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Calcium Retards NH_2OH Inhibition of O_2 Evolution Activity by Stabilization of Mn^{2+} Binding to Photosystem II†

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ABSTRACT: Calcium is required for oxidation of water to molecular oxygen by photosystem II; the Ca^{2+} demand of the reaction increases upon removal of 23- and 17-kDa extrinsic polypeptides from detergent-derived preparations of the photosystem. Employing the manganese reductant NH_2OH as a probe to examine the function of Ca^{2+} in photosystem II reveals that (1) Ca^{2+} slows the rate of NH_2OH inhibition of O_2 evolution activity, but only in photosystem II membranes depleted of extrinsic proteins, (2) other divalent cations (Sr^{2+}, Cd^{2+}) that compete for the Ca^{2+} site also slow NH_2OH inhibition, (3) Ca^{2+} is noncompetitive with respect to NH_2OH, (4) in order to slow inhibition, Ca^{2+} must be present prior to the initiation of NH_2OH reduction of manganese, and (5) Ca^{2+} appears not to interfere with NH_2OH reduction of manganese. We conclude that the ability of Ca^{2+} to slow the rate of NH_2OH inhibition arises from the site in photosystem II where Ca^{2+} normally stimulates O_2 evolution and that the mechanism of this phenomenon arises from the ability of Ca^{2+} or certain surrogate metals to stabilize the ligation environment of the manganese complex.

The formation of molecular oxygen from water by photosystem II (PSII)[1] is proposed to occur by means of a linear, four-electron oxidation process involving five so-called S-state intermediates [$S_0 \rightarrow S_4$ (Joliot & Kok, 1975)]. In dark-adapted material, S_1 predominates, and evidence exists to indicate that a concerted oxidation of water occurs after formation of the S_4 state (Radmer & Ollinger, 1986), sug-

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; Mn, manganese ligated to photosystem II in oxidation states higher than +2; PS, photosystem; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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gesting that the S-state cycle may operate as a charge-accumulating device. Among the components of the O_2-evolving complex of PSII that are essential for efficient operation of the S-state cycle are three extrinsic polypeptides (17, 23, and 33 kDa, respectively) and the inorganic cofactors Mn, Ca^{2+}, and Cl^-. Extraction of any of these cofactors results in a loss of O_2 evolution activity (Ghanotakis & Yocum, 1990). Exposure of detergent-isolated PSII membranes to high concentrations (1–2 M) of NaCl extracts the 17- and 23-kDa species without concurrent loss of functional Mn (Sandusky et al., 1983). In the absence of extrinsic polypeptides, the Mn atoms are rendered susceptible to attack by large reductants, such as hydroquinone or TMPD, dependnet on experimental conditions (Ghanotakis et al., 1984d; Tamura et al., 1986, 1990). Sensitivity of PSII in inhibition by a smaller reductant, NH_2OH, is also enhanced by polypeptide removal, as evidenced by a lowering of the NH_2OH concentration required to effect strong inhibition of the O_2-evolving reaction (Ghanotakis et al., 1984d; Tamura & Cheniae, 1985). The end product of the inhibitory action of reductants on PSII is the formation of Mn^{2+}, which may be detected as the hexaquo species $[Mn(H_2O)_6]^{2+}$ owing to its characteristic six-line EPR signal. Between three and four Mn atoms associated with each PSII reaction center are released by the action of reductants (Yocum et al., 1981; Ghanotakis et al., 1984d; Tamura & Cheniae, 1985). Reductant inhibition of PSII activity is countered by illumination, which is presumed to promote photooxidation of the Mn^{2+} created by reductant action (Sharp & Yocum, 1981; Ghanotakis et al., 1984d).

Addition of Ca^{2+} is required to restore O_2 evolution activity to PSII preparations from which the extrinsic 17- and 23-kDa polypeptides have been released (Ghanotakis et al., 1984b; Miyao & Murata, 1984; Nakatani, 1984). These polypeptides are proposed to form part of a structure that facilitates retention of Ca^{2+} at its site of action in the O_2-evolving complex (Ghanotakis et al., 1984c). Recent investigations of the affinity of Ca^{2+} in restoring steady-state O_2 evolution activity to polypeptide-depleted PSII have produced K_M values for the metal in the range of 20–50 μM (Cammarata & Cheniae, 1987; Waggoner et al., 1989) for PSII membranes, although a lower affinity Ca^{2+} site (K_M = 1–2 mM) can also be detected in the same samples exhibiting a high-affinity site (Boussac et al., 1985a; Cammarata & Cheniae, 1987; Homann, 1988). Recent investigations with more purified preparations from which Ca^{2+} has been rigorously extracted ($\ll$1 Ca^{2+} retained/reaction center) reveal an even lower K_M value (1–4 μM; Kalosaka et al., 1990) in addition to the higher values seen in other preparations.

