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Stoichiometry, inhibitor sensitivity, and organization of manganese associated with photosynthetic oxygen evolution

(photosynthesis/electron paramagnetic resonance)

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ABSTRACT **Chloroplast thylakoid membranes isolated in the presence of EDTA retain high rates of O_2 evolution (≥340 μmol·h^{-1}·mg chlorophyll^{-1}) but contain no Mn^{2+} that is detectable by electron paramagnetic resonance (EPR) at room temperature. The total Mn^{2+} content of these preparations is 4.6 per 400 chlorophylls; 0.6 Mn^{2+} can be released by addition of Ca^{2+}, a treatment that does not affect O_2 evolution. The remaining Mn^{2+} (4 per 400 chlorophylls) appears to be functionally associated with O_2 evolution activity. Inhibition by Tris, NH_2OH, or heat will release a small fraction of Mn^{2+} from these membranes (≈25% with Tris, for example). Addition of Ca^{2+} further enhances Mn^{2+} release so that for Tris and for NH_2OH, 2 and 3, respectively, Mn^{2+} per 400 chlorophylls are extracted from the O_2-evolving complex. Based on the microwave power-saturation properties of the EPR signal IIf, which arises from an intermediate electron carrier in the water splitting process, it appears that one of the four Mn^{2+} associated with photosystem II is uniquely sensitive to Tris. A new model is proposed for the organization and inhibitor sensitivity of manganese in the O_2-evolving complex.**

A substantial body of evidence (for review, see ref. 1) supports a role for manganese in the catalysis of O_2 evolution by photosystem II. Attempts to quantify the Mn^{2+} associated with isolated chloroplast tylakoid membranes have produced estimates that vary widely (2), although there is general agreement that several populations of the ion exist, with at least two of these populations presumed to be functional in the O_2-evolving reaction (1). One approach to the assessment of manganese function in O_2 evolution has been to use magnetic resonance techniques to measure spin–spin and spin–lattice relaxation times (T_2^{-1}, T_1^{-1}) in NMR experiments or the characteristic six-line hyperfine spectrum of Mn^{2+} in EPR experiments. Both magnetic resonance techniques have detected changes in chloroplast-associated manganese on continuous or short-flash illumination (3, 4); these data have been taken as evidence for light-induced valence-state changes of manganese functional in the water splitting process. This interpretation has been challenged by results showing that chloroplast thylakoid membranes can bind significant amounts of manganese during chloroplast isolation, which is not involved in the O_2-evolving complex. Robinson *et al.* (5) have shown that this adventitious Mn^{2+} can be converted to higher oxidation states by O_2^- generated by photosystem I, an effect that leads to intensity changes in Mn^{2+} magnetic resonance signals unrelated to O_2 evolution. Removal of spurious Mn^{2+}, either by divalent cation treatment (6) or with EDTA during chloroplast isolation (7, 8), abolishes room temperature changes in Mn^{2+} magnetic resonance signals, although it has recently been suggested that the ion can be detected by EPR at cryogenic temperatures (9).

An alternative method for the magnetic resonance detection of manganese functional in photosystem II was first used by Lozier *et al.* (10), who used EPR to examine that Mn^{2+} released from thylakoid membranes inhibited by chaotropic agents or Tris. Blankenship and Sauer (11) used the latter reagent to examine Mn^{2+} binding sites and their thylakoid membrane topology, while Robinson *et al.* (12) have used NMR measurements to obtain data on Mn^{2+} topology in NH_2OH-treated thylakoid membranes. In the experiments described here, we have extended the former approach by combining new techniques for the isolation of highly active thylakoid membranes devoid of loosely bound nonfunctional Mn^{2+} with recent results (13, 14) on the mode of action of Tris and NH_2OH to assess the stoichiometry and organization of manganese associated with O_2 evolution.

