, were not detected in illuminated samples retaining a functional Mn complex; NH_2OH reduction of Mn was therefore proposed to occur by a two-electron mechanism (Beck & Brudvig, 1987). These EPR data agree with results obtained from optical and O_2 flash yield measurements (Saygin & Witt, 1985; Kretschmann et al., 1991), which also indicate that low NH_2-OH concentrations reduce the Mn complex by two electrons to form S_{-1} rather than S_0–NH_2OH.

Removal of 23- and 17-kDa polypeptides increases the sensitivity of PSII to inhibition by NH_2OH and exposes Mn to reduction and extraction by species (H_2Q, *p*-phenylenediamines) substantially larger than NH_2OH (Ghanotakis et al., 1984c; Tamura et al., 1986; Mei & Yocum, 1990). It has also been shown that certain bulky, charged reductants (ascorbate, ferrocyanide) can reduce and extract Mn from PSII, but only after removal of the 33-kDa extrinsic polypeptide (Tamura et al., 1990). These observations would indicate that the inhibitory potency of the range of reductants tested in PSII is subject to limitations imposed by their size and charge rather than by their redox potentials.

We have shown that Ca^{2+} is able to stabilize the structural environment of the Mn complex and in doing so slows Mn^{2+} release during exposure of PSII to NH_2OH (Mei & Yocum, 1991). In this paper, we report the results of an examination of the effect of Ca^{2+} on reductant-mediated inhibition of O_2 evolution activity using H_2Q, a large hydrophobic reductant. A comparison of the inhibitory action of this reductant with that of NH_2OH indicates that the Ca^{2+}-stabilized structure of the O_2-evolving complex can retain Mn^{2+} near its native binding sites in a stable, shielded environment. In the presence of Ca^{2+}, a unique NH_2OH site in the Mn complex is detected that is not susceptible to rapid hydroquinone reduction.

MATERIALS AND METHODS

Preparation and storage of PSII membranes depleted of extrinsic 23- and 17-kDa proteins has been described (Mei & Yocum, 1991). Polypeptide-extracted membranes were washed once in 50 mM MES buffer (pH 6) prior to storage to remove residual Cl^- and Ca^{2+}; preparations so treated retain a residual amount of activity (about 20%) that is observed in the absence of added Ca^{2+} or Cl^- (Mei & Yocum, 1991). Preliminary assays identified a H_2Q concentration (0.2 mM) that permitted sufficient time for the manipulations necessary to determine inhibition kinetics. Salt-washed PSII membrane suspensions (2 mg of Chl/mL) containing various concentrations of Ca-$(MES)_2$ or $(CH_3)_4NCl$ were incubated with H_2Q in darkness for fixed (30 min) or varied times periods; activity inhibition was followed by assays of O_2 evolution activity (Mei & Yocum, 1991). The assay mixture contained 10 mM $CaCl_2$, 50 mM MES buffer (pH 6.0), and 0.31 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) as the acceptor. Where indicated, samples were exposed to EDTA prior to O_2 evolution assays by diluting 0.01 mL of the incubation mixture into 1.6 mL of pH 6 MES buffer containing 0.2 mM EDTA and 0.31 mM DCBQ. After 30 s, 10 mM $CaCl_2$ was added for assays of remaining O_2 evolution activity.