Estimates of the stoichiometry of Ca^{2+} associated with the O_2-evolving activity of PSII vary. Tamura and Cheniae (1988) and Ono and Inoue (1988) report that the minimal number of Ca^{2+} required for activity is 2 atoms per PSII reaction center whereas Shen et al. (1988) report a value of 1 atom per reaction center. Release of one of two Ca^{2+} from spinach PSII membranes produces a significant depression of activity (Ono & Inoue, 1988). Removal of the second atom of Ca^{2+} requires more stringent conditions, such as exposure to lowered pH and repetitive washings (Kalosaka et al., 1990); it is not entirely clear whether the second atom of the metal is directly involved in O_2 evolution activity. After extraction of Ca^{2+}, other metals can occupy the binding site. Ghanotakis et al. (1984b) showed that Sr^{2+} would restore O_2 evolution activity at lower rates than can be observed in the presence of Ca^{2+}; this appears to be due to slower turnover of the S states (Boussac & Rutherford, 1988b). It has also been shown that Cd^{2+} and La^{3+} can compete with Ca^{2+} (Waggoner & Yocum, 1990; Ghanotakis et al., 1985) and these metals, as well as monovalent cations [for example, Na^+ (Waggoner et al., 1989)], are inhibitory.

The exact role of Ca^{2+} in the sequential one-electron oxidation steps that lead to the formation of O_2 from water remains obscure. Some investigations have shown that in the absence of Ca^{2+} the amplitude of a $g = 2$, $s = {}^1/_2$ multiline EPR signal, ascribed to the formation of the S_2 state (Dismukes & Siderer, 1981), is decreased in intensity (de Paula et al., 1986) or eliminated (Ghanotakis et al., 1987; Kalosaka et al., 1990; Ono & Inoue, 1990), whle Boussac and Rutherford (1988) have suggested that the metal may not be required for formation of the multiline species. More recently, it has been reported (Boussac et al., 1989; Sivaraja et al., 1989; Ono & Inoue, 1990) that the multiline signal formed in the absence of Ca^{2+} differs from the signal observed in the presence of Ca^{2+}; carboxylate chelators (EGTA, citrate) used for Ca^{2+} removal have been shown to be responsible for modification of the multiline signal (Boussac et al., 1990). Replacement of Ca^{2+} by Sr^{2+} also alters the multiline signal, producing narrowed line widths (Boussac & Rutherford, 1988a). Alternative approaches employing measurements of delayed luminescence of thermoluminescence have produced divergent findings. Delayed fluorescence measurements indicate that Ca^{2+} depletion blocks S-state advancement beyond S_3 (Boussac et al., 1985b), whereas thermoluminescence measurements suggest that S-state advancement may be blocked at S_2 (Ono & Inoue, 1989a,b).

This paper reports the results of experiments employing the Mn reductant NH_2OH as a probe to examine Ca^{2+} function in PSII. We show that Ca^{2+} slows the rate of NH_2OH inhibition of O_2 evolution activity in PSII membranes from which the 23- and 17-kDa extrinsic polypeptides have been removed. The effect we describe derives from stabilization of Mn^{2+} binding to PSII, and is reflected in a slower rate of NH_2OH inhibition. The Mn^{2+} created by NH_2OH action in the presence of Ca^{2+} appears to be retained at or near its native binding site so long as adequate conditions of Ca^{2+} are present.

Materials and Methods

Photosystem II preparations were isolated from spinach by using the method of Berthold et al. (1981) with modifications (Ghanotakis et al., 1984a). Salt-washed PSII membranes were obtained by exposing PSII membranes to 2 M NaCl (30 min in darkness, 1.5 mg of Chl/mL) followed by a wash step in 50 mM MES buffer (pH 6.0). All preparations were suspended in 0.4 M sucrose–50 mM MES buffer (pH 6) and stored at −70 °C before use. The salt-washed preparations show lowered O_2 evolution activity that correlates with removal of the 17- and 23-kDa extrinsic proteins, as assessed by polyacrylamide gel electrophoresis. Residual activity (about 20%) is slowly inhibited by low (0.1 mM) concentrations of NH_2OH and shows a normal Cl^- requirement, consistent with the retention by PSII of some 23-kDa protein after the extraction procedure. Native and salt-washed PSII membranes (2 mg of Chl/mL) in storage buffer were incubated with NH_2OH in darkness for varying periods of time under conditions outlined in the legends to the figures and tables. Calcium was added either as the chloride salt or as a solution of $Ca(OH)_2$ neutralized to pH 6 with unneutralized MES buffer [$Ca(MES)_2$]. The inhibition time course was followed by assays of steady-state O_2 evolution activity, using 0.01 mL of the incubation mixture diluted into a 1.6-mL assay mixture in a stirred, thermostated (25 °C) cell fitted with a Clark-type O_2 electrode. The assay buffer contained 10 mM $CaCl_2$, 50