MATERIALS AND METHODS

Thylakoid membranes were isolated from market spinach by a variety of procedures. Salt/EDTA membranes were isolated as described (5). Sucrose/$MgCl_2$ and sucrose/EDTA chloroplasts were prepared by grinding leaves in sucrose buffer (0.4 M sucrose/20 mM Hepes, pH 7.5/15 mM NaCl)/2 mM $MgCl_2$ or 1 mM EDTA. The pellets from these initial steps were washed with sucrose buffer/1 mM EDTA and then with sucrose buffer/2 mM $MgCl_2$ and then suspended in sucrose buffer. Finally, membranes were isolated by grinding leaves in sucrose buffer, pelleting the membranes, and suspending the pellet in sucrose buffer without further washing. The final suspensions [2.4–4.5 mg of chlorophyll (Chl)/ml] were either stored at −35°C or used immediately. When assayed for activity, these preparations gave gramicidin-uncoupled rates of O_2 evolution >300 μmol·hr^{-1}·mg Chl^{-1}; one exception, the sucrose buffer-isolated and washed preparation, is below. The possibility that residual amounts of EDTA might contaminate these preparations was examined by addition of $MnCl_2$ to the membrane suspensions before and after additional washes with 150 mM NaCl/4 mM $MgCl_2$ or sucrose buffer. The levels of EPR-detectable Mn^{2+} found in these experiments indicated that the concentration of residual EDTA was <0.5 μM. For Tris inactivation, a thylakoid suspension (0.6 ml) was mixed with 0.2 ml of 3.2 M Tris, pH 8 (at 25°C), allowed to stand in room light for 20 min at 3°C, and then transferred to the EPR flat cell. When NH_2OH was the inactivating reagent, the suspension (0.6 ml) was mixed with 6 μl of a 500 mM stock solution of NH_2OH in 0.01 M HCl and then incubated in the dark for 20 min. Heat inactivation at 57°C was carried out in an EPR flat cell as described (15). Inactivation of O_2 evolution by these inhibitory treatments was assessed by the appearance of signal IIf or by direct assay of O_2 evolution activity (or both).

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Abbreviation: Chl, chlorophyll.

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EPR measurements were carried out with a Bruker model ER200D spectrometer operated at X band (9.43 GHz) with 100 KHz modulation; a Varian TM_{110} mode cavity (model E238) was fitted to the microwave bridge of the instrument by using appropriately modified waveguide and impedance matching circuitry. Except where noted, the microwave power was 100 mW and the modulation amplitude was 9.5 G as determined by the procedure of Poole (16); samples were contained in a Scanco quartz TM aqueous flat cell (model S-813). To ensure reproducibility, the flat cell orientation and depth of insertion into the cavity were maintained constant for each experiment.

To use EPR for the quantitation of Mn^{2+}, a standard curve for the ion was constructed for each of the experimental conditions used. The points on these curves were determined from the peak-to-trough amplitude of the third hyperfine line (low-field side of $g = 2.0$; see Fig. 1) and represented the averages of amplitudes obtained for stock solutions of $MnCl_2$ in sucrose buffer, 0.5 M HCl, H_2O, or sucrose buffer/Tris. For stock $MnCl_2$ solutions in 0.8 M Tris/sucrose buffer, the six-line amplitudes were decreased to approximately one-half the intensity observed in sucrose buffer or in 0.5 M HCl. The curves were linear from 2.5 to 100 μM $MnCl_2$ and extrapolated to zero at $[Mn^{2+}] = 0$. For determination of total Mn^{2+} concentration, thylakoid suspensions were acidified with HCl to a final concentration of 0.5 M (50 mM Ca^{2+} was also present); higher concentrations of HCl produced no further increase in the amplitude of the six-line spectrum. Quantitation of Mn^{2+} by this EPR procedure produced values estimated to be within an error limit of 10%. The acidification/EPR procedure for total chloroplast manganese quantitation gave results in agreement with those obtained by neutron activation analysis for chloroplast manganese (unpublished observations). Since Tris inactivation in these experiments was carried out in the light, it is possible that some Mn would escape detection as the Mn^{2+} form, owing to the possible existence of stable higher oxidation states of manganese. To test this possibility thylakoid membranes were first exposed to Tris (pH 8.4) at 0°C and then to H_2O_2 at pH 6.0, a procedure that reduces MnO_2 to Mn^{2+} (15). No increase in the intensity of EPR signals from hexaquo Mn^{2+} was observed when H_2O_2 was added after Tris inactivatioin in the presence of Ca^{2+}. Measurements of signal IIf were carried out as described (17).

