Articles

Mechanism of Irreversible Inhibition of O_2 Evolution in Photosystem II by Tris(hydroxymethyl)aminomethane†

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ABSTRACT: The dark reaction of tris(hydroxymethyl)aminomethane (Tris) with the O_2-evolving center of photosystem II (PSII) in the S_1 state causes irreversible inhibition of O_2 evolution. Similar inhibition is observed for several other amines: NH_3, CH_3NH_2, $(CH_3)_2NH$, ethanolamine, and 2-amino-2-ethyl-1,3-propanediol. In PSII membranes, both depleted of the 17- and 23-kDa polypeptides and undepleted, the rate of reaction of Tris depends inversely upon the Cl^- concentration. However, the rate of reaction of Tris is about 2-fold greater with PSII membranes depleted of the 17- and 23-kDa polypeptides than with undepleted PSII membranes. We have used low-temperature electron paramagnetic resonance (EPR) spectroscopy to study the effect of Tris on the oxidation state of the Mn complex in the O_2-evolving center, to monitor the electron-donation reactions in Tris-treated samples, and to observe any loss of the Mn complex (forming Mn^{2+} ions) after Tris treatment. We find that Tris treatment causes loss of electron-donation ability from the Mn complex at the same rate as inhibition of O_2 evolution and that Mn^{2+} ions are released. We conclude that Tris reduces the Mn complex to labile Mn^{2+} ions, without generating any kinetically stable, partially reduced intermediates, and that the reaction occurs at the Cl^--sensitive site previously characterized in studies of the reversible inhibition of O_2 evolution by amines.

The oxidation of H_2O by photosystem II (PSII)[1] occurs at the O_2-evolving center which contains a polynuclear Mn complex [reviewed by Brudvig et al. (1989)]. Light-driven charge separations at the PSII reaction center advance the O_2-evolving center through its five oxidation states S_0 through S_4, with release of an O_2 molecule occurring during the S_4 to S_0 transition. The Mn complex appears to contain four strongly exchange-coupled Mn ions (Dismukes et al., 1982; de Paula et al., 1986), which have been proposed to exist in the S_2 state as an oxo-bridged cubanelike tetramer (Brudvig & Crabtree, 1986).

A number of small molecules have been observed to interact with the O_2-evolving center, with results that include delays in the S-state cycle, inhibition of steady-state O_2 evolution, and release of the Mn complex as Mn^{2+} ions. One such molecule, whose reaction with the O_2-evolving center has been well characterized, is hydroxylamine. At high concentrations, hydroxylamine causes release of Mn^{2+} ions and inhibition of O_2 evolution (Cheniae & Martin, 1971). However, at lower concentrations, hydroxylamine merely induces a two-step delay in the S-state cycle (Velthuys & Kok, 1978) and does not inhibit steady-state O_2-evolution activity. EPR and optical spectroscopic studies have suggested that the initial reaction of hydroxylamine with the O_2-evolving center is to reduce the dark-stable S_1 state of the Mn complex to an S_{-1} state (Saygin & Witt, 1985; Beck & Brudvig, 1987). The X-ray absorption edge for Mn does not, however, exhibit a shift when hydroxylamine is added to PSII membranes in the dark, leading to the suggestion that reduction of the Mn complex only takes place upon illumination (Guiles et al., 1990). At higher concentrations, hydroxylamine further reduces the high-valent Mn complex to labile Mn^{2+} ions, which are then lost into solution (Beck & Brudvig, 1988).

The inhibition of O_2 evolution by various primary amines, particularly tris(hydroxymethyl)aminomethane (Tris), has been the subject of a great deal of study (Hind & Whittingham, 1963; Yamashita & Horio, 1968). Amines can cause reversible inhibition of O_2 evolution by binding to PSII in competition with Cl^- (Izawa et al., 1969; Sandusky & Yocum, 1983, 1984, 1986). This behavior is observed for a variety of amines including Tris, NH_3, methylamine, and 2-amino-2-ethyl-1,3-propanediol. NH_3 is also observed to bind to another site in PSII in a manner not competitive with Cl^-. This binding of NH_3 to the Cl^--independent site on the Mn complex has been shown to occur after formation of the S_2 state, resulting in an altered S_2-state multiline EPR signal (Beck et al., 1986; Britt et al., 1989). Under similar conditions, other amines do not appreciably perturb the line shape of the S_2 multiline EPR signal; instead, ammonia and methylamine cause the S_2 multiline EPR signal to be converted into the S_2 $g = 4.1$ EPR signal. This conversion is suppressed by high Cl^- concentrations (Beck & Brudvig, 1986). It therefore seems likely that reversible amine inhibition of O_2 evolution takes place at the Cl^--binding site and that only NH_3 can also bind at the Cl^--independent site located on the Mn complex.

In contrast, Tris treatment in the light has been observed to cause irreversible inhibition of O_2 evolution and loss of the Mn complex as Mn^{2+} (Yamashita & Horio, 1968; Homann, 1968; Lozier et al., 1971; Blankenship & Sauer, 1974). The flash studies of Frasch and Cheniae (1980) show that inactivation of O_2 evolution takes place much more rapidly when

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[1] Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; EPR, electron paramagnetic resonance; kDa, kilodalton(s); MES, 2-(*N*-morpholino)-ethanesulfonic acid; PSII, photosystem II; Tris, tris(hydroxymethyl)-aminomethane; Y_D, redox-active tyrosine residue giving rise to EPR signal II.