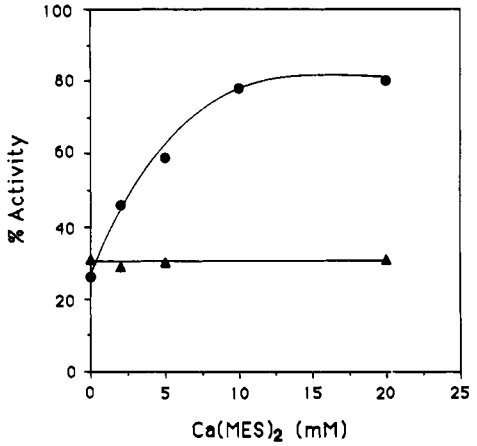


FIGURE 1: Effect of Ca^{2+} on inhibition of oxygen evolution activity by NH_2OH in intact and salt-washed PSII membranes. Calcium hydroxide adjusted to pH 6 with unneutralized MES was used for all additions of the metal. Intact and salt-washed PSII membranes, both in 0.4 M sucrose/50 mM MES (pH 6.0) (2 mg of Chl/mL), were incubated in darkness (4 °C) for 30 min with 1 mM NH_2OH and for 3 min with 0.1 mM NH_2OH, respectively, and then diluted to 12.5 μg of Chl/mL and examined for remaining oxygen evolution activity with 0.31 mM DCBQ as the acceptor and 10 mM $CaCl_2$/50 mM MES as the assay buffer. Control activity (100%) of intact and salt-washed PSII was 650 and 450 μmol of O_2 h^{-1} mg of Chl)$^{-1}$, respectively; control activities of salt-washed preparations were obtained by assay in the presence of 10 mM $CaCl_2$. Triangles and circles are for intact and salt-washed PSII membranes, respectively.

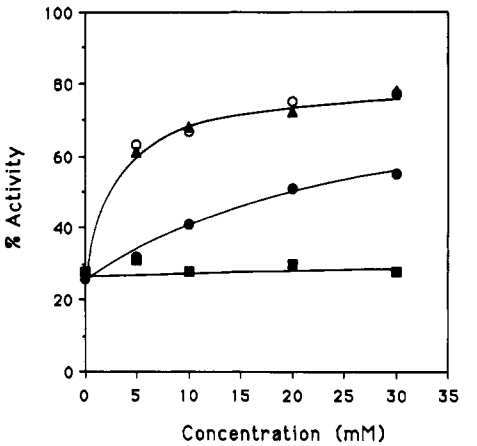


FIGURE 2: Effect of divalent metals on NH_2OH inhibition of salt-washed PSII membranes. Samples of salt-washed PSII membranes were incubated in 0.1 mM NH_2OH for 3 min with the concentrations of metal ions shown. Assay conditions and control activities (100%) are as indicated in Figure 1. The counteranion Cl^- was the same in all additions of metal ions. Open circles are for $CaCl_2$, triangles for $CdCl_2$, solid circles for $SrCl_2$, and squares for $MgCl_2$.

mM MES buffer (pH 6.0) and 0.31 mM 2,6-dichloro-*p*-benzoquinone as the acceptor. In some experiments, the NH_2OH-exposed samples were diluted 160-fold in the assay cuvette and incubated in the dark for 30 s in the presence or absence of Ca^{2+} or with EDTA prior to illumination.

The effects of NH_2OH and Ca^{2+} on Mn reduction were monitored at room temperature by using a Bruker ER-200D EPR spectrometer operated at X-band (Yocum et al., 1981). The spectrometer was fitted with a TM cavity accommodating a large aqueous flatcell; concentrations of aqueous Mn^{2+} as low as 2 μM can be detected by this arrangement. A linear relationship between the amplitude of EPR signals and the concentration of Mn^{2+} was established by measuring the hexaaquo six-line signals (using the intensity of the third line from the low-field side) in a series of $MnCl_2$ solutions (2–60 μM). The standard curve was used to determine the amount of Mn^{2+} in the samples treated with NH_2OH. In these experiments, salt-washed PSII membranes (2 mg of Chl/mL; 4 Mn/250 Chl) were incubated with Ca^{2+} and NH_2OH and centrifuged to separate supernatants from membranes. The pellets were resuspended in 50 mM MES buffer (pH 6.0). Both supernatants and resuspended pellets were scanned for EPR signals. Loss of Mn from material diluted 40-fold in 50 mM MES (pH 6), containing 10 mM $CaCl_2$ or 2 mM EDTA, and resuspended in the same buffers after centrifugation (40000*g*, 6 min) was assessed by acidifying the resuspended pellets to pH 1 to release residual Mn as Mn^{2+}.