RESULTS

Stoichiometry and Binding of Mn^{2+} to Thylakoid Membranes. EPR spectra of chloroplast thylakoid membranes that represent the extremes of Mn^{2+} content observed in these investigations are shown in Fig. 1. Although both preparations are capable of high rates of O_2 evolution, the unperturbed sucrose buffer preparation, from which EDTA and washing were omitted, contains EPR-detectable Mn^{2+}; Ca^{2+} releases more Mn^{2+} and acidification shows that the total Mn^{2+} content exceeds that of the salt/EDTA membranes by a factor of 3. These data provide evidence for the existence of EPR-detectable Mn^{2+} that is not associated with photosystem II activity and may be either partially or completely displaced by Ca^{2+} without affecting O_2 evolution. The properties of several thylakoid membrane preparations with regard to their Mn^{2+} content are summarized in Table 1. The preparations in which 1 mM EDTA was present during leaf homogenization show levels of Mn^{2+} of <5 per 400 Chl, and a Ca^{2+}-displaceable fraction of the ion to which we assign a nonfunctional role based on the fact that Ca^{2+} does not inhibit oxygen evolution. Note that repeated exposure to sucrose buffer produces membranes that have a lower Mn^{2+} content and that 25% of the ion may be displaced by Ca^{2+}. These preparations are the least active in O_2 evolution (100–125 μmol of $O_2 \cdot hr^{-1} \cdot mg$ of Chl^{-1}), a result that suggests that this isolation method has removed both functional and nonfunctional Mn^{2+}. Based on the data of Table 1, we conclude that the Mn^{2+} content

