Photoinhibition of Hydroxylamine-Extracted Photosystem II Membranes: Studies of the Mechanism†

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ABSTRACT: The effects of photosystem II (PSII) exogenous electron donors and acceptors on the kinetics of weak light photoinhibition of NH_2OH/EDTA-extracted spinach PSII membranes were examined. Under aerobic conditions, Mn^{2+} (~1 Mn/reaction center; K_m ~400 nM) inhibited photoinactivation and ~1 Mn/reaction center plus 100 μM NH_2NH_2 gave almost complete protection. In the absence of electron donors, strict anaerobiosis greatly inhibited photoinactivation even in the presence of an electron acceptor. Under aerobic conditions, the addition of electron acceptors (FeCN, DCIP), oxyradical scavengers, or superoxide dismutase strongly suppressed rates of photodamages. Increase in the concentrations of superoxide above those produced by illuminated NH_2OH/EDTA-photosystem II membranes increased the rates of damage in the light but gave no damage in the dark. Scavengers of hydroxyl radicals and singlet oxygen did not suppress the rates of aerobic photoinhibition. These findings, along with others, lead us to conclude that photodamage of the secondary donors of the PSII reaction center occurs by two mechanisms: (1) a rapid superoxide and tyrosine Y_Z^+ dependent process and (2) a slower process in which P_{680}^+/Chl^+ catalyze the damages.

Photosystem II (PSII)[1] is a large membrane-bound multisubunit complex that catalyzes the light-driven reduction of plastoquinone (PQ) with electrons derived from the oxidation of water to molecular O_2. Its reaction center (RC) consists of a heterodimer of homologous membrane-spanning polypeptides, D_1 and D_2, which together bind all the redox components required for efficient charge separation and stabilization. The absorption of a light quantum by the PSII RC results in a rapid transient charge separation between the primary electron donor P_{680} and the intermediate electron acceptor, pheophytin (Pheo). This transient state (P_{680}^+/$Pheo^-$) is rapidly stabilized by the oxidation of $Pheo^-$ by the primary electron acceptor, Q_A, which in turn is oxidized by the secondary electron acceptor, Q_B, and by the reduction of P_{680}^+ by the secondary donor tyrosine Y_Z of D_1. The resulting Y_Z^+ is reduced by the tetra-Mn water-oxidizing complex which cycles through a series of oxidation states leading to molecular oxygen. Tyrosine Y_D of the D_2 protein is a sidepath electron donor to P_{680}^+. The function(s) of Cyt *b*-559, which is closely associated with the RC, is(are) not well established. [For reviews of PSII, see Babcock (1987), Babcock et al. (1989), Hansson and Wydrzynski (1990), Ghanotakis and Yocum (1990) and Andersson and Styring (1991).]

PSII is peculiarly susceptible to photoinhibition (photoinactivation) [for reviews, see Kyle (1987), Critchley (1988), and Andersson and Styring (1991)]. In oxygen-evolving systems, very high light intensities are generally required and the photoinactivation mechanism has a very low quantum yield of ~1 × 10^{-7} RC/quantum (Eckert et al., 1991). Following inactivation/inhibition of the oxygen-evolving complex by treatments which deplete Mn/Cl^-, PSII is much more susceptible to photoinactivation even by very weak light intensities and the quantum yield of the inactivation is increased greatly. For example, the optimum quantum yield for photoinhibition of Mn-depleted thylakoids and PSII membranes is about 5 × 10^{-5} RC/quantum (Callahan et al., 1986) and 1 × 10^{-4} RC/quantum (Eckert et al., 1991), respectively; moreover, only 6–10 quanta $PSII^{-1}$ s^{-1} (Eckert et al., 1991) and 18 quanta $PSII^{-1}$ s^{-1} (Callahan et al., 1986) are necessary to give half-maximal rates of photoinhibition of Mn-depleted PSII membranes and thylakoids, respectively.