RESULTS

Figure 1 shows the effect of increasing concentrations of Ca^{2+} on NH_2OH inactivation of O_2 evolution in intact and salt-washed PSII membranes. In this experiment, the membrane suspensions containing the indicated amounts of Ca^{2+} were exposed to NH_2OH for 3 min (salt-washed membranes) or 30 min (intact membranes) in darkness and assayed as described under Materials and Methods. Because NH_2OH inhibition is enhanced by removal of the extrinsic polypeptides (Ghanotakis et al., 1984d; Tamura & Cheniae, 1985), 0.1 and 1 mM NH_2OH were used with the salt-washed and intact preparations, respectively. Under these conditions, the data of Figure 1 show that Ca^{2+} can confer protection against NH_2OH inactivation of O_2 evolution only in salt-washed membranes. This result suggests that in order for Ca^{2+} to affect the inhibitory action of NH_2OH, the added metal must be able to equilibrate rapidly with a site that is screened by the presence of the extrinsic polypeptides (Ghanotakis et al., 1984c; Ono & Inoue, 1988).

The experiments shown in Figure 2 compare the protective effects of several divalent cations on the NH_2OH inactivation process in salt-washed PSII membranes. These data show that Cd^{2+} and Sr^{2+} are also effective in protecting the O_2-evolving reaction against NH_2OH inhibition. The protective effect conferred by Cd^{2+}, a divalent cation that does not activate O_2 evolution but competes for the Ca^{2+} site (Waggoner & Yocum, 1990) and blocks formation of the S_2 EPR signal (Ono & Inoue, 1989b), is about the same as that observed for Ca^{2+}. Strontium, whose ionic radius is larger (1.13 Å) than that of Ca^{2+} or Cd^{2+} (1.00 Å), is also effective, but only at higher concentrations than the divalent metals of smaller ionic radii. Other divalent metals [Mg^{2+}, as well as Mn^{2+} (not shown)] of smaller radii are ineffective in alleviating the normal course of NH_2OH inhibition. These data indicate that the ability of metal ions to affect NH_2OH inhibition of O_2 evolution is limited to those species that are at present known to compete for the Ca^{2+} site in PSII, regardless of whether they activate O_2 evolution activity. We have also tested monovalent cations such as Na^+, which are weak competitive inhibitors of activity (Waggoner et al., 1989); no protective effect was found (data not shown). Taken together, the results just described suggest that ionic radius, charge, and specificity for interaction with the Ca^{2+} site in PSII are the factors that determine whether a metal can slow the rate of NH_2OH inhibition of PSII.

In the experiments shown in Figure 2, the Cl^- concentrations were the same; Cl^- has no effect, at the highest concentrations examined (100 mM), on NH_2OH inhibition in salt-washed PSII membranes (Mei & Yocum, 1990). This finding contrasts with the results of Beck and Brudvig (1988), who showed that Cl^- retarded Mn reduction by N-methylated hydroxylamines and proposed that NH_2OH-mediated Mn reduction occurs through a Cl^--sensitive site. It should be noted, however, that the latter investigations utilized native PSII mem-

Table I: Effect of Ca^{2+} on the Rate of NH_2OH Inactivation of O_2 Evolution Activity[a]

$Ca(MES)_2$ concn (mM)	k_{obs} (min^{-1})	$Ca(MES)_2$ concn (mM)	k_{obs} (min^{-1})
0	0.40	1.00	0.16
0.05	0.24	5.00	0.10
0.15	0.22	10.00	0.05
0.50	0.18		

[a] Samples (2 mg of Chl/mL) of salt-washed PSII membranes were incubated with the indicated Ca^{2+} concentrations for 30 s, after which 0.1 mM NH_2OH was added to initiate the inhibition process. The observed rate constants are the averages from three experiments.

branes rather than polypeptide-depleted material; we are conducting further investigations on the origin of this apparent discrepancy.

The results of experiments examine the effect of varied concentrations of Ca^{2+} on the rate of NH_2OH inhibition in salt-washed PSII membranes are presented in Table I. The NH_2OH inactivation reaction, carried out in the dark except for brief (<10 s) exposures to very dim light during sample withdrawals for assay, obeys the same type of pseudo-first-order kinetics observed earlier with thylakoid membranes (Cheniae & Martin, 1971; Sharp & Yocum, 1981). As shown in Table I, a decrease in the rate constant for NH_2OH inhibition is observed at the lowest Ca^{2+} concentration tested (50 μM), and further decreases in k_{obs} are observed up to 10 mM Ca^{2+}. The nature of the interaction between NH_2OH and Ca^{2+} was also examined in the presence of varied concentrations of NH_2OH and Ca^{2+}; the time of exposure to NH_2OH was the same (3 min). Double-reciprocal plot analyses of the results indicated that Ca^{2+} is noncompetitive with respect to NH_2OH; an estimated Ca^{2+} K_M of about 2.5 mM was determined from these experiments (data not shown). Therefore, Ca^{2+} does not interfere directly with the reaction between NH_2OH and the O_2-evolving complex by competing with NH_2OH for a site at or near Mn, nor does Ca^{2+} slow inhibition by binding NH_2OH.