Reduction of PSII-associated Mn was monitored at room temperature using a Bruker ER-200D EPR spectrometer operated at X-band (Yocum et al., 1981; Mei & Yocum, 1991); instrument conditions are given in relevant table and figure legends. Samples for Mn analyses were prepared using the incubation conditions described above, followed by centrifugation. Pellets were resuspended to 2 mg of Chl/mL in 50 mM MES (pH 6)/10 mM $CaCl_2$/0.5 mM $Fe(CN)_6^{3-}$; supernatants and pellets were scanned for Mn^{2+} six-line signals. Any ferrocyanide created by H_2Q oxidation precipitates Mn^{2+} from solution (Blankenship & Sauer, 1974; Hoganson et al., 1991). Activity was assayed after EPR measurements. Illumination of PSII membranes exposed to H_2Q in darkness for 30 min was carried out after 40-fold dilution in 10 mM $CaCl_2$/50 mM MES (pH 6)/0.31 mM DCBQ. These samples were illuminated (2 min at 4 °C), centrifuged, and resuspended to 2 mg of Chl/ml for EPR measurements. To determine Mn loss from reduced PSII membranes that had been diluted 40-fold in 10 mM $CaCl_2$ or 2 mM EDTA buffered with MES (pH 6), the material was centrifuged and resuspended pellets were acidified with HCl to pH 1 to release residual Mn as Mn^{2+}. Estimates of Mn^{2+}/reaction center ratios in reduced samples were corrected for loss of the metal from inactivated centers as well as for a population of reaction centers (about 20%) that are resistant to reduction (Mei & Yocum, 1991).

Water proton spin–lattice relaxation rates were determined at 8 °C in samples incubated with H_2Q plus or minus Ca^{2+}. The intrinsic relaxivity of salt-washed PSII membranes was determined before reductant addition, after which the reductant was added to determine the effect on R_1. Details of the NMR instrumentation and the pulse sequences employed are described by Srinivasan and Sharp (1986).

RESULTS

The absence of extrinsic 23- and 17-kDa proteins permits Ca^{2+} to equilibrate rapidly with its site of action in PSII and slow the rate of NH_2OH inhibition of O_2 evolution activity (Mei & Yocum, 1991). This function of Ca^{2+} was characterized using the larger reductant, H_2Q. Figure 1A shows that Ca^{2+} attenuates H_2Q inhibition of O_2 evolution activity in salt-washed PSII membranes. Without Ca^{2+}, the observed half-time of H_2Q inactivation was 14 min; with 10 mM Ca^{2+} present, the half-time increased to 140 min (Figure 1B). Divalent metals (Sr^{2+}, Cd^{2+}) that compete with Ca^{2+} for its site of action in PSII also slow the rate of H_2Q inhibition (data not shown). In contrast to the noncompetitive interaction previously documented for Ca^{2+} and NH_2OH (Mei & Yocum, 1991), double-reciprocal plots show the interaction between Ca^{2+} and H_2Q to be mixed-competitive (data not shown). Although Cl^- does not interfere with NH_2OH inhibition in salt-washed PSII membranes, the extent of H_2Q inhibition in these preparations is affected by the anion (Figure 1A,B). In the absence of Ca^{2+}, 15 mM $(CH_3)_4NCl$ increased the observed half-time for H_2Q inactivation to 40 min (Figure 1B). This result is consistent with the Cl^- sensitivity detected for binding of other large species to PSII.

Investigations documenting inhibition of PSII activity with large reductants (H_2Q, TMPD) also report a corresponding