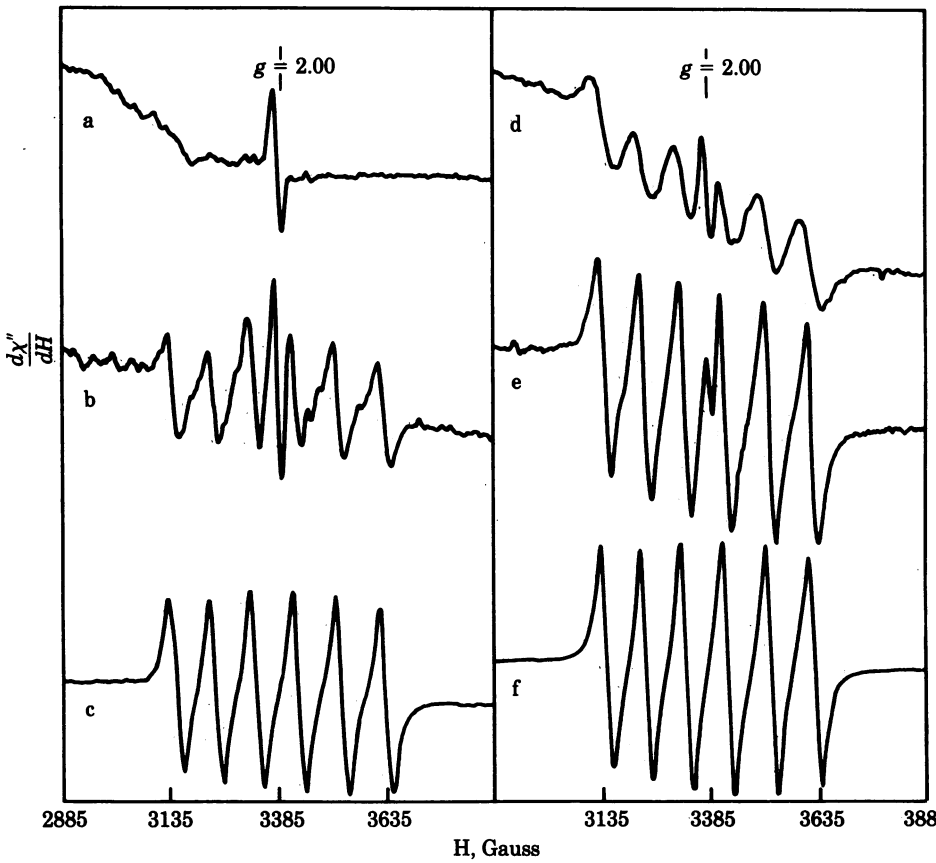

FIG. 1. (*Left*) Salt/EDTA chloroplasts (3 mg of Chl/ml). Curves: a, no additions, <0.1 Mn^{2+}/400 Chl; b, added 40 mM Ca^{2+}, 0.6 Mn^{2+}/400 Chl; c, added 40 mM Ca^{2+}/0.5 M HCl, 4.6 Mn^{2+}/400 Chl; gain settings were 5, 10, and 2.5 × 10^5 for a, b, and c, respectively. (*Right*) Sucrose chloroplasts (3.99 mg of Chl/ml). Curves: d, no additions, 0.6 Mn^{2+}/400 Chl; e, added 40 mM Ca^{2+}, 2.5 Mn^{2+}/400 Chl; f, added 40 mM Ca^{2+}/0.5 M HCl, 13.4 Mn^{2+}/400 Chl; gain settings were 5, 4, and 0.8 × 10^5 for d, e, and f, respectively.

Table 1. EPR-detectable Mn^{2+} associated with various thylakoid membrane preparations: Effect of Ca^{2+} addition or of acidification

Membrane preparation		EPR-detectable Mn^{2+} per 400 Chl		
Homogenization mixture	Wash solution(s)	No treatment	40 mM Ca^{2+}	0.5 M HCl/ 40 mM Ca^{2+}
0.4 M NaCl/EDTA	150 mM NaCl/4 mM $MgCl_2$	0	0.4	4.6
Sucrose buffer/EDTA	Sucrose buffer/EDTA, sucrose buffer/$MgCl_2$	0	0.3	4.9
Sucrose buffer/$MgCl_2$	Sucrose buffer/EDTA, sucrose buffer/$MgCl_2$	0	0.4	5.9
Sucrose buffer	Sucrose buffer, sucrose buffer	0.3	1.0	3.9
Sucrose buffer	None	0.6	2.5	13.4

Chloroplast membranes were isolated from different batches of spinach leaves; where present, EDTA was 1 mM.

of highly active O_2-evolving thylakoid membranes is <5 Mn^{2+} per 400 Chl; the majority of this Mn^{2+} is functional in O_2 evolution and resides at an EPR-silent binding site or sites.

Characterization of Photosystem II Mn^{2+}: Action of Inhibitors of Oxygen Evolution. In view of the fact that salt/EDTA thylakoid membranes are minimally contaminated by nonfunctional Mn^{2+} (0.4–0.6 per 400 Chl) while retaining high rates of O_2 evolution activity (≥340 μmol·hr^{-1}·mg of Chl^{-1}), experiments were undertaken to determine whether any of the Mn^{2+} functional in O_2 evolution could be converted to an EPR-detectable form by inhibitory treatments that destroy O_2 production. In Table 2 we show that only exposure to Tris in the light will produce EPR-detectable Mn^{2+} in amounts exceeding those detected by a noninhibitory treatment (exposure to Ca^{2+}). We present here, for comparison, results with unwashed membranes, in which substantial amounts of Mn^{2+} are detected after inhibitory treatment. These data suggest that only a small fraction of the Mn^{2+} functional in O_2 evolution is released into an EPR-detectable form after inhibition of O_2 evolution and that Mn^{2+} from other binding sites may be released by the inhibitors. We confirmed this latter possibility by carrying out experiments (data not shown) that showed that both Tris and NH_2OH released intentionally added Mn^{2+} from salt/EDTA membranes; a similar observation was made by Blankenship and Sauer (11) for Tris.