In the preceding experiments, Ca^{2+} was incubated with salt-washed PSII membranes prior to NH_2OH addition. To determine whether Ca^{2+} could affect NH_2OH inhibition if the metal was added after the reductant, an order-of-addition experiment was carried out in which membranes were preincubated with Ca^{2+}, followed by NH_2OH addition, or alternatively incubated with NH_2OH followed by addition of Ca^{2+}. The time between first and second additions to the incubation mixture was fixed at 30 s. The data (Figure 3) show that Ca^{2+} must be present prior to NH_2OH in order to affect NH_2OH inhibition; addition of the metal cannot affect the rate of inhibition once it has been initiated.

Previous investigations on NH_2OH inhibition of O_2 evolution activity have shown that the inhibitory process correlates with the appearance of loosely bound Mn^{2+} that can be detected by water proton relaxation enhancements (Sharp & Yocum, 1981) or by the appearance of EPR-detectable Mn^{2+} six-line spectra (Yocum et al., 1981). Room temperature EPR was utilized to determine in more detail whether Ca^{2+} interferes with reduction of Mn, or whether Mn^{2+}, created by the NH_2OH reaction, is retained by Ca^{2+}-supplemented PSII membranes. Figure 4 presents the results of Mn^{2+} analyses by EPR from an order-of-addition experiment similar to that described in Figure 3. The spectra shown are from supernatants after 10-min centrifugation of samples exposed to Ca^{2+} before (spectrum A) or after (spectrum B) treatment with NH_2OH for 3 min. As noted in the legend to Figure 4, substantial amounts of EPR-detectable Mn^{2+} remain associated with the pellet exposed to Ca^{2+} prior to NH_2OH treatment, and this pellet retains greater amounts of residual O_2 evolution activity than the pelleted membranes incubated with Ca^{2+} after NH_2OH exposure.

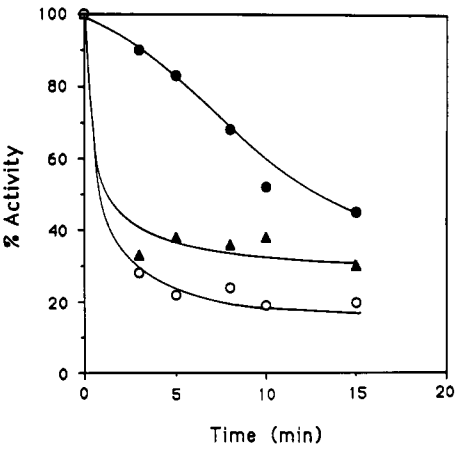


FIGURE 3: Effect of the order of addition of Ca^{2+} and NH_2OH on inhibition of oxygen evolution activity. Samples of salt-washed PSII membranes were incubated in 0.1 mM NH_2OH for the times shown. Assay conditions and control activity are as indicated in Figure 1. Solid circles are for 10 mM Ca^{2+} added 30 s before NH_2OH, triangles for 10 mM Ca^{2+} added 30 s after NH_2OH, and open circles for no Ca^{2+} addition.

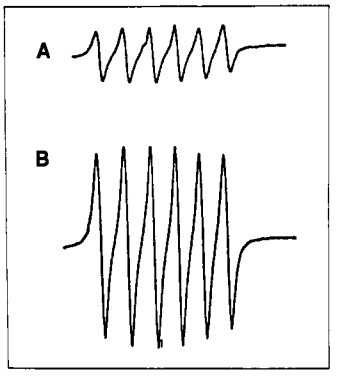


FIGURE 4: Effect of Ca^{2+} addition on Mn^{2+} release from salt-washed PSII membranes caused by NH_2OH exposure. Hexaaquo Mn^{2+} six-line spectra from supernatants produced by 10 min of centrifugation after 3-min incubation with 0.1 mM NH_2OH. Spectrum A: 10 mM Ca^{2+} added before NH_2OH incubation for 3 min. Spectrum B: 10 mM Ca^{2+} added after NH_2OH incubation for 3 min. The Mn^{2+} concentrations were (spectrum A) 8 μM and (spectrum B) 22 μM; The corresponding pellets contained 12 (A) and 3 (B) μM Mn^{2+}, respectively, that was EPR-detectable, with accompanying residual rates of O_2 evolution of 310 (A) and 150 (B) μmol h^{-1} (mg of Chl)$^{-1}$. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 Gpp; gain, 2×10^5; time constant, 100 ms; sweep time, 100 s; sweep width, 1000 G. Other conditions are given under Materials and Methods.