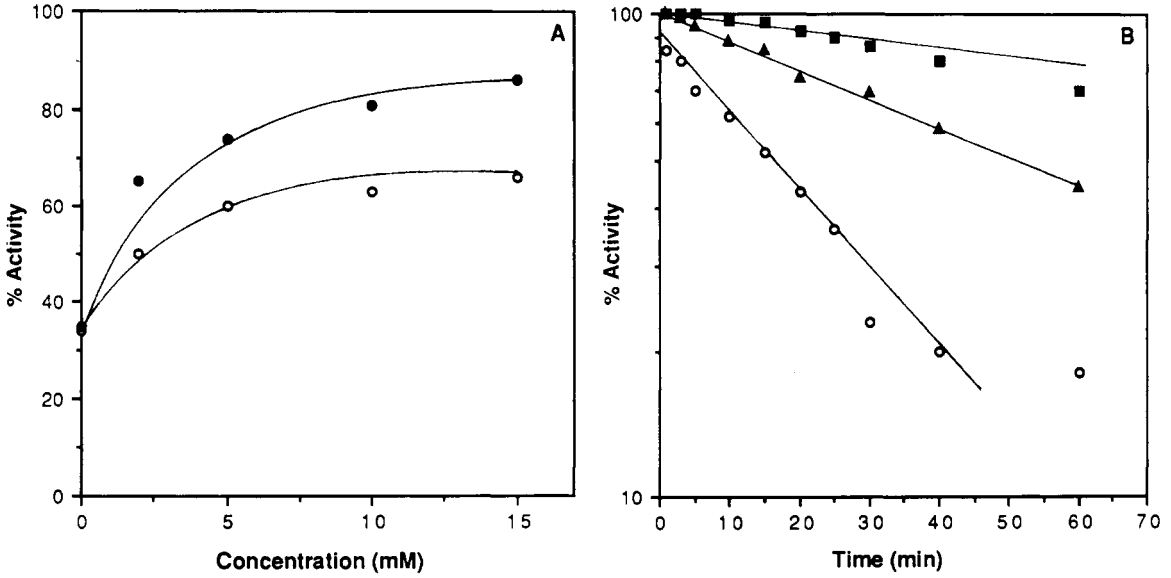

FIGURE 1: Slowing of hydroquinone inhibition of O_2 evolution activity by addition of Ca^{2+} or Cl^-. In all experiments shown, $Ca(MES)_2$ (pH 6) or $(CH_3)_4NCl$ were used for additions of Ca^{2+} and Cl^- ions, respectively. Control (100%) activity was 450 μmol of O_2 h^{-1} (mg of Chl)$^{-1}$. (A) Effect of inorganic ion concentration. Salt-washed membrane suspensions containing the indicated concentrations of Ca^{2+} or Cl^- were incubated in darkness for 30 min with 0.2 mM H_2Q. Solid circles, Ca^{2+}; open circles, Cl^-. (B) Effect of Ca^{2+} and Cl^- on inactivation half-times. Data shown are for 0.2 mM H_2Q inhibition in the presence of 10 mM $Ca(MES)_2$ (squares), 30 mM $(CH_3)_4NCl$ (triangles), or no addition (open circles).

Table I: Effect of Ca^{2+} on Mn Retention by H_2Q-Reduced, Salt-Washed PSII Membranes[a]

addition	EPR-detectable Mn^{2+} (μM)		O_2 evolutn activ (μmol h^{-1} (mg of Chl)$^{-1}$)
	supernatant	pellet	
none	0	36	450
0.2 mM H_2Q	24	12	133
0.2 mM H_2Q + 10 mM $Ca(MES)_2$	6	30	382

[a] Salt-washed PSII membranes (2 mg of Chl/mL) were incubated for 30 min at 4 °C with the additions shown; $Ca(MES)_2$ was added before H_2Q. Manganese(II) quantitation (after acidification) and O_2 evolution assays were performed as described in Materials and Methods. EPR operating conditions: microwave power, 50 mW; modulation amplitude, 10 G(pp); gain, 2×10^5; time constant, 100 ms; sweep time, 100 s; sweep width, 1000 G.

loss of Mn (Ghanotakis et al., 1984c; Tamura et al., 1986). The Ca^{2+} effect on this process was investigated by exposing salt-washed PSII membranes to H_2Q for 30 min in darkness; after centrifugation, pellets and supernatants were analyzed by room-temperature EPR for Mn^{2+} six-line signals. Table I shows that, without Ca^{2+}, extensive Mn and activity losses occur in salt-washed PSII membranes. The presence of Ca^{2+} attenuates this phenomenon and induces a 4-fold decrease in Mn^{2+} release; Mn retention shows a close correlation with O_2 evolution activity.