Photoinhibition of various O_2-evolving (Ohad et al., 1984; Mattoo et al., 1984; Virgin et al., 1988; Hundal et al., 1990; Aro et al., 1990) and non-O_2-evolving (Callahan et al., 1986; Jegerschold & Styring, 1991; Jegerschold et al., 1990) PSII preparations may cause an accelerated degradation/turnover of D_1 and D_2 proteins. The degradation of D_1>D_2 proteins is catalyzed by proteolytic activity of the PSII complex itself (Virgin, et al., 1990; Shipton & Barber, 1991). Abundant evidence exists showing that photoinhibition/photoinactivation events precede any proteolysis of the RC polypeptide(s).

Studies made with O_2-evolving preparations have led to a general consensus that the RC acceptor side is initially affected during photoinhibition. Nevertheless, appreciable uncertainty exists with respect to the first site of inhibition and to the molecular mechanism(s) causing the inhibition and the damage to D_1>D_2 polypeptides. One school of thought advocates Q_A to be the initial site of photoinactivation (Allakhverdief et al., 1987; Styring et al., 1990; Setlik et al., 1990; Vass et al., 1988,

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[1] Abbreviations: Car, carotenoid; Chl, chlorophyll; Cyt-*c*, cytochrome *c*; D_1 and D_2, homologous 32-kD_a polypeptides which, as a dimer, form the PSII RC core; DABCO, 1,4-diazabicyclo[2.2.2]octane; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, diphenylcarbazide; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; EDTA/NH_2OH-PSII, PSII membranes extracted with NH_2OH and EDTA to inactivate water oxidation and to completely remove the Mn cluster; EPR, electron paramagnetic resonance; FeCN, ferricyanide; His, histidine; LP Cyt-*b*-559, low-potential cytochrome *b*-559; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P_{680}, PSII primary electron donor chlorophyll; PBQ, phenyl-*p*-benzoquinone; PQ, plastoquinone; Pheo, pheophytin; PSII, photosystem II; Q_A and Q_B, primary and secondary PQ electron acceptors of PSII, respectively; RC, reaction center; S_3, an oxidation state of the water-oxidizing complex; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)-aminomethane; Y_Z and Y_D, redox-active tyrosine-161 and -160, respectively, of the D_1 and D_2 RC proteins.

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1992). According to Vass et al. (1992), protonation of reduced Q_A results in an inhibition of electron transfer which is reversible; however, double reduction of Q_A promotes its dissociation and results in an irreversible inhibition without oxidative damage to D_1 and D_2 polypeptides. These ideas offer an explanation for the previously observed acceleration of photoinhibition by strict anaerobic or reducing conditions (Trebst, 1962; Satoh & Fork, 1982; Krause et al., 1985; Setlik et al., 1990). Subsequently, photoinduced Chl triplets are formed which react with oxygen to give singlet oxygen. Singlet oxygen is postulated to cause oxidative damage to $D_1 > D_2$ proteins in the near vicinity of P_{680} and to promote the subsequent turnover of $D_1 > D_2$ proteins (Vass et al., 1992). These results and ideas agree well with those of Nedbal et al. (1990) and Setlik et al. (1990), who described fast, slow, and very slow kinetic phases of photoinhibition. The fast component, which was observed only under anaerobic or strongly reducing conditions, probably relates to formation of protonated Q_A^- and/or Q_A^{2-}.