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the Mn complex is in the S_2 state than in S_1. The observation that reductants, such as reduced 2,6-dichloroindophenol, can moderate the inhibitory effect of Tris is consistent with this observation (Cheniae & Martin, 1978). Such reductants are capable of reducing S_2 to S_1, either directly or indirectly, leaving the O_2-evolving center much less susceptible to inactivation by Tris.

Nevertheless, irreversible inhibition of O_2 evolution by Tris can take place even when the Mn complex remains in the dark-stable S_1 state. This inhibition has been previously observed by Ikehara and Sugahara (1969) and Cheniae and Martin (1978), but was not extensively studied at the time. An advantage of studying the reaction of Tris with dark-adapted PSII is that only the S_1 state is present at the start of the reaction, whereas in the light there are multiple S states present simultaneously, and they are continuously undergoing transitions to form other S states, either forward or backward.

In this paper, we have used low-temperature EPR and O_2-evolution activity measurements to study the irreversible inhibition of O_2 evolution that occurs when PSII is treated with Tris in the dark. We have previously demonstrated the irreversible inhibition of O_2 evolution in PSII by treating the S_1 state with a variety of amines, including Tris, methylamine, 2-amino-2-ethyl-1,3-propanediol, and NH_3 (Beck et al., 1989). In this work, we have used EPR spectroscopy to monitor directly the state of the Mn complex and to characterize the electron-donation reactions in Tris-treated samples, using the methods of de Paula et al. (1985). Our results suggest that Tris reduces the Mn complex to a highly reduced form, containing labile Mn^{2+}, without any detectable partially reduced intermediates, and that the reaction occurs at the Cl^--sensitive site previously characterized in studies of the reversible inhibition of O_2 evolution by amines.

Experimental Procedures

Preparation of PSII Samples. O_2-Evolving PSII membranes were isolated from spinach leaves by using a modified version of the Berthold et al. (1981) procedure (Beck et al., 1985). PSII membranes were stored at 77 K as a suspension of 5–10 mg of chlorophyll/mL in a buffer containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–NaOH, pH 6.0, 15 mM NaCl, and 30% (v/v) ethylene glycol. All steps during isolation and handling of PSII membranes were performed under a dim green safe light. This allowed the membranes to remain dark-adapted, ensuring that the O_2-evolving centers were in the S_1 state prior to treatment.

Tris Treatment. Tris(hydroxymethyl)aminomethane (Tris) was used as received from Sigma. PSII samples were resuspended at 0.5 mg of chlorophyll/mL in a buffer solution containing 0.8 M Tris–H_2SO_4, pH 8.0, 15 mM NaCl, and 30% (v/v) ethylene glycol. This solution was kept at 0 °C and was stirred in the dark. Aliquots were removed during a 90-min incubation and were assayed for O_2-evolution activity or for electron-transfer properties as described below.

Polypeptide Depletion. PSII samples were resuspended to 0.5 mg of chlorophyll/mL in a buffer containing 20 mM MES–NaOH, pH 6.0, 2 M NaCl, and 30% (v/v) ethylene glycol, stirred in the dark at 0 °C for 45 min, and then spun down. The pellet was resuspended in the same buffer and immediately spun down. The membranes were then washed several times with a buffer solution containing 20 mM MES–NaOH, pH 6.0, 15 mM NaCl, and 30% (v/v) ethylene glycol.

Oxygen-Evolution Rates. O_2-Evolution rates were measured at 25 °C with a Clark electrode. All assays consisted of a suspension of PSII membranes at 2.5 μg of chlorophyll/mL in a buffer solution containing 20 mM MES–NaOH, pH 6.0, 5 mM $CaCl_2$, and 250 μM 2,5-dichloro-*p*-benzoquinone (DCBQ). Untreated PSII membrane samples typically had O_2-evolution activities of 400–500 μmol of O_2 (mg of chlorophyll)$^{-1}$ h^{-1}. The residual Tris (4 mM) in the O_2 assay was insufficient to cause any inhibition of O_2-evolution activity under these conditions. The level of Ca^{2+} present was sufficient to allow maximal O_2-evolution activity even in samples that were depleted of the extrinsic 17- and 23-kDa polypeptides.

Chloride Dependences. PSII membranes were washed several times in a buffer containing only 15 mM MES–NaOH, pH 6.0, and 30% (v/v) ethylene glycol to remove Cl^- from the solution. The samples were then resuspended to 0.5 mg of chlorophyll/mL in a buffer containing 0.8 M Tris–H_2SO_4, pH 8.0, 30% (v/v) ethylene glycol, and NaCl to bring the Cl^- concentration to that desired. The kinetics of inhibition of O_2-evolution activity by Tris treatment were then measured in these samples as described above.

Manganese Quantitation. Samples of PSII membranes were treated with Tris in the dark as described above for 90 min and then centrifuged. The resulting pellet was resuspended to 4 mg of chlorophyll/mL, mixed with an equal volume of 1 N HCl, and placed in an EPR tube. After 20-min exposure to HCl, the sample was frozen in liquid nitrogen. The EPR spectrum was measured at 15 K to quantitate the amount of Mn^{2+}.