The Ca^{2+}-dependent correlation between activity and Mn retention (Table I) was characterized in more detail using EPR spectroscopy to detect any Mn^{2+} that might be associated with reduced samples. Calcium-supplemented PSII membranes were reduced with 0.2 mM H_2Q in darkness; after 30 min, reduction was terminated by addition of 0.5 mM $Fe(CN)_6^{3-}$. Our experiments (data not shown) confirm the finding that ferrocyanide, produced by H_2Q oxidation, precipitates free Mn^{2+} in solution (Blankenship & Sauer, 1974; Hoganson et al., 1991), so this treatment should suppress any residual adventitious Mn^{2+}. The reduced PSII membranes were centrifuged and resuspended in a buffer containing 50 mM MES/10 mM $CaCl_2$/0.5 mM $Fe(CN)_6^{3-}$/ and divided into two samples. One sample was stored in the dark while the other was diluted to 50 μg of Chl/mL, illuminated (2 min), centrifuged, and resuspended to the original Chl concentration. The EPR spectra of both samples, shown as parts A and B of Figure 2, reveal that about 11 μM Mn^{2+} is EPR detectable in the dark-adapted sample. This corresponds to 2 Mn^{2+}/reaction center after the corrections described in Materials and Methods. Spectrum B (Figure 2) demonstrates that more than 80% of the Mn^{2+} in the dark-adapted sample disappears after illumination; O_2 evolution activity is unchanged by the illumination step. Thus, Mn reduction by H_2Q in Ca^{2+}-stabilized PSII membranes does occur. However, the resulting Mn^{2+} is retained by these membranes and is readily reincorporated into the O_2-evolving complex in an EPR-silent form.

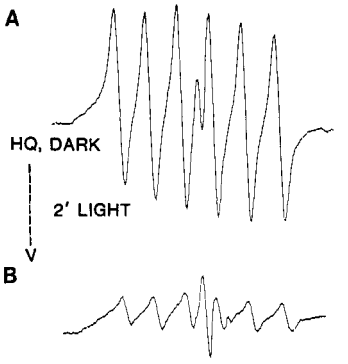

FIGURE 2: Reversal of hydroquinone reduction of Mn in the presence of Ca^{2+} by illumination. Hexaquo Mn^{2+} six-line spectra are shown for samples before and after illumination. Details are given in Materials and Methods and the text. (A) A sample held in darkness throughout: EPR-detectable Mn^{2+}, 11 μM; O_2 evolution, 360 μmol of O_2^{-1} h (mg of Chl).$^{-1}$ (B) An equivalent sample after 2-min illumination as described in Materials and Methods: Mn^{2+}, 2 μM; O_2 evolution activity, 360 μmol of O_2 h^{-1} (mg of Chl)$^{-1}$. EPR spectrometer operating conditions for both samples: microwave power, 200 mW; modulation amplitude, 10 G(pp); gain, 2×10^5; time constant, 100 ms; sweep time, 100 s; sweep width, 1000 G.

The effect of Ca^{2+} on H_2Q-catalyzed Mn reduction was also probed using NMR to detect H_2O proton spin–lattice relaxation enhancements (R_1) that might arise from formation of Mn^{2+} (Sharp & Yocum, 1981). Figure 3 shows that a significant R_1 enhancement is induced by H_2Q reduction of

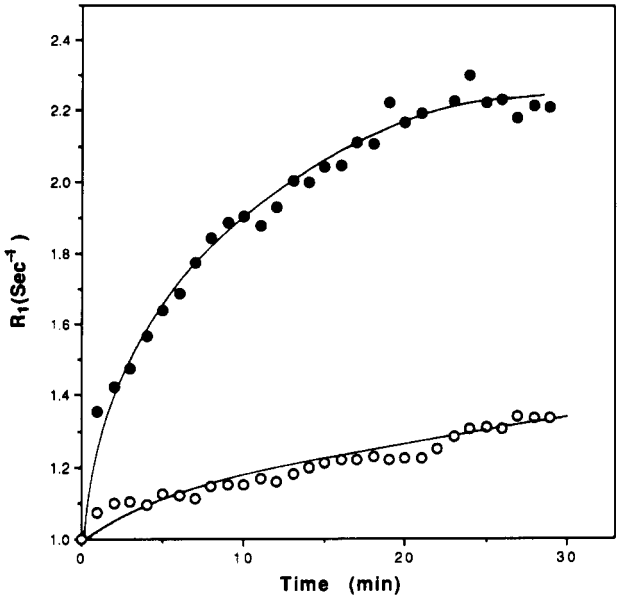

FIGURE 3: Enhancement of water proton spin–lattice relaxation rates (R_1) by H_2Q reduction of Mn: effect of Ca^{2+}. Salt-washed PSII membranes were incubated in 0.2 mM H_2Q for the times shown. Solid circles, no Ca^{2+} addition; open circles, 10 mM Ca^{2+} added before H_2Q.