Another school advocates Q_B to be the initial site of photoinactivation. One hypothesis suggests that inactivation occurs from formation of oxygen radicals from the oxidation of Q_B^-/Q_B^{2-} by molecular oxygen (Kyle et al., 1984; Kyle, 1987). Another holds that destabilization of Q_B^- binding occurs, followed by an irreversible modification of the D_1 protein (Ohad et al., 1988, 1990). Additionally, arguments have been advanced indicating that the presence of PQ at the Q_B site mediates these reactions (Critchley, 1988; Trebst et al., 1990; Gong & Ohad, 1991). Such photoinactivation of the RCII acceptor side apparently is followed by inactivation of RC donor side reactions leading to increased lifetimes of the highly oxidizing cation radicals of the RC ($Y_Z^+/P_{680}^+/Chl^+$) (Gong & Ohad, 1991). These cation radicals are suggested to damage the D_1 protein such that it becomes susceptible to proteolytic degradation. It is not entirely clear whether the Q_A or the Q_B hypothesis best relates to the partial protection from aerobic photoinhibition which is observed with the addition of chemical scavengers of oxyradicals (Barenyi & Krause, 1985; Sopory et al., 1990) or by additions of SOD and catalase (Barenyi & Krause, 1985; Richter et al., 1990; Setlik et al., 1990).

Studies of photoinhibition of Mn-depleted (Callahan et al., 1986; Blubaugh & Cheniae, 1990b; Blubaugh et al., 1991; Klimov et al., 1990; Eckert et al., 1991; Ono & Inoue, 1991b) and Cl^--depleted (Jegerschold & Styring, 1991; Theg et al., 1986; Jegerschold et al., 1990) PSII preparations have led to a general agreement that the donor side of the RC becomes selectively photodamaged during illumination. According to Blubaugh et al. (1991), the order of susceptibility of PSII components to photodamage during weak light illumination of wheat NH_2OH-PSII is Chl/Car > Y_Z > Y_D ≫ P_{680}, Pheo, Q_A. Loss of a 4-μs decay of P_{680}^+, presumably reflecting $Y_Z \rightarrow P_{680}^+$, a partial loss of photoactivation capability, and the formation of a quencher of Chl *a* variable fluorescence all proved kinetically correlated with photodamage to Chl/Car. A similar loss of a rapid component of decay of P_{680}^+ occurs on illumination of Tris-PSII (Eckert et al., 1991). Loss of the remaining photoactivation capability and partial loss of capacity for photooxidation of exogenous donors (Mn^{2+}/DPC) in limiting light decayed in parallel with loss of Y_Z (Blubaugh & Cheniae, 1990b).

In similar experiments also made in weak light but with spinach NH_2OH-PSII, Ono and Inoue (1991b) observed parallel loss of Y_Z, photoactivation capability, and the A_T-band thermoluminescence emission. This emission is thought to reflect charge recombination between Q_A^- and a putative oxidized His residue (Ono & Inoue, 1991a); however, it is not known whether this His residue is the same redox-active His functioning at the S_3 state of water oxidation (Boussac et al., 1990).

On the other hand, strong light photoinhibition of NH_2-OH-PSII rapidly impairs both the capabilities of A_T-band emission and photoactivation in parallel and significantly faster than the loss of Y_Z^+ or Y_D^+ formations (Ono & Inoue, 1991b). Moreover, Mn^{2+} photooxidation capability in strong light was lost much more slowly than the capabilities of A_T-band emission and photoactivation. Previously, Klimov et al. (1990) indicated that strong light treatment of Tris-PSII abolished photoactivation capability without inhibiting Y_Z^+ or Y_D^+ formations. Ono and Inoue (1991a) have postulated that a putative redox-active His of D_1 normally is oxidized by Y_Z^+ and the resulting His^+ oxidizes exogenous Mn^{2+} to initiate assembly of the Mn cluster via the photoactivation process [see, however, Blubaugh and Cheniae (1990b) and Nixon and Diner (1992)]. The contrasting effects of weak vs strong light inactivation of Y_Z formation and capability of A_T-band emission suggest that the putative His residue is more susceptible to photoinactivation by strong light than Tyro Y_Z.