In order to determine whether membrane-associated Mn^{2+} correlates with O_2 evolution activity, further experiments were carried out in which salt-washed PSII membranes were incubated with Ca^{2+} and NH_2OH, diluted 40-fold with buffered $CaCl_2$ or EDTA to terminate NH_2OH inhibition, centifuged, and resuspended in the dilution buffers. Table II summarizes the results from this experiment, which show that in contrast to the sample with Ca^{2+}, EDTA exposure causes a concurrent loss of both Mn and activity. Table III summarizes the results of an experiment to determine whether the presence of Ca^{2+} during the NH_2OH incubation step decreases the rate of formation of EDTA-labile activity shown in Table II. The results of Table III were obtained by first incubating samples with Ca^{2+} and NH_2OH for various times and then diluting

Table II: Effect of EDTA Treatment on Mn^{2+} Retention in Ca^{2+}/NH_2OH-Treated PSII Membranes[a]

treatment	EPR-detectable Mn^{2+} after acidification (μM)	O_2 evolution act. [μmol of O_2 h^{-1} (mg of Chl)$^{-1}$]
none	36 (100)	465 (100)
diluted/resuspended in 10 mM $CaCl_2$	33 (92)	414 (91)
diluted/resuspended in 2 mM EDTA	15 (42)	160 (35)

[a] Salt-washed PSII membranes (2 mg of Chl/mL) containing 10 mM $CaCl_2$ were exposed to 0.1 mM NH_2OH for 3 min, diluted 40-fold with 50 mM MES buffer (pH 6) containing 10 mM $CaCl_2$ or 2 mM EDTA, and centrifuged for 6 min at 40000g. The resulting pellets were resuspended in the same buffer systems and assayed for activity. Following assay, the samples were acidified with HCl to pH 1, and Mn^{2+} was quantified as described under Materials and Methods. The values in parentheses are the percent of control. A small (3 μM) amount of Mn^{2+} was detected prior to acidification of the sample resuspended in $CaCl_2$ (data not shown).

Table III: Effects of Ca^{2+} and EDTA on O_2 Evolution Stability Following Exposure of PSII to Ca^{2+} and NH_2OH[a]

incubn time with Ca^{2+} + NH_2OH (min)	[μmol of O_2 h^{-1} (mg of Chl)$^{-1}$] act. after dilution and 30-s incubn with: 10 mM $CaCl_2$	no addition	200 μM EDTA
0	445 (100)	445 (100)	449 (100)
1	431 (96)	274 (62)	107 (24)
3	398 (87)	211 (47)	94 (21)
5	338 (75)	169 (38)	80 (18)
10	265 (59)	125 (28)	73 (16)

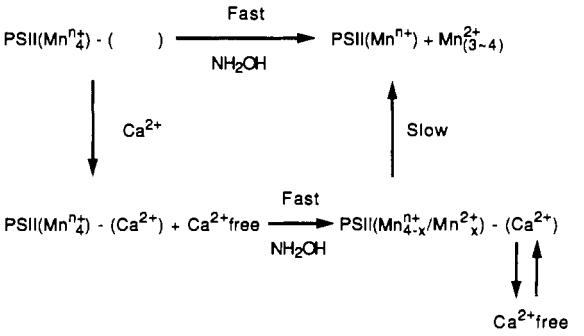
[a] Prior to assay, salt-washed PSII membranes (2 mg of Chl/mL) were incubated with 10 mM Ca^{2+} and 0.1 mM NH_2OH for the times shown, diluted 160-fold in 50 mM MES, pH 6, and incubated in the dark for a further 30 s with the additions shown plus DCBQ, after which $CaCl_2$ was added as necessary for assay of O_2 evolution activity. The rates are the averages of three separate experiments; the percent of control rates is given in parentheses.

aliquots 160-fold in the O_2 assay cuvette with the additions shown. After a second 30-s incubation in the dark, activity was assayed. As can be seen from the data, aliquots diluted in the presence of Ca^{2+} for the second incubation step retain activity, some of which is lost if the metal is omitted during incubation. A 30-s exposure to EDTA after NH_2OH treatment, however, creates a very strong inhibition of activity, evident after 1 min of exposure to NH_2OH. This indicates that the presence of Ca^{2+} during NH_2OH incubation has not interfered with formation of labile Mn^{2+}, even though it facilitates retention of activity by these salt-washed PSII membranes.