EPR Spectroscopy. PSII samples containing 0.8 M Tris did not seem to be capable of performing a stable charge separation upon illumination, since no Fe(II)–Q_A^- EPR signal was observed. Therefore, EPR samples of Tris-treated PSII were made by removing aliquots from the Tris treatment mixture and immediately diluting them with a buffer containing 20 mM MES–NaOH, pH 6.0, 15 mM NaCl, and 30% (v/v) ethylene glycol and centrifuging the samples. The resulting pellets were resuspended in the same buffer, recentrifuged, and then resuspended to 4–6 mg of chlorophyll/mL before being loaded into EPR tubes. This procedure quenches the Tris reaction.

All EPR spectra were obtained at liquid helium temperatures, using a home-built X-band EPR spectrometer (Beck et al., 1991). Sample illuminations took place at 200–210 K, for 2 min. Difference spectra were obtained by computer subtraction of a spectrum taken immediately before illumination from the illuminated spectrum.

Results

Inhibition of Oxygen Evolution. When PSII membranes are treated with Tris in the dark, their rate of O_2 evolution decreases with time (Figure 1). A variety of other amines also cause irreversible inhibition of O_2 evolution. The rate of this inhibition was measured for a range of amines, in order to compare the effects of structural differences among the amines on the rate of inhibition, as well as to compare the rates to those observed for hydroxylamines (Table I).

The high ionic strength of the amine treatment buffer solutions raises the possibility that inhibition of O_2 evolution occurs because the 17- and 23-kDa polypeptides have been depleted as a result of the high ionic strength. However, the main effect of removing these polypeptides is to increase the Ca^{2+} requirement for O_2 evolution. If Ca^{2+} is present in higher concentrations, as it is in our O_2-evolution assays, polypeptide depletion has little effect upon O_2-evolution activity. To confirm this, PSII membranes were treated with high-salt buffers to deplete the extrinsic polypeptides. No inhibition of O_2-evolution activity was observed (Figure 1), further indicating that the inhibition caused by primary amine treatment

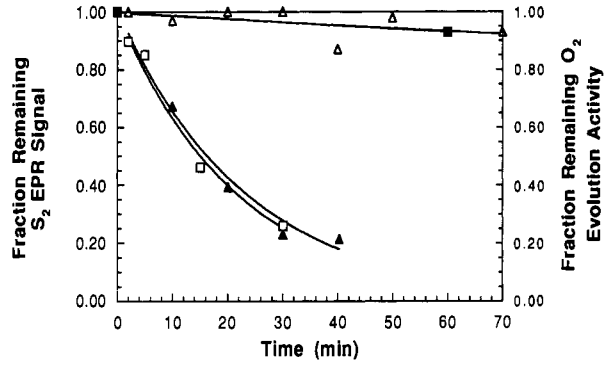

FIGURE 1: Comparison of the rate of decay of the yield of the S_2 multiline EPR signal and irreversible loss of O_2 evolution in PSII membranes (0.5 mg of chlorophyll/mL) treated with 0.8 M Tris, 0.8 M NaCl, or 0.8 M Na_2SO_4 at pH 8.0, 0 °C, and in darkness. The intensity of the S_2 multiline signal was determined from the sum of the peak-to-peak heights of three hyperfine lines to lower field and three lines to higher field of $g = 2.0$. The yield of the multiline signal in each sample was normalized relative to the yield of the $Fe^{II}Q_A^-$ EPR signal at $g = 1.9$, and to the yield of the multiline signal observed in an untreated control sample. The O_2-evolution activities were normalized to the activity measured after 2 min of incubation. (▲) O_2-Evolution activity in a Tris-treated sample; (△) O_2-evolution activity in an Na_2SO_4-treated sample; (□) S_2 multiline EPR signal in a Tris-treated sample; (■) S_2 multiline EPR signal in a NaCl-treated sample. The solid lines through the data points are exponential curve fits which yielded the following half-times for decay: 15 and 16 min for the S_2 EPR and O_2 evolution, respectively, of the Tris-treated samples and 580 and 630 min for the S_2 EPR and O_2 evolution, respectively, of the control samples.

Table I: Rates for Irreversible Inhibition of O_2 Evolution in the S_1 State by Amines

amine	pK_a (0 °C)	k_{obs} (M^{-1} s^{-1})[a]	rank
Tris	8.8	3.4×10^{-3}	6
AEPD	9.5	5.7×10^{-3}	5
NH_3	10.0	12×10^{-3}	4
ethanolamine	10.2	37×10^{-3}	3
CH_3NH_2	11.4	52×10^{-3}	2
$(CH_3)_2NH$	11.6	300×10^{-3}	1

[a] Rate constants were obtained from single-exponential curve fits as described in Figure 1 and have been corrected for the amount of the amine present in the treatment buffer at pH 8.0 and 0 °C.

is not simply occurring due to removal of the extrinsic polypeptides by the high ionic strength. O_2-evolution and EPR measurements were also made with samples incubated in either 0.8 M NaCl or 0.8 M Na_2SO_4. No significant loss of activity was observed in either case, ruling out that the inactivation was due to a high concentration of either Cl^- or SO_4^{2-}.