To examine further the properties of functional Mn^{2+} after Tris or NH_2OH treatment, we sought to convert the photosystem II-associated ion into an EPR-detectable form. In thylakoid membrane suspensions treated with NH_2OH (in the dark) or Tris (in the light), addition of 40 mM Ca^{2+} produced a substantial increase in the amount of EPR-detectable Mn^{2+} (Fig. 2). Amounts of Ca^{2+} >40 mM produced no further increase in the six-line amplitudes of hexaquo Mn^{2+}.

To quantify the extent of Mn^{2+} released by these inhibitors, we used both the Ca^{2+}-release technique and centrifugation-washing procedures. Representative data from these experiments are given in Table 3 and show that Tris/Ca^{2+} releases two and NH_2OH/Ca^{2+} releases three of the four Mn^{2+} associated with the O_2-evolving reaction. These findings suggest that the manganese atoms associated with the O_2-evolving complex behave in different ways on inhibition of the water splitting process and that the specific pattern of manganese perturbation is a function of the inhibitory treatment.

Because Tris inhibits most effectively in the light (13) while NH_2OH requires darkness for maximum inhibition (14) the data above suggest that one of the four Mn^{2+} in the O_2-evolving complex may be uniquely susceptible to attack by Tris in the light. This possibility was further explored by investigating the power-saturation properties of Z^+ the oxidized form of the donor to $P680^+$ (18, 19), in NH_2OH- and Tris-inhibited thylakoid membranes. Warden *et al.* (20) have shown that the EPR signal from Z^+, referred to as signal IIvf in O_2-evolving chloroplasts, is difficult to saturate in unperturbed chloroplast preparations but that signal IIf, arising from Z^+ in inhibited preparations, saturates at 20 mW. We have extended these observations by showing that the power saturation of Z^+ is sensitive to the extent to which Mn^{2+} functional in photosystem II has been perturbed (21). The results of an experiment to determine the power saturation properties of signal IIf in NH_2OH/EDTA-washed thylakoid membranes are shown in Fig. 3; this result is compared with our data for Tris (21) in Table 4. Both NH_2OH/EDTA and Tris treatments, where either three or one Mn^{2+} have been

Table 2. Quantitation of Mn^{2+} released from thylakoid membranes by Ca^{2+} and by treatments that inactivate O_2 evolution

Treatment	EPR-detectable Mn^{2+} per 400 Chl	
	Salt/EDTA membranes	Unwashed sucrose buffer membranes
None	0	0.6
50 mM Ca^{2+}	0.4	2.5
0.8 M Tris/light	1.2	3.0
5 mM NH_2OH	0.4	1.1
Heat (57°C) for 2 min	0.4	0.9