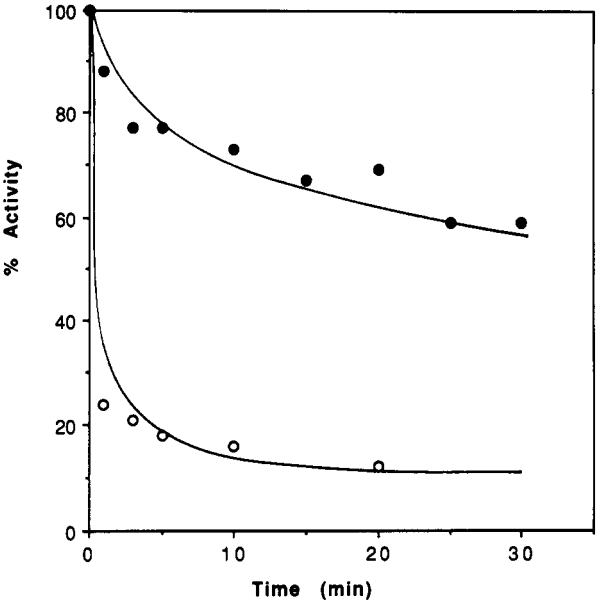

FIGURE 4: Differential EDTA sensitivity of the oxygen-evolving complex after reduction by H_2Q or NH_2OH in the presence of Ca^{2+}. Salt-washed membranes plus Ca^{2+} (10 mM) were incubated with 0.2 mM H_2Q or 0.1 mM NH_2OH for the times shown and diluted 160-fold into 0.2 mM EDTA/MES buffer containing 0.31 mM DCBQ. After a 30-s dark incubation, 10 mM $CaCl_2$ was added for assay of O_2 evolution activity. The control (100%) activity is given in Figure 1. Solid circles, H_2Q; open circles, NH_2OH.

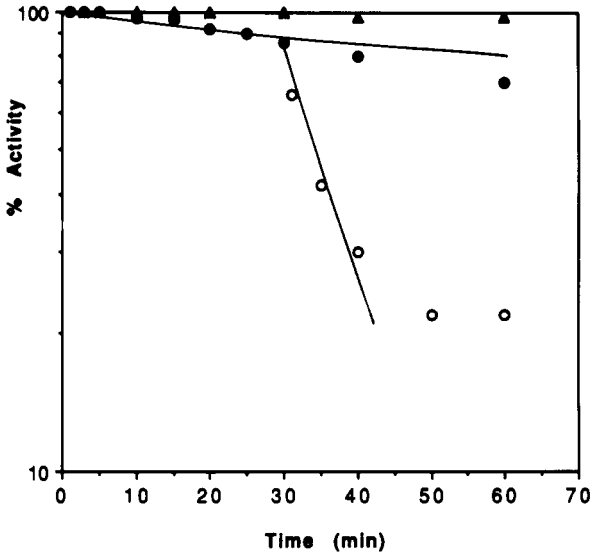

FIGURE 5: Synergy of hydroquinone and NH_2OH with respect to inhibition of O_2 evolution activity in Ca^{2+}-containing, salt-washed PSII membranes. The membrane suspensions, containing 10 mM Ca^{2+}, were incubated for the times shown with 20 μM NH_2OH (triangles) or 0.2 mM H_2Q (closed circles). Open circles represent a sample incubated with 0.2 mM H_2Q for 30 min prior to 20 μM NH_2OH addition. The control (100%) activity is given in Figure 1.