The free base of an amine has been identified as the active species in reversible and irreversible inhibition of O_2-evolution activity by amines (Hind & Whittingham, 1963; Izawa et al., 1969; Sandusky & Yocum, 1983, 1984, 1986). The rate constants given in Table I have therefore been corrected for the amount of amine present as the free base in the treatment buffer at pH 8.0 and 0 °C. Rate constants for the inhibition of O_2 evolution by hydroxylamines are distinctly larger (Tamura & Cheniae, 1985), and the rate constants for reduction of the S_1 state to S_{-1} by hydroxylamines are about 3 orders of magnitude larger (Beck & Brudvig, 1988).

Several reactions involving the Mn complex have been observed to have an inverse dependence upon the Cl^- concentration, including the reversible inhibition of O_2 evolution by amines (Sandusky & Yocum, 1983, 1984, 1986), the irreversible inhibition of O_2 evolution by hydroxylamines (Kelley & Izawa, 1978), and the reduction of the S_1 state of the Mn complex to the S_{-1} state by *N,N*-dimethylhydroxylamine (Beck & Brudvig, 1988). In order to determine the Cl^- dependence

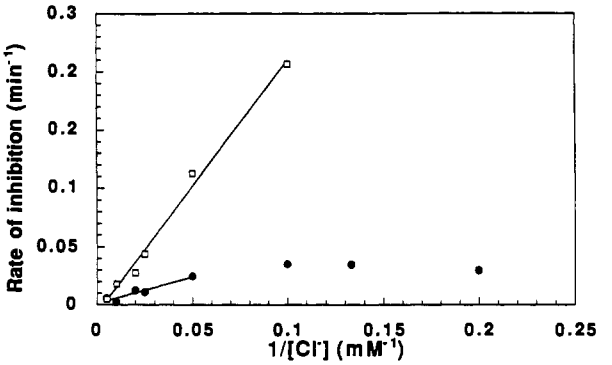

FIGURE 2: Rates for the irreversible inhibition of O_2-evolution activity in PSII membranes (●) and 17- and 23-kDa polypeptide-depleted PSII membranes (□) suspended at 0.5 mg of chlorophyll/mL and treated with 0.8 M Tris at pH 8.0, 0 °C, and in darkness, plotted as a function of the inverse of the Cl^- concentration present during the Tris treatment. Rates were obtained by fitting the O_2-evolution activities measured over a 40-min period to a single-exponential decay, and are not corrected for the concentration of Tris present as the free base. Solid lines are the linear least-squares fits to the data, including only the initial points for the undepleted samples.

of the irreversible inhibition of O_2 evolution by amines, PSII membranes were treated with Tris over a range of Cl^- concentrations, and the rates of inhibition of O_2 evolution were measured (Figure 2), We observe an effect similar to those seen in both the irreversible inhibition by hydroxylamines and the reversible inhibition by amines. High Cl^- concentrations have a protective effect.

Recent studies have suggested a link between the effects of Cl^- on PSII and the binding of the 17- and 23-kDa polypeptides (Homann, 1988). In order to study this, PSII membranes which had been depleted of these polypeptides were treated with Tris at varying concentrations of Cl^- (Figure 2). These samples exhibited a much larger and more linear response to different Cl^- concentrations.

Loss of Formation of the S_2 Multiline EPR Signal. After illumination at 210 K for 2 min, untreated PSII membranes show the S_2 multiline EPR signal centered at $g = 2$, and also a signal at $g = 1.9$, which is caused by the primary electron acceptor, Q_A^-, coupled to Fe(II). It is clear that in the untreated samples, illumination caused one charge separation to occur, oxidizing the O_2-evolving center to the S_2 state and reducing Q_A. At these temperatures, diffusion is limited, and so Q_A cannot readily be reoxidized, lowering the possibility of performing multiple charge separations.

When hydroxylamine-treated PSII membranes are examined by EPR, the yield of the S_2 multiline EPR signal decreases over time (Beck & Brudvig, 1988). The $Fe^{II}Q_A^-$ EPR signal, however, does not significantly change, indicating that a stable charge separation takes place, as in the untreated membranes. Similar behavior is observed for Tris-treated membranes (Figure 3). An $Fe^{II}Q_A^-$ EPR signal is observed in all samples, demonstrating that a stable charge separation can occur in these membranes, but the size of the S_2 multiline EPR signal observed decreases as the duration of the Tris treatment increases. After 90 min of Tris treatment, only a small fraction of the S_2-state multiline signal is observed (Figure 1). Since the S_2 state is not observed, at least not in the normal multiline configuration, then either the Mn complex is in a state other than S_1 after Tris treatment in the dark or an alternative electron donor is being oxidized in place of the Mn complex.