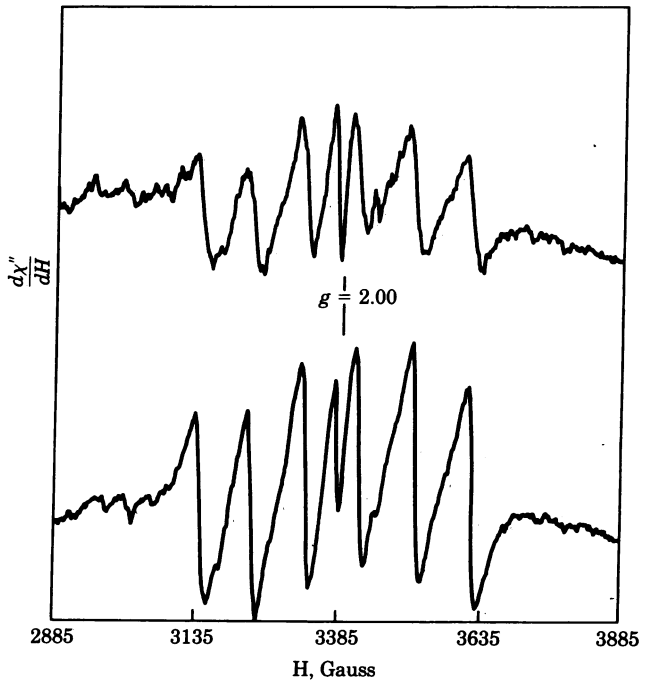

FIG. 2. EPR spectra of thylakoid membranes (3.0 mg Chl/ml) exposed to Tris (curve a; 1.2 Mn^{2+}/400 Chl) or to Tris/40 mM Ca^{2+} (curve b; 2.7 Mn^{2+}/400 Chl). The gain settings were 10 × 10^5.

Table 3. Effects of Ca^{2+} or washing on EPR-detectable Mn^{2+} in Tris- and NH_2OH-inactivated thylakoid membranes

Treatment	EPR-detectable Mn^{2+} per 400 Chl
0.8 M Tris/light	1.2
0.8 M Tris/light, 40 mM Ca^{2+}	1.9*
5 mM NH_2OH	0.4
5 mM NH_2OH, 40 mM Ca^{2+}	3.0*
Tris/EDTA/light, sucross buffer, acid	2.0
5 mM NH_2OH/EDTA, sucrose buffer, acid	1.2

* Corrected for Mn^{2+} released by Ca^{2+} in the absence of Tris or of NH_2OH.

released from photosystem II, respectively, produce a condition whereby signal IIf saturates at a microwave power of ≈39 mW. However, when a second Mn^{2+} is released with Ca^{2+} (or by washing with EDTA) from Tris-inhibited membranes, the microwave power required to saturate IIf is decreased to 20 mW.

DISCUSSION

Two results important to the understanding of manganese function in photosynthetic O_2 evolution are conveyed by these data. The first concerns the number of Mn^{2+} associated with the O_2-evolving reaction. In highly active chloroplast thylakoid membranes (350 μmol of $O_2 \cdot hr^{-1} \cdot mg\ Chl^{-1}$) prepared with EDTA to suppress the level of exogenous Mn^{2+}, the major fraction of Mn^{2+} (4 per 400 Chl) was in an EPR-silent form that was resistant to release by Ca^{2+}. The Ca^{2+}-labile fraction of Mn^{2+} in these highly active membranes (0.4–0.6 per 400 Chl) accounts for the rest of the Mn^{2+} found associated with these membranes. Based on these data and others, we conclude that four Mn^{2+} per photosystem II trap are required for optimal functioning of photosynthetic O_2 evolution. This number assumes one photosystem II trap per 400 Chl and is based on earlier studies on Mn^{2+} quantitation (2); the actual trap concentration may vary from the value used here (22).