Discussion

Previous investigations (Sharp & Yocum, 1981; Ghanotakis et al., 1984c) have shown that reductant-mediated inhibition of O_2 evolution activity is slowed or prevented by light, presumably through a mechanism that involves photooxidation of Mn^{2+} created by the reductants. The results presented here show that the rate of NH_2OH inhibition of O_2 evolution activity in the dark can also be slowed, but not prevented, by Ca^{2+} addition to salt-washed PSII membranes. This effect on NH_2OH inhibition requires removal of the extrinsic 23- and 17-kDa proteins (Figure 1), in agreement with other results demonstrating that extrinsic proteins can impede rapid access of Ca^{2+} to its site in PSII (Ghanotakis et al., 1984c; Ono & Inoue, 1988). It is therefore likely that the Ca^{2+} site that affects the rate of NH_2OH inhibition is situated at a site topologically similar to the Ca^{2+} site that activates O_2 evolution

Scheme I: Mechanism for Ca^{2+}-Induced Slowing of the Rate of NH_2OH Inhibition of O_2 Evolution[a]



[a] See Discussion in the text for further details.

activity. The data of Figure 2 show that other metals (Sr^{2+}, Cd^{2+}) that retard NH_2OH inhibition are species that compete effectively for the Ca^{2+} site that activates O_2 evolution while the data of Table I show that the range of Ca^{2+} concentrations that slow inhibition is similar to the concentrations that activate O_2 evolution activity (Boussac et al., 1985; Cammarata & Cheniae, 1987; Ghanotakis et al., 1984b; Homann, 1988). Taken together, these results suggest that the Ca^{2+} site responsible for the effect on NH_2OH inhibition exhibits the same metal ion specificity as the site that activates O_2 evolution activity. The decrease in the apparent affinity of PSII for Ca^{2+} in the dark (K_M = 2.5 mM; not shown) with NH_2OH and the inability of the metal to slow inhibition if it is added to incubation mixtures after NH_2OH would suggest that the Ca^{2+} site is modified by reduction of higher oxidation states of Mn to Mn^{2+} and/or that the site has a lower Ca^{2+} affinity in dark-adapted PSII. These proposals are consistent with other results showing that exposure of PSII to NH_2OH decreases the binding affinity of Ca^{2+} (Tamura & Cheniae, 1988; Tamura et al., 1989); the suggestion that a Ca^{2+} site is associated with S_1 is consistent with data (Boussac & Rutherford, 1988c) showing that the metal is most easily released from PSII in S_3, rather than from lower S states.

A number of reductants, including NH_2OH, react effectively with higher oxidation states of Mn to produce Mn^{2+} (Davies, 1969). As Figure 4 and Tables II and III show, exposure of Ca^{2+}-supplemented PSII membranes to NH_2OH induces EDTA-sensitive activity, consistent with the creation of a population of extractable Mn. On the basis of our EPR data (Figure 4; Table II) as well as previous findings (Cheniae & Martin, 1971; Sharp & Yocum, 1981; Yocum et al., 1981), we would conclude that the EDTA-extractable species is most likely Mn^{2+}. The data of Table III further indicate that the rate of formation of the labile pool of Mn^{2+} by NH_2OH is not affected by the presence of Ca^{2+} under our incubation and assay conditions. The most probable explanation for retention of activity in Ca^{2+}/NH_2OH-exposed material containing EDTA-sensitive Mn is that Ca^{2+} creates a stable environment within the oxygen-evolving complex that promotes retention of Mn^{2+}. This hypothesis is illustrated by Scheme I.

The capacity of PSII membrane preparations to bind Mn^{2+} in an EPR-silent form (Hoganson et al., 1989) precludes an accurate assessment of the amount of Mn^{2+} in our samples. In concentrated samples subjected to long-term (10 min) centrifugation, a minimal estimate of Mn^{2+} retention is provided in the legend to Figure 4. Other investigations have shown that 3–4 Mn/reaction center are removed from thylakoids and PSII membranes (Yocum et al., 1981; Ghanotakis et al., 1884d; Tamura & Cheniae, 1985), and our results, obtained by EDTA treatment of Ca^{2+}/NH_2OH-incubated

samples, are not substantially different from those estimates. For example, the EDTA-treated sample of Table II retaining 42% Mn but only 35% O_2 evolution activity of the control appears to have lost between 3 and 4 Mn/reaction center. At the same time, it is unlikely that PSII membranes containing EDTA-labile Mn^{2+} have been reduced to the level of the so-called S_{-1} state of the O_2-evolving enzyme system. Other EPR studies employing NH_2OH and its derivatives to reduce Mn failed to detect Mn^{2+} associated with S_{-1} (Beck & Brudvig, 1988a). It is more probable that Ca^{2+}/NH_2OH-treated samples represent a transiently stable form of an even lower oxidation state of the O_2-evolving complex, such as S_{-3}, a labile species proposed by Beck and Brudvig (1987) to be formed by the action of hydroxylamines as an intermediate preceding inactivation of the O_2-evolving complex.