High salt concentrations have been known to remove the 17- and 23-kDa polypeptides, causing Ca^{2+} depletion and blocking the S-state transitions. Unlike the hydroxylamine treatments, treatment with Tris and the other amines takes

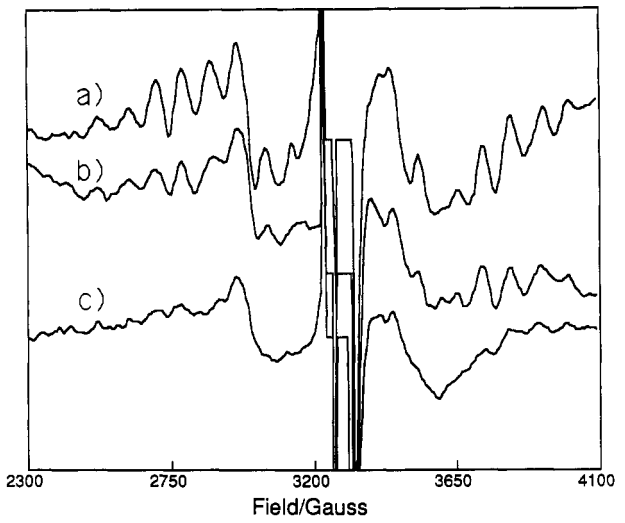

FIGURE 3: Effect of Tris treatment on the yield of the S_2-state multiline EPR signal produced by illumination at 210 K of PSII membranes (4–6 mg of chlorophyll/mL) at pH 6.0. Prior to being washed and frozen in liquid N_2, the samples (0.5 mg of chlorophyll/mL) were incubated in 0.8 M Tris at pH 8.0, 0 °C, and in darkness. (a) 2-min incubation; (b) 10-min incubation; (c) 60-min incubation. EPR spectrometer conditions: microwave frequency, 9.0450 GHz; microwave power, 0.08 mW; field modulation amplitude, 20 G; sample temperature, 8 K. The spectra shown are the difference between the spectrum obtained following illumination and the spectrum obtained prior to illumination.

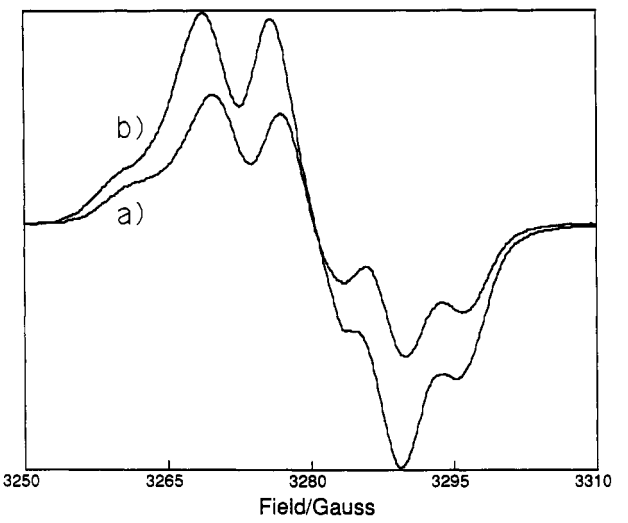

FIGURE 4: Effect of Tris treatment upon signal II in PSII membranes. Prior to being washed and frozen in liquid N_2, the sample (0.5 mg of chlorophyll/mL) was incubated in 0.8 M Tris at pH 8.0, 0 °C, and in darkness, for 25 min. (a) Dark scan; (b) after illumination at 210 K for 2 min. EPR spectrometer conditions: microwave frequency, 9.0509 GHz; microwave power, 0.5 μW; field modulation amplitude, 4 G; sample temperature, 15 K.

place in a high ionic strength buffer, and removal of these polypeptides would be one explanation of the observed decay in the S_2 multiline EPR signal. However, since the major effect of removal of the 17- and 23-kDa polypeptides is to increase the Ca^{2+} requirement, no decay should be observed after Tris treatment when excess Ca^{2+} is present, if polypeptide removal is indeed the cause of the decay of the S_2 multiline EPR signal yield. The presence of 5 mM Ca^{2+} in the buffer does not affect the decay (data not shown), demonstrating that removal of the 17- and 23-kDa polypeptides is not the cause of the inability of Tris-treated membranes to produce the S_2 multiline EPR signal.

Redox Reactions of the Electron-Donor Side of Tris-Treated Photosystem II. It has been previously observed that low concentrations of hydroxylamine will leave the Mn complex in a state which is EPR-silent after one illumination. If Q_A^- is reoxidized after each illumination by warming the samples to 0 °C in the presence of DCBQ, then multiple illumination cycles can be performed on a sample. Hydroxylamine-treated samples give rise to the S_2-state multiline signal after three illuminations, indicating the presence of an intermediate which has been reduced by two electrons from the S_1 state (Beck & Brudvig, 1987). In contrast, there is no additional S_2-state multiline EPR signal or $g = 4.1$ EPR signal produced in Tris-treated membranes after three or even five illumination cycles. The size of the $Fe^{II}Q_A^-$ signal, while diminishing on warming in the dark in the presence of DCBQ, returns to full intensity with each illumination, showing that Q_A^- is reoxidized on warming and that multiple charge separations do occur in the samples. As can be seen in Figure 1, loss of the ability to produce the S_2 multiline EPR signal occurs at the same rate as the irreversible inhibition of O_2-evolution activity, suggesting that the Mn complex has been irreversibly damaged.