The second finding concerns the inhibitor lability of the Ca^{2+}-insensitive Mn^{2+} associated with salt/EDTA membranes. We show (Fig. 2 and Table 3) that neither Tris nor NH_2OH inhibition of O_2 evolution under the conditions we used converts a substantial fraction (≤25%) of this Mn^{2+} into an EPR-detectable form. We also show, however, that addition of Ca^{2+} to membranes in the presence of inactivating amines increases the amount of EPR-detectable Mn^{2+}. For NH_2OH, three Mn^{2+} are released from inhibitor-sensitive sites whereas, for Tris, one Mn^{2+} is released by Tris alone and a second is released by Tris/40 mM Ca^{2+}. Washing of inhibited membranes (Table 4) with the divalent chelator EDTA produces a similar release of Mn^{2+}. The differential release of Mn^{2+} by the two inhibitors most probably reflects differences in the modes of action of NH_2OH and Tris. The fact that Tris requires light to produce an inhibition by attack at the S_2 state (13) whereas NH_2OH inhibiton appears to involve only the lowest S state (14) suggests that one of the four photosystem II-associated Mn^{2+} may be uniquely sensitive to Tris attack. This appears to be so; the data of Fig. 3 and Table 4 show that Tris/Ca^{2+} or Tris/EDTA extraction produces the low microwave-power-saturation form of signal IIf whereas $Z^{\cdot}$ in NH_2OH/EDTA-washed membranes, which retain 1 Mn^{2+} per 400 Chl, requires higher microwave-power levels for saturation (≈40 mW).

This interpretation of our EPR data—i.e., that specific Mn^{2+}–$Z^{\cdot}$ interaction controls $Z^{\cdot}$ saturation—is the most straightforward at present. However, we note that the interaction between $Z^{\cdot}$ and manganese appears to be fairly weak under all conditions thus far observed. For example, if the basis for the effect is dipole–dipole in nature, then a strong field (comparable with the applied magnetic field) at $Z^{\cdot}$ arising from manganese should result in a broadened signal IIf spectrum (23). We have not observed this predicted broadening and conclude that the physical separation between $Z^{\cdot}$ and manganese

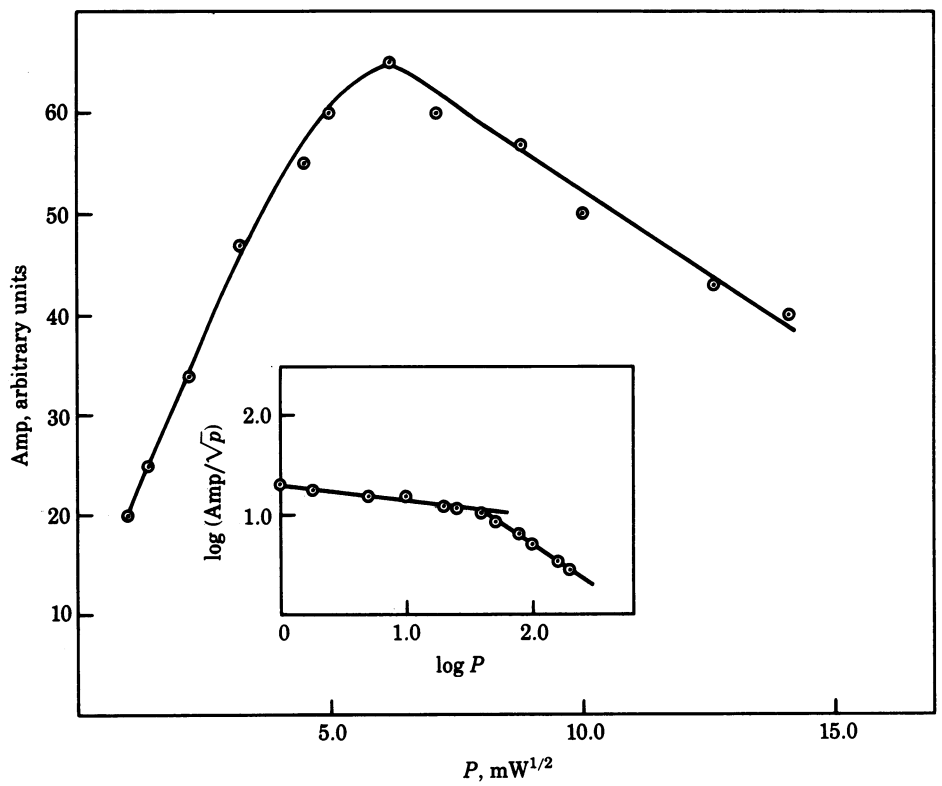

FIG. 3. Effect of microwave power (*P*) on the amplitude of signal IIf (Amp) in NH_2OH/EDTA-washed thylakoid membranes. The magnetic field was locked at signal IIs (3376 G), The Chl concentration was 3 mg/ml, the instrument time constant was 200 msec, and the modulation amplitude was 4 Gpp.