The molecular mechanism underlying these photoinactivations of the PSII RC donor side components of Mn-depleted or Cl^--depleted preparations is unknown. Most workers assume the photodamages are oxidative in nature and are caused by the highly oxidizing cation radicals such as P_{680}^+, Chl^+, and/or Y_Z^+, whose lifetimes are greatly increased following Mn and/or Cl^--depletion (Conjeaud & Mathis, 1980). This idea is supported by reports showing partial protection from photoinhibition by exogenous electron donors to PSII (Klimov et al., 1990; Blubaugh & Cheniae, 1990b; Eckert et al., 1991), acceleration of photoinactivation by exogenous electron acceptors to PSII (Klimov et al., 1990), and fast oxygen-independent degradation of the D_1 protein during illumination of Cl^--depleted or Tris-washed thylakoids (Jegerschold et al., 1990; Jegerschold & Styring, 1991).

Here we report studies of the effects of exogenous PSII donors/acceptors, anaerobiosis, oxy-radical scavengers, and superoxide dismutase on the kinetics of weak light photoinhibition of NH_2OH-PSII made highly depleted of Mn with use of EDTA and Chelex-treated buffers. The data show the mechanism of photodamages to the PSII RC donor side components involves more than simple oxidative damages caused by $P_{680}^+/Chl^+/Y_Z^+$. They indicate that the most rapid photodamages are inhibited either by Mn^{2+} binding at a high-affinity site (K_m ~ 400 nM) or by the addition of PSII exogenous electron acceptors, oxygen radical scavengers, or superoxide dismutase. Similarly, strict anaerobiosis slows the photoinhibition of NH_2OH-PSII donor site components. We are led to suggest that minimally two mechanisms underly the photoinactivation of NH_2OH-PSII donor side components: (1) a rapid process requiring both superoxide and a cation radical(s) of the RC and (2) a slower process driven only by the cation radical(s) of the RC.

MATERIALS AND METHODS

Sample Preparation. Spinach NH_2OH/EDTA-PSII were prepared from spinach PSII membranes ($VO_2 \geq 700$ μmol O_2/(mg of Chl·h)) as described by Blubaugh and Cheniae (1990b) with the following exceptions: (1) 1 mM EDTA was included during both the NH_2OH extraction and the first wash of the extracted membranes; (2) Chelex-100 treated 0.4

M sucrose/50 mM Mes–NaOH/15 mM NaCl, pH 6.5 (buffer B) was used for the subsequent four repetitive washes before final resuspension of the membranes (≥2 mg of Chl/mL) in Chelex-100-treated 0.8 M sucrose/50 mM Mes–NaOH/15 mM NaCl, pH 6.5 (buffer A); and (3) all labware used for preparation and photoinhibition of the membranes had been presoaked in 2 N HNO_3 or 10 mM EDTA and then rinsed with Milli-Q water just prior to use.

Photoinhibition. For aerobic photoinhibition, NH_2OH/EDTA-PSII were diluted to 250 μg of Chl/mL in buffer A with additions as noted in the figure legends. The samples (0.8 mL in 15-mm-diameter glass vials) routinely were illuminated from below with diffused fluorescent cool-white light of uniform intensity (50–60 μE m^{-2} s^{-1}) while being shaken continuously at 23 °C. Anaerobic photoinhibition was done similarly with the following exceptions: (1) Buffer A containing 5 mM glucose/20 mM $NaHCO_3$ was bubbled (gas-dispersion tube) with 99.999% N_2 for 15 min before addition to vials containing NH_2OH/EDTA-PSII, glucose oxidase, and catalase to give final concentrations of 250 μg of Chl/mL, 0.4 mg/mL, and 1.1 × 10^4 IU catalase/mL, respectively; and (2) then serum stoppers, equipped with gas inlet/outlet ports, were quickly inserted into the vials. A gentle stream of 99.999% N_2 was then directed onto the surface of the mixture during a 15-min dark preincubation at 23 °C and during the subsequent illumination regimes. The O_2 concentration in the gas stream from the outlet ports was determined polarographically to be ≤2 μM.

Following photoinhibition at aerobic/anaerobic conditions, the membranes were transferred to microfuge tubes in ice, pelleted, then washed twice with cold Chelexed buffer A before resuspension (≥250 μg of Chl/mL) in the same buffer prior to assays. These washes were necessary to remove from the membranes any additions of glucose/glucose oxidase and chemical scavengers of oxy radicals which otherwise gave significant reduction of DCIP in activity measurements.