If there is no oxidation of the Mn complex to the S_2 state in these samples, then an alternative electron donor must be oxidized to form a stable charge separation upon illumination. Since we observe the $Fe^{II}Q_A^-$ EPR signal in Tris-treated samples, a charge separation must occur, and so the presence or absence of an alternative donor will tell us whether or not the Mn complex is being oxidized by $P680^+$. After hydroxylamine treatment, a small fraction of the PSII membranes cannot produce S_2 after three illuminations, and in these centers, the alternative electron donor is cytochrome *b*-559. In Tris-treated membranes, however, the high ionic strength of the buffer solution has for the most part destabilized the binding of the 17- and 23-kDa polypeptides, causing cytochrome *b*-559 to be present in its low-potential form. Low-potential cytochrome *b*–559 is readily autoxidized, and is already mostly oxidized in Tris-treated membranes before illumination, preventing it from being the predominant alternative electron donor. Some photooxidation of cytochrome *b*-559 does occur, however, as shown in Figure 3 (the g_y turning point of the cytochrome *b*-559 EPR signal occurs at $g = 2.2$, approximately 3000 G in Figure 3). In contrast, Y_D is significantly reduced in the dark in Tris-treated membranes. As can be seen in Figure 4, the size of signal II increases greatly after the first illumination, indicating that Y_D is the predominant electron donor in Tris-treated membranes. These results make it clear that the Mn complex in Tris-treated PSII membranes is no longer being oxidized by $P680^+$. More specifically, this behavior is very distinct from that observed in hydroxylamine-treated PSII samples, were the Mn complex can be reduced to an S_{-1} state and then oxidized to S_2 after three charge separations.

Effects of Tris Treatment on the Mn Complex. Tris treatment in the light has been known to disrupt the Mn complex and cause the release of Mn^{2+} ions from PSII. A small amount of the six-line Mn^{2+} EPR signal, centered at $g = 2.0$, is present in EPR spectra of PSII prepared by Tris treatment followed by centrifugation and resuspension in buffer to remove the Tris. Because Mn^{2+} quantitation cannot be performed on samples prepared in this fashion (due to washing), aliquots of Tris-treated PSII membranes were placed directly in EPR tubes. Again, only a very small amount of the six-line EPR signal from the Mn^{2+} ion was observed. This result raises the possibility that the Mn complex has not been totally disrupted, and is still bound to the protein in some form, although no longer able to act as an electron donor. We

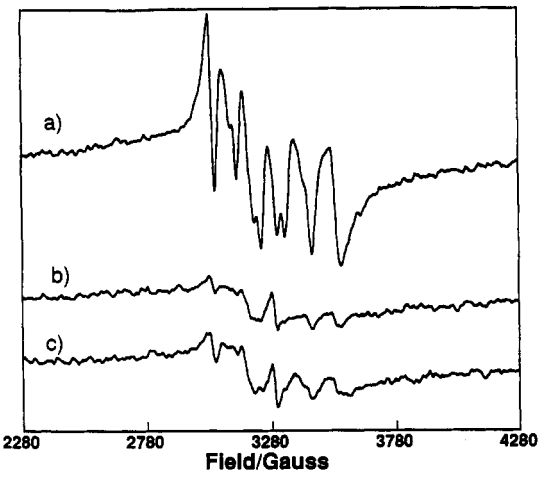

FIGURE 5: EPR assays of Mn bound to PSII membranes before and after amine treatment. PSII membranes (0.5 mg of chlorophyll/mL) were treated with (a) pH 6.0 buffer, (b) 0.8 M Tris, pH 8.0, or (c) 0.8 M ethanolamine, pH 8.0, at 0 °C for 75 min. After being pelleted and resuspended at 4 mg of chlorophyll/mL, the samples were mixed with an equal volume of 1 N HCl, left at room temperature for 20 min, and then frozen in liquid N_2. EPR spectrometer conditions: microwave frequency, 9.0447 GHz; microwave power, 4 μW; field modulation amplitude, 20 G; sample temperature, 15.5 K.

decided, therefore, to assay the amount of Mn remaining in the PSII membranes. HCl treatment of untreated PSII membrane releases the Mn complex as free $Mn^{2+}(H_2O)_6$ ions. As can be seen in Figure 5, however, when Tris-treated PSII membranes are pelleted, resuspended, and treated with HCl, the amount of Mn^{2+} retained with the membranes is only a very small fraction of that in an untreated PSII sample. This demonstrates that the Mn had already been released by Tris treatment but that it was not readily observable by EPR in the reaction mixture itself. From Figure 5, it can be seen that Tris treatment in the dark followed by pelleting and resuspending the sample in a Tris-free buffer removes >90% of the Mn.