Table 4. Effect of Mn^{2+} release from thylakoid membranes on power saturation of signal IIf

Treatment	Mn^{2+} released* per 400 Chl	Power saturation, mW
NH_2OH/EDTA	3.0	39
Tris/light	1.2	39
Tris/light, 40 mM Ca^{2+}	2.0	20

* Corrected for Mn^{2+} released by Ca^{2+} alone.

is at least several Ångstroms. The saturation effects of manganese on $Z^{\ddagger}$ thus indicate that the two species are in close proximity but that intervening atoms separate the two.

Based on the data discussed above, we propose the model in Fig. 4 for the organization of the components involved on the oxidizing side of photosystem II. P680 represents the reaction center chlorophyll and Z is the associated donor that gives rise to signal IIvf or IIf on one-electron oxidation. The EPR properties of $Z^{\ddagger}$ indicate that it is an organic radical and Kohl's work with quinone model complexes provides evidence that it may be derived from a plastoquinone species (24). The question as to whether there are other donors to $P680^{+}$ under steady-state O_2-evolving conditions either in series or in parallel with Z (25, 26) is, at present, ambiguous and the model in Fig. 4 is intended to leave this question open. We note, however, that Z appears to be closely associated with P680 in that signal II is observed in photosystem II reaction center preparations (27, 28) and the intimate association of Z with manganese is supported by data reported here and elsewhere (21) that indicate that the power-saturation properties of $Z^{\ddagger}$ are correlated with perturbations of functional manganese. The association between Z and Mn may also be reflected in the decay time of $P680^{+}$. Mathis and coworkers (19) have shown that in O_2-evolving chloroplasts, P^{+}_{680} is reduced with submicrosecond kinetics whereas, in Tris- or NH_2OH-inhibited chloroplasts, this decay is extended into the microsecond range and is pH dependent. A reasonable interpretation of these data in the context of Fig. 4 is that the alteration of Z–Mn interaction induced by inhibitor treatment also affects the Z–P680 interaction. In summary, our present results indicate that four manganese, as shown in Fig. 4, are associated with each photosynthetic O_2-evolution center. Moreover, from the EPR-saturation properties of $Z^{\ddagger}$ (Table 4) it appears that two of these four are in closer physical proximity to Z and that one of these, denoted by the asterisk in Fig. 4, is uniquely susceptible to Tris attack when the O_2-evolving complex is in the S_2 state.

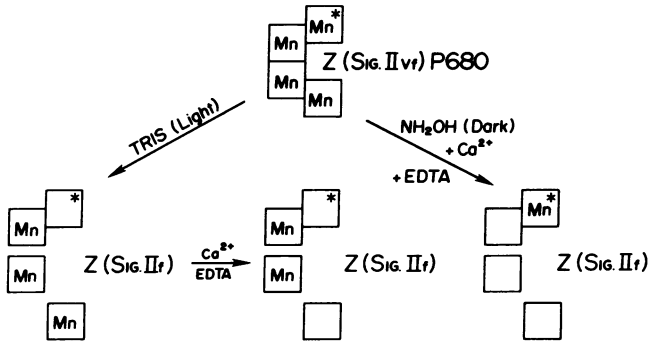

FIG. 4. Model for the organization and inhibitor sensitivity of Mn^{2+} associated with photosynthetic O_2 evolution. The model assumes one photosystem II trap per 400 Chl; the Mn denoted by (*) is the atom uniquely susceptible to attack by Tris in the light.

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