Photoactivation. NH_2OH/EDTA-PSII were treated exactly as for photoinhibition. Subsequently, the sample mixtures (0.3 mL), containing 250 μg of Chl/mL, 50 μM DCIP, 1 mM $MnCl_2$, and 25 mM $CaCl_2$, were preincubated (10 min) in darkness at 23 °C and then illuminated under 40 μE m^{-2} s^{-1} for 30 min to give the maximum extent of photoactivation.

Activity Assays. Determinations of rates of oxygen evolution of photoactivated samples and rates of PSII exogenous donor photooxidations of nonphotoactivated samples were made essentially as described previously (Blubaugh & Cheniae, 1990b). Unless otherwise noted, however, a limiting light intensity (48 μE m^{-2} s^{-1}) was used here in assays of exogenous donor(s) photooxidation by nonphotoactivated membranes. The assay mixture (20 μg of Chl/mL in buffer A) routinely contained 2 μM Mn^{2+}/3 mM H_2O_2 as exogenous electron donors and 50 μM DCIP as the electron acceptor. At these conditions, rates of DCIP photoreduction by control or photoinhibited membranes were linear for at least 2–3 min. Though 3 mM H_2O_2 itself was an ineffective PSII donor, it increased the quantum yield of Mn^{2+} photooxidation of nonphotoinhibited NH_2OH/EDTA-PSII to a value equivalent to that observed with DPC (Inoue & Wada, 1987).

Production of superoxide during aerobic photoinhibition of NH_2OH/EDTA-PSII was estimated using Cyt-*c* (McCord & Fridovich, 1968, 1969). A suspension of NH_2OH/EDTA-PSII (250 μg of Chl/mL in buffer A containing 80 μM Cyt-*c* plus additions where noted in the figure legends) was illuminated at conditions used for aerobic photoinhibition and then the membranes were pelleted. The absorbance (550 nm) of the supernatants from illuminated versus dark incubated samples was then determined directly without dilutions. The amount of Cyt-*c* reduced by superoxide was calculated using the differential extinction coefficient (21.0 mM^{-1}) for reduced minus oxidized Cyt-*c* at 550 nm (Massey, 1959).

Other Procedures and Source of Enzymes. The Mn content of NH_2OH/EDTA-PSII was determined by flameless atomic absorption spectrophotometry. Membranes were diluted to ≤130 μg of Chl/mL with MQ water and then 10 μL of the sample and 5 μL of a palladium nitrate/magnesium nitrate modifier reagent (Welz et al., 1988) were injected into the graphite furnace. Peak area absorbance (279.5 nm) was linear with Mn concentration up to 10 ng of Mn/mL.

The Cu, Zn-SOD from bovine ethryocytes and the buttermilk xanthine oxidase were purchased from Sigma Chemical Co., St. Louis, MO. Catalase (bovine liver) was obtained from Calbiochem Corp., San Diego, CA. Inactivated, denatured SOD was obtained by boiling the enzyme for 60 min.

RESULTS

High-Affinity Mn-Binding Affects the Kinetic Components of Photodamage to the PSII RC. Weak light illumination of wheat NH_2OH-PSII in the absence of externally added Mn^{2+} leads to rather rapid ($t_{1/2}$ ~ 2–3 min) loss of Y_Z^+ formation, the loss of high quantum yield photooxidations of exogenous electron donors (Mn^{2+}/DPC/I^-), and the loss of ~50% of the capability to carry out photoactivation (Blubaugh & Cheniae, 1990b; Blubaugh et al., 1991). The loss of the other ~50% of the photoactivation capability occurred more rapidly ($t_{1/2}$ ~ 0.8 min) and was correlated with a slowing of $Y_Z \rightarrow P_{680}^+$ electron transfer and the formation of a quencher of flash induced Chl *a* variable fluorescence. In similar studies of effects of weak light illumination on spinach NH_2OH-PSII, Ono and Inoue (1991b) found that the loss of Y_Z^+ formation and the capability of photoactivation as well as A_T-band emission all decayed in parallel during illumination. They found no evidence for the faster component of photodamage reported by Blubaugh et al. (1990b; 1991).