If the Mn ions are released from PSII by the Tris treatment, then why was only a very small amount of free Mn^{2+} observed in the Tris-treated samples? One possibility is that the Mn ions are released from PSII as some sort of higher valence complex which is EPR-silent. However, addition of dithionite to the supernatant from a Tris-treated sample did not increase the amount of Mn^{2+} visible by EPR. Another possibility is the formation of MnO_2. MnO_2 would not be observable by this treatment because it is insoluble and would remain in the pellet. However, Yocum et al. (1981) demonstrated that release of Mn as MnO_2 did not take place during Tris treatment. A final explanation is that the Tris buffer contains a chelator which binds Mn^{2+} and lowers its symmetry, thus suppressing the six-line Mn^{2+} EPR signal. This was confirmed by measuring the intensity of the Mn^{2+} EPR signal in Tris buffer compared to the normal resuspension buffer and by titrating Tris buffer with Mn^{2+}. The titration data were well fit by assuming a simple equilibrium between bound and unbound ligand. The ligand concentration was found to be 25–30 μM in 0.8 M Tris, and therefore cannot be Tris itself, but more likely some very low level contaminant. However, such a concentration of ligand would be sufficient to chelate most of the Mn^{2+} released from PSII by 0.8 M Tris treatment, thus explaining the small yield of the six-line Mn^{2+} EPR signal in the Tris reaction mixture.

Discussion

We have characterized the inactivation of O_2 evolution and disruption of the Mn complex that occurs upon the reaction of Tris with the dark-stable S_1 state of the O_2-evolving center of PSII. Irreversible inhibition of O_2 evolution upon incubation of PSII in 0.8 M Tris in the dark was reported earlier by Ikehara and Sugahara (1969) and Cheniae and Martin (1978), but the mechanism was not studied in detail. More work has been focused on the light-induced inactivation of O_2 evolution by Tris (Yamashita & Horio, 1968). Frasch and Cheniae (1980) showed that inhibition of O_2 evolution by Tris occurs much more rapidly in the S_2 state, causing loss of the Mn complex. It has been generally believed that Tris disrupts the Mn complex by chelating Mn ions, removing them from the membrane, where they would subsequently be reduced. However, our results make this explanation unlikely, since we find that Tris does not noticeably chelate Mn^{2+} ions in solution. The irreversible inhibition has been observed to proceed at a much higher rate with the S_2 state than the S_1. Since the more highly oxidized Mn ions present in the S_2 state would be less labile than those in the S_1 state, the chelation mechanism would be expected to be slower for S_2 than S_1. More generally, a number of other amines show similar behavior, including amines such as methylamine, which are only monodentate, and could not therefore chelate a metal ion.

The observation that a variety of amines react similarly with PSII also rules out the possibility that Tris inhibits O_2-evolution activity through a direct covalent interaction with protein functional groups. Given the variation in the structure of the amines shown here to inhibit O_2-evolution activity, including one secondary amine, it would be unreasonable to expect them all to be able to react with the protein in a similar manner.

Our data, together with previous studies, suggest that amines bind to the O_2-evolving center in the S_1 state which leads to reduction and release of the Mn ions. We observe an inverse dependence of the rate of inactivation on the Cl^- concentration. Although our studies do not demonstrate competitive binding of amines and chloride, we suggest that the irreversible inhibition of O_2 evolution by amines occurs because of a slow reaction when amines are bound at the same Cl^--sensitive site identified in previous studies of the reversible inhibition of O_2 evolution by amines (Sandusky & Yocum, 1983, 1984, 1986). This mechanism is given in eq 1–2.

$$S_1(Cl^-) + RNH_2 \overset{K_{eq}}{\rightleftharpoons} S_1(RNH_2) + Cl^- \quad (1)$$

$$S_1(RNH_2) \xrightarrow{k_1} Mn^{2+} \text{ release} \quad (2)$$

The inactivation of O_2 evolution and loss of ability to form the S_2 state occur at the same rate, indicating that eq 2 is the rate-limiting step in this model. The observed rate constant, k_{obs}, would then be defined in this model by eq 3. This model

$$k_{obs} = k_1 K_{eq}/[Cl^-] \quad (3)$$

explains the inverse dependence of k_{obs} on the Cl^- concentration (Figure 2). k_{obs} also increases with increasing basicity of the amine (Table I). One or both of two factors could account for this trend.

The first is that binding of the amines, as shown in eq 1, is expected to be increasingly favorable with increasing basicity of the amine. A trend similar to that shown in Table I has been observed for the reversible inhibition of O_2 evolution (Ghanotakis et al., 1983). Therefore, the effect of the basicity of the amine on binding (K_{eq}) probably is an important factor in the rate of inactivation of O_2 evolution.

The second is that oxidation of amines (see below) is expected to be increasingly favorable with increasing basicity and with increasing N-substitution of the amine. N-Substi-

tution, in particular, has a large effect on the potential for oxidation of amines. For example, the oxidation of dimethylamine occurs at about 0.4 V lower potential than methylamine (Siegerman, 1975). This may explain why the rate of reaction of dimethylamine is significantly faster than expected based only on the pK_a's, compared to the rates of reaction of the primary amines.

It is known that amines bind to the S_2 state more tightly than to S_1 (Beck & Brudvig, 1986). Hence, the observation (Frasch & Cheniae, 1980) that the inactivation of O_2 evolution by Tris takes place much more rapidly in the S_2 state than in S_1 could be explained by the increased binding of Tris in S_2. However, an explanation of the Tris-induced inactivation of O_2 evolution must also account for the release of the Mn complex as Mn^{2+} ions. In order to produce Mn^{2+} ions, the high-valent Mn complex must be reduced.