Our results demonstrating the ability of Ca^{2+} to stabilize PSII preparations containing Mn^{2+} would indicate that binding of the former species is contributing to the structural organization of the oxygen-evolving complex. Such a function of Ca^{2+} in PSII is in accord with the involvement of the metal in stabilizing the structures and catalytic activities of a number of enzymes (Einspahr & Bugg, 1984). Our results confirming the observations of Tamura and Cheniae (1988), namely, that the integrity of the Mn atoms in PSII influences the affinity of Ca^{2+} for its site, suggest that the existence of a complex interaction between these two metals is ultimately responsible for the active form of the O_2-evolving complex. Since it has also been proposed (Rutherford, 1989) that Ca^{2+} may ligate the H_2O utilized as substrate for O_2 evolution, it is possible that this metal is critical for both structural organization and substrate binding at the active site of the O_2-evolving enzyme.

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Stimulation of Transcript Elongation Requires both the Zinc Finger and RNA Polymerase II Binding Domains of Human TFIIS†

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ABSTRACT: The eukaryotic transcriptional factor TFIIS enhances transcript elongation by RNA polymerase II. Here we describe two functional domains in the 280 amino acid human TFIIS protein: residues within positions 100–230 are required for binding to polymerase, and residues 230–280, which form a zinc finger, are required in conjunction with the polymerase binding region for transcriptional stimulation. Interestingly, a mutant TFIIS with only the polymerase binding domain actually inhibits transcription, whereas a mutant in which the polymerase binding and zinc finger domains are separated by an octapeptide is only weakly active. The zinc finger itself has no effect on transcription, but in contrast to the wild-type protein, it binds to oligonucleotides. These findings suggest that TFIIS may interact with RNA polymerase II such that the normally masked zinc finger can specifically contact nucleotides in the transcription elongation zone at a position juxtaposed to the polymerization site.

Regulation of transcript elongation by RNA polymerase II (pol II)[1] is emerging as an important mechanism for gene control in eukaryotic cells. A number of cellular and viral genes (Bentley & Groudine, 1986; Wright & Bishop, 1989; Bender et al., 1987; Reddy & Reddy, 1989; Spencer & Groudine, 1990) are now known to be regulated at the level of transcript elongation. Specific cis-acting transcriptional blocks, located within the transcriptional unit and generated under specified cellular conditions, have been shown to mediate the control of transcript elongation in these examples (Bentley & Groudine, 1986; Reines et al., 1989). That purified pol II can preferentially recognize some of these transcriptional block sites (Diedrick et al., 1987; Kerppola & Kane, 1988) suggests that occasional read-through of such sites in vivo may be mediated by elongation factors (Reines et al., 1989). Involvement of a factor(s) that is capable of influencing elongation is further indicated by the fact that the rate of RNA synthesis by purified pol II is 20–30-fold slower than the in vivo rate of 1000 nucleotides/min. (Ucker & Yamamoto, 1984). One such elongation factor, TFIIS (initially named SII; Natori, 1982), which is capable of stimulating purified pol II transcription, has been identified in mouse (Natori et al., 1973), calf thymus (Rappaport et al., 1987), human (Reinberg & Roeder, 1987), yeast (Sawadogo et al., 1980), and *Drosophila* (Sluder et al., 1989) cell extracts.

Biochemical analysis of TFIIS indicates that it is a phosphoprotein that can stimulate pol II transcription of calf thymus DNA 2–3-fold (Sekimizu et al., 1979) and that of dC-tailed DNA 4–5-fold (SivaRaman et al., 1990). Moreover, purified TFIIS can promote read-through at specific sites within viral and cellular genes whose transcription had been initiated by defined initiation factors (Reines et al., 1989). Thus, TFIIS can stimulate transcription and promote read-through of elongation blocks.

It has been suggested that TFIIS exerts its influence on transcript elongation by binding to pol II without interacting with DNA or NTP (Reinberg & Roeder, 1987; Horikoshi et al., 1984). Further, it has been found that TFIIS binds specifically to the phosphorylated form of the C-terminal domain (CTD) of the largest subunit of pol II a/o (Sawadogo et al., 1980). The potential significance of the interaction between the CTD and TFIIS is indicated by recent data implicating involvement of the CTD in transcriptional elongation (Laybourn & Dahmus, 1989; Sawadogo & Sentenac, 1990; Corden, 1990). While the CTD may be the major contact site for TFIIS, other regions of the subunit may also be involved in TFIIS interactions based on studies with a fusion protein containing a fragment of the largest pol II subunit (Rappaport et al., 1988). Binding of TFIIS to DNA under low salt conditions has been observed previously (Sawadogo et al., 1981), but the physiological significance of this binding is unclear because TFIIS is a basic protein (p*I* 8.7).

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[1] Abbreviations: pol II, RNA polymerase II; TFIIS, transcript elongation factor (or SII); CTD, C-terminal domain of the RNA polymerase II largest subunit; cDNA, complementary DNA; Δ, indicates deletion; ∇, indicates insertion; T7, T7 phage RNA polymerase; BSA, bovine serum albumin; EXAFS, extended X-ray absorption fine structure; dsDNA, double-stranded DNA; ssDNA or ssRNA, single-stranded DNA or RNA; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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