salt-washed PSII membranes without added Ca^{2+}, consistent with release of Mn^{2+} from th O_2-evolving complex and its subsequent rebinding to nonfunctional sites. With Ca^{2+} present prior to H_2Q reduction, a much smaller R_1 enhancement is seen (Figure 3). This finding would indicate that, in the presence of Ca^{2+}, H_2Q-generated Mn^{2+} remains in an environment that does not equilibrate rapidly with the surrounding medium. Evidence that H_2Q-generated Mn^{2+} is not readily exposed to the external medium was also obtained from the results of experiments analyzing the inhibitory effect of EDTA on O_2 evolution. Salt-washed PSII membranes with added Ca^{2+} were reduced with H_2Q or NH_2OH for increasing periods of time and diluted 160-fold into an O_2 assay cuvette containing 0.2 mM EDTA/MES buffer for a 30-s dark incubation preceding illumination. As shown in Figure 4, samples exposed to NH_2OH for short periods of time were readily inactivated by EDTA. The same experimental protocol utilizing H_2Q failed to produce comparable EDTA sensitivity, even at long (30 min) incubation times with reductant.

The origin of contrasting properties of PSII samples reduced with either NH_2OH or H_2Q with Ca^{2+} present was sought by conducting experiments using the reductants together. Figure 5 shows the slow H_2Q inactivation of O_2 evolution activity observed in the presence of Ca^{2+}; a low concentration of NH_2OH (20 μM) alone caused little detectable inhibition. A sequential combination of reductants, however, with NH_2OH following H_2Q, induces rapid activity inhibition. The data of Table II demonstrate that the duration of H_2Q incubation preceding NH_2OH addition is essential to observe rapid inactivation with the latter reductant, and Table III documents the effects of these reductant treatments on Mn retention by PSII. For Mn quantitation by EPR, incubation mixtures were diluted 40-fold in either 10 mM $CaCl_2$ or 2 mM EDTA and centrifuged; pellets were resuspended in the corresponding dilution buffer, assayed for activity, and then acidified to quantitate Mn. The data (Table III) show that activity and Mn content are minimally affected by 20 μM NH_2OH and that 0.2 mM H_2Q creates less EDTA-sensitive Mn^{2+} than was observed earlier with 100 μM NH_2OH (Mei & Yocum, 1991). For a sample preincubated with H_2Q, however, addition of 20 μM NH_2OH produces extensive Mn^{2+} release (60–75%) from active centers. The alternative exposure protocol (20 μM NH_2OH added before H_2Q) results in retention of both O_2 evolution activity and Mn over the periods of reductant exposure shown in Table III.

DISCUSSION

Reductant-mediated inhibition of PSII proceeds in sequential steps. Reductants bind to the O_2-evolving complex and reduce Mn to Mn^{2+}. This reaction is followed by an inhibitory release of Mn^{2+} atoms from the active site of H_2O oxidation. Reduction of the Mn complex no doubt occurs through a series of intermediate steps, including the formation of S_{-1} (Beck & Brudvig, 1987). In light of the data we present here,

Table II: Effect of Preincubation with H_2Q on the Rate of NH_2OH Inactivation of O_2 Evolution Activity[a]

incub time with H_2Q before NH_2OH additn (min)	k_{obs}, NH_2OH inhibitn (min^{-1})	incub time with H_2Q before NH_2OH additn (min)	k_{obs}, NH_2OH inhibitn (min^{-1})
0	0.046	30	0.099
10	0.066	40	0.154

[a] Salt-washed PSII membranes (2 mg of Chl/mL) were exposed to 0.2 mM H_2Q for the indicated times followed by addition of 20 μm NH_2OH. Activity assays were performed as described in Materials and Methods.

Table III: Effects of the Combination of NH_2OH and H_2Q on Activity and Mn Loss from Ca^{2+}-Stabilized, Salt-Washed PSII Membranes[a]

	Diluent			
	10 mM $CaCl_2$		2 mM EDTA	
30-min reductant treatment	V_{O_2}[b]	$[Mn^{2+}]$[c]	V_{O_2}[b]	$[Mn^{2+}]$[c]
none	462	36	462	36
20 μM NH_2OH	462	34	434	33
0.2 mM H_2Q	416	31	277	23
0.2 mM H_2Q, then 20 μM NH_2OH, 10 min	139	11	116	8
20 μM NH_2OH, then 0.2 mM H_2Q, 10 min	425	30	323	27