One possibility for reduction of the Mn complex is that the binding of an amine induces a disproportionation reaction within the Mn complex whereby the valence of the Mn ion(s) coordinated to the amine increases and the valence of the uncoordinated Mn ion(s) decreases. Such a disproportionation reaction could result in the formation of Mn^{2+} which could be released from the protein. Subsequent reduction of the remaining high-valent Mn would be required to account for the observation that all of the Mn is released as Mn^{2+} upon inactivation by Tris. Increasing the basicity of the amine would possibly accelerate the rate of reaction because the more basic amines would better stabilize a higher valence state of Mn. However, oxidation of the Mn complex to the S_2 state would not be expected to enhance such a disproportionation reaction.

Alternatively, the release of the Mn complex by amines could be due to oxidation of the amine by the Mn complex. This is in accord with the observation of Frasch and Cheniae (1980), because the S_2 state is a stronger oxidant and would be more readily reduced than the S_1 state (Hanssum & Renger, 1985; Andréasson & Hansson, 1987). Such a reduction would also account for the release of the Mn complex as Mn^{2+} ions and would be very similar to that postulated for the reaction of hydroxylamines with the Mn complex (Beck & Brudvig, 1987). The reaction of Tris is generally similar to the reaction observed between hydroxylamines and the Mn complex in PSII, with one difference: no two-electron-reduced intermediate can be isolated.

In the case of hydroxylamines, the initial reaction with the O_2-evolving center involves two electrons, forming S_{-1} upon reaction with the dark-stable S_1 state and S_0 upon reaction with the S_2 state. The subsequent reaction is much slower, allowing the S_{-1} state to be produced in high yield as an intermediate in the dark reaction of hydroxylamine with PSII. The lack of a two-electron-reduced intermediate in the reaction of amines could be explained if the amines rapidly deliver four or more electrons to the O_2-evolving center. An initial two-electron oxidation of an amine would produce a hydroxylamine derivative in situ which would be expected to react rapidly in a second two-electron reaction. A four-electron reduction of the Mn complex would produce an "S_{-3}" state upon reaction with the S_1 state or an "S_{-2}" state upon reaction with the S_2 state. "S_{-2}" and "S_{-3}" represent highly reduced states of the Mn complex which rapidly decompose to release Mn^{2+}. Such a mechanism explains the lack of observation of a partially reduced intermediate in the reaction of Tris. This mechanism also explains the different effects of light on the reactions of hydroxylamines and amines. In the case of the reaction of hydroxylamine, light has a protective effect because the initial product of the reaction, S_{-1} or S_0, is stable and can be photooxidized to the higher S states. However, in the case of the reaction of Tris, light accelerates the inactivation. This could be explained if Tris reacts more rapidly with the light-induced S_2 state than the dark-stable S_1 state and if the product of the reaction of a single molecule of Tris with S_2 is reduced below S_{-1} and rapidly decomposes to release Mn^{2+}. In the case of hydroxylamine, the reaction does occur much faster with S_2 than S_1 (Andréasson & Hansson, 1987). Hence, the proposed mechanisms of the reactions of amines and hydroxylamines with the O_2-evolving center appear to be generally similar with the main difference being the number of electrons delivered in the initial reaction.

Our results (Figure 2) show that, in 17- and 23-kDa polypeptide-depleted samples, Cl^- has a large effect upon the rate of inhibition of O_2 evolution by Tris. These observations are consistent with those made by Mei and Yocum (1990) of the inhibitory effects of hydroquinone and phenylenediamine upon O_2 evolution by PSII.

It has been recently found that Cl^- can stabilize the binding of the extrinsic polypeptides (17 and 23 kDa), which can become dissociated from the PSII complex in solutions of high ionic strength (Homann, 1988). Homann (1988) has suggested that this is the cause of the Cl^- effect upon inhibition of PSII by reductants. In Figure 2, k_{obs} is linear with respect to $1/[Cl^-]$ in the region of high $[Cl^-]$ but levels off at low $[Cl^-]$ for the undepleted samples. In contrast, removing the 17- and 23-kDa extrinsic polypeptides results in a linear dependence of k_{obs} with respect to $1/[Cl^-]$ over the entire plot, and the maximal rate observed is much higher than those observed for samples with the polypeptides intact. This suggests that the extrinsic polypeptides limit the maximal rate of inhibition of O_2 evolution by Tris and provides additional evidence that the extrinsic polypeptides affect the accessibility of the Mn complex to reductants. Effects upon the stability of the binding of the extrinsic polypeptides may contribute to the Cl^- effect on inhibition of O_2 evolution by Tris, particularly at low Cl^- concentrations. However, since a large Cl^- effect is also observed in the absence of the extrinsic polypeptides, the effect of Cl^- on the binding of the 17- and 23-kDa polypeptides cannot be a major factor in the observed effect of Cl^- on the rate of irreversible inhibition of O_2 evolution by Tris.

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Registry No. Tris, 77-86-1; Mn^{2+}, 7439-96-5; O_2, 7782-44-7; Cl^-, 16887-00-6; NH_3, 7664-41-7; CH_3NH_2, 74-89-5; $(CH_3)_2NH$, 124-40-3; 2-amino-2-ethyl-1,3-propanediol, 115-70-8; ethanolamine, 141-43-5.

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