On the other hand, illumination of spinach NH_2OH/EDTA-PSII at conditions essentially equivalent to those used by Blubaugh et al. (1991) caused a very rapid ($t_{1/2}$ ~ 0.3 min) photodamage resulting in virtually complete (≥90%) loss of photoactivation capability and high quantum yield photooxidation of exogenous donors through the Y_Z-dependent site 1 locus (Blubaugh & Cheniae, 1990b) (data not shown). If the photodamages to the PSII RC donor side components/reactions are caused by PSII cation radicals (P_{680}^+/Chl^+/Y_Z^+), then the kinetic differences observed by various workers may relate to differences among membrane preparations of the abundance of adventitiously bound Mn^{2+} capable of reduction of Y_Z^+. Mn^{2+} bound with high affinity (K_m ~ 1 μM) at aspartate-170 of D_1 has been shown to reduce Y_Z^+ (Nixon & Diner, 1992), and additions of Mn^{2+} to NH_2OH-PSII (Blubaugh & Cheniae, 1990b) or Tris-PSII (Klimov et al., 1990) greatly diminish their susceptibility to photoinhibition.

In the experiments of Figure 1, spinach NH_2OH/EDTA-PSII containing only 0.19 ± 0.05 Mn/220 Chl was preincubated in darkness with various low Mn^{2+} concentrations and then illuminated for the times shown. Subsequently, the capacity of the membranes to photooxidize Mn^{2+}/H_2O_2 at limiting light conditions through the Y_Z-dependent site 1 was determined. In the absence of Mn^{2+} during illumination, the relative quantum yield of Mn^{2+}/H_2O_2 photooxidation declined rapidly and approached zero after only 5 min of weak light

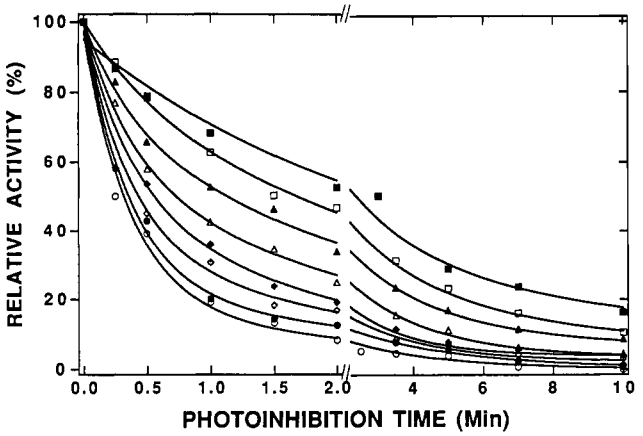

FIGURE 1: Effects of addition of increasing $MnCl_2$ concentrations on the time course of photoinhibition of spinach NH_2OH/EDTA-PSII by weak light. The $MnCl_2$ concentrations during photoinhibition were as follows: 0 (○), 50 nM (●), 200 nM (◇), 500 nM (◆), 700 nM (△), 1000 nM (▲), 1500 nM (□), and 2000 nM (■). (For clarity of presentation, data obtained with the addition of 100 and 300 nM $MnCl_2$ are not shown.) Following preillumination for times shown, the membranes were pelleted and washed before assay(s) (Materials and Methods). The lines are best fits by IGOR software (Lake Oswego, OR) to decay curves having multiple first-order components.