[a] Salt-washed PSII membranes (2 mg of Chl/mL) were exposed (30 min) to the indicated reductant concentrations. Thereafter, the second reductant was added as indicated for the time shown. At the end of this incubation period the samples were diluted, centrifuged, and treated as described in Materials and Methods. EPR conditions as in Table I.[b] In μmol of O_2 h^{-1} (mg of Chl)$^{-1}$. [c] In μM.

the inhibitory release of Mn^{2+} from Ca^{2+}-stabilized PSII depends on the number of Mn^{2+} present per reaction center and on the reduction of at least one critical Mn atom in the O_2-evolving complex. In the presence of Ca^{2+}, hydroquinone reduction of PSII produces samples retaining 80% of control O_2 evolution activity and EPR-detectable Mn^{2+} that is quenched by illumination (Figure 2). Results to be reported elsewhere show that illumination also reverses a downward shift in XANES edge energies, caused by H_2Q reduction, that correlate with the formation of about 2 Mn^{2+}/reaction center (Riggs, P., and Penner-Hahn, J. E., personal communication). Thus, H_2Q-generated Mn^{2+} atoms remain in close proximity to their binding sites so long as Ca^{2+} is present. The observations that this Mn^{2+} is detected in PSII membranes after ferrocyanide exposure, produces a minimal enhancement of H_2O proton spin–lattice relaxation rates (R_1) in Ca^{2+}-stabilized samples (Figure 3), and is difficult to extract with EDTA (Figure 4) provide evidence that it must reside in a shielded environment. At the same time, our data seem to indicate that this Mn^{2+} is not tightly ligated; we find no evidence of ligand-induced broadening of the EPR signal [the EPR Mn^{2+} quantitations agree with XANES data (not shown)] and NMR measurements indicate that the metal is not ligated to sites of reorientationally restricted mobility to create an efficient relaxation pathway for H_2O protons.

Since Mn^{2+} lacks ligand-field stabilization energy, increases in the amount of Mn^{2+} present in a multinuclear complex of the metal should increase its lability. The differences in EDTA sensitivity observed after reduction of the O_2-evolving complex with either H_2Q or NH_2OH (Figure 4) correlate well with the amounts of Mn^{2+} present in reduced samples [about 3 labile Mn^{2+}/reaction center with NH_2OH (Mei & Yocum, 1991) and about 2 stably-retained Mn^{2+}/reaction center with H_2Q (Figure 2)]. Thus, while Ca^{2+} binding to PSII influences the stability of the reduced O_2-evolving complex, the magnitude of this effect is also determined by the extent of reduction of the Mn cluster itself.

The synergistic interactions between H_2Q and NH_2OH shown in Figure 5 and the differential effects of Cl^- (Figure 1) provide evidence that these reductants act on the Mn cluster at topologically and chemically distinct sites. In the presence of Ca^{2+}, a Mn site can be detected which reacts about 30 times more rapidly with NH_2OH than with H_2Q (Figure 5). This contrasts with the observation that H_2Q reduction of at least one higher oxidation state of Mn (Mn^{3+}) occurs 3 times faster in solution than the corresponding reaction with NH_2OH (Davies, 1969). These opposing observations may provide evidence that the intrinsic protein matrix of PSII along with the extrinsic 33-kDa protein imposes barriers that limit access of large reductants to the Mn complex. This structural influence of the protein matrix may explain why double reciprocal plot analyses of interactions between Ca^{2+} and NH_2OH or H_2Q show that Ca^{2+} is noncompetitive with respect to NH_2OH (Mei & Yocum, 1991) but mixed-competitive with respect to H_2Q (data not shown).

Our data indicate that the differential sensitivity of PSII Mn to reductants may arise in part from the structure and/or metal oxidation states of the Mn cluster. Models for the structure of the four Mn atoms in the S_2 state of PSII suggest that they are ligated in an interactive environment that forms a tetranuclear cluster or alternative arrangements (monomer–trimer of dimer–dimer). The reductant-dependent extents of Mn^{2+} formation reported here after reduction of the dark-stable S_1 state show that these metals can behave either as a dimer of dimers (H_2Q reduction) or as a monomer–trimer (NH_2OH reduction). A further heterogeneity is evident in the case of NH_2OH. Prereduction of Mn for 30 min with H_2Q enables 20 μM NH_2OH to inactivate H_2O oxidation with a half-time of about 7 min (Table II). This is more rapid than the corresponding half-time (13.5 min) in the presence of Ca^{2+} for inhibition by 100 μM NH_2OH alone (Mei & Yocum, 1991) and indicates that higher oxidation states of individual atoms of the Mn cluster might differ in their susceptibility to reduction and/or their contribution to the stability of the O_2-evolving complex.

Demonstrated differences in stability of reduced PSII derivatives obtained after exposure to H_2Q and NH_2OH may point to a connection between Mn oxidation states and reductant susceptibility. A model for the S_2 state proposing that the Mn oxidation states are 3 Mn^{4+}/Mn^{3+} (de Paula & Brudvig, 1985) agrees with XANES data indicating that the S_1 state is comprised of 2 Mn^{3+}/2 Mn^{4+} [Guiles et al. (1990) and Riggs, P., and Penner-Hahn, J. E., personal communication]. As the strongest oxidant in the S_1 state, Mn^{4+} may be the first species reduced by NH_2OH. In PSII preparations containing added Ca^{2+} or extrinsic polypeptides, low NH_2OH concentrations may only be sufficient to catalyze a single two-electron reaction (Beck & Brudvig, 1987; Kretschmann et al., 1991) that reduces Mn^{4+} to Mn^{2+} to form a S_{-1} state containing Mn^{4+}/2 Mn^{3+}/Mn^{2+}. Retention of several Mn atoms in higher oxidation states would maintain the overall integrity of the Mn complex, consistent with our data (Figure 5, Table III) and the earlier findings that noninhibitory concentrations of NH_2OH produce a two-flash delay in O_2 yields (Bouges, 1971; Kok & Velthuys, 1977). At higher concentrations, NH_2OH would also form S_{-1}, but also reduce additional Mn atoms to form an unstable S_{-3} state containing three labile Mn^{2+} (Beck & Brudvig, 1987).

The stability of H_2Q-reduced PSII derivatives could be explained by assuming that this reductant converts Mn^{3+} to Mn^{2+} to create an S_{-1} state (2 Mn^{2+}/2 Mn^{4+}) that differs

from S_{-1} produced by low NH_2OH concentrations (Mn^{4+}/2 Mn^{3+}/Mn^{2+}). The H_2Q-reduced S_{-1} state would retain a comparable structural integrity on account of the stability and exchange-inert nature of ligand-associated Mn^{4+} (Pecoraro, 1988). In this context, loss of activity and Mn^{2+} caused by addition 20 μM NH_2OH to H_2Q-reduced samples (Figure 5) would arise from Mn^{4+} reduction to produce the same unstable S_{-3} state generated by higher concentrations of NH_2-OH alone.

Any model for the formation and stability of reduced derivatives of PSII is to an extent speculative. The properties of synthetic complexes of Mn^{4+} lend support to our proposals, in that they exhibit greater stability relative to similar complexes containing either Mn^{3+} or Mn^{2+} and require substantial structural rearrangements of the ligand environment relative to complexes containing Mn in lower oxidation states (Pecoraro, 1988). On the other hand, so far as we can determine no Mn^{2+} EPR signals have been detected in PSII samples exposed to low concentrations of NH_2OH. Also, it would seem likely that reduced intermediates containing Mn^{2+} and Mn^{4+} would undergo a dismutation reaction to produce Mn^{3+}, rather than the EPR-active Mn^{2+} detected after H_2Q reduction. This reaction may not be facile, however; a synthetic tetranuclear Mn cluster has been prepared that contains Mn^{4+}/Mn^{3+}/2 Mn^{2+} (Chan & Armstrong, 1990).

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Registry No. Ca^{2+}, 7440-70-2; Mn^{2+}, 7439-96-5; NH_2OH, 7803-49-8; hydroquinone, 123-31-9.