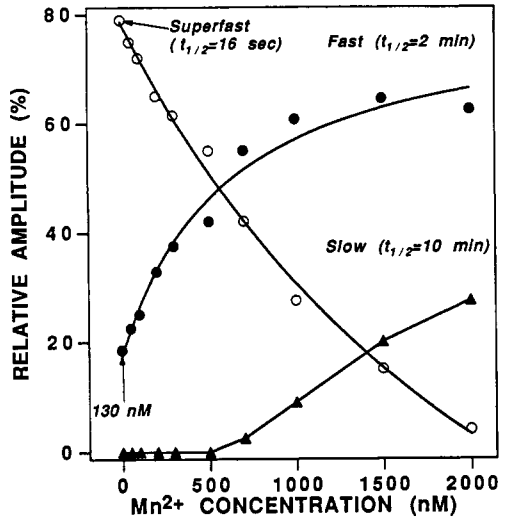

FIGURE 2: Analyses of the effects of $MnCl_2$ concentrations on the amplitudes and half-times of the kinetic components of photoinhibition of NH_2OH/EDTA-PSII. The results from the best-fit analysis for each of the decay curves of Figure 1 are plotted vs the $MnCl_2$ concentration giving the decay curve. The 130 nM Mn^{2+} value shown was obtained assuming Michaelis–Menten kinetics and extrapolation of the solid circle curve to zero relative amplitude. This value compares favorably to the experimentally determined value (170 nM Mn^{2+}). For other details, see text.

exposure. With increasing Mn^{2+} concentrations during illumination, both the initial rate and the extent of decline of the quantum yield of Mn^{2+}/H_2O_2 decreased. Best fit analyses (Figure 1 legend) of the data points of Figure 1 yielded the solid lines shown. The photodamages were best described by 2 or 3 kinetic components having different half-times and amplitudes dependent on the Mn^{2+} concentration present.

In Figure 2, we plotted the relative amplitudes of kinetic components having half-times of 16 s (superfast), 2 min (fast), and 10 min (slow) versus the concentrations of Mn^{2+} present during the illumination. This plot shows (1) in the absence of added Mn^{2+} (open circles), the amplitude of the superfast and fast components of photodamage are 80% and 20%, respectively, with no contribution from the slow component; (2) the amplitude of the superfast component markedly decreases while the amplitude of the slow component markedly

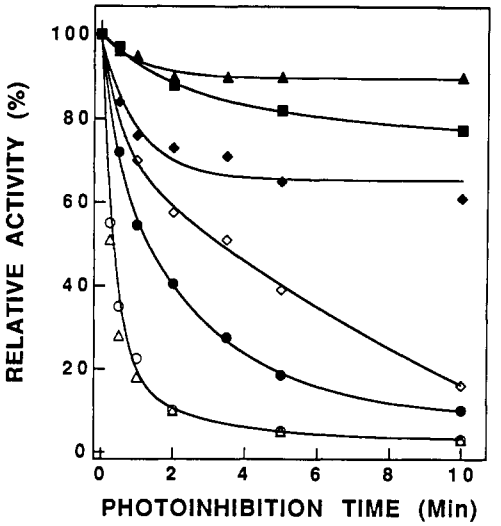

FIGURE 3: Electron donation to PSII by benzidine, NH_2OH, or a high affinity-bound Mn^{2+} protects against weak light photoinactivation. NH_2OH/EDTA-PSII was preilluminated in the absence of any additions (○) or in the presence of 40 μM benzidine (■), 100 μM NH_2NH_2 (▲), 30 μM NH_2OH (◇), 1 μM $MnCl_2$ (●), or 1 μM $MnCl_2$ plus either 30 μM NH_2OH (◆) or 100 μM NH_2NH_2 (▲). Subsequently, the membranes were washed before determination of DCIP photoreduction by Mn^{2+}/H_2O_2 (Materials and Methods).

increases with Mn^{2+} additions over the range of 0–500 nM; and (3) further increase of Mn^{2+} concentrations results in additional decreases and increases of the amplitudes of the superfast and fast components, respectively, and a slowly increasing contribution from a slow ($t_{1/2} \sim 10$ min) component of photoinhibition.

We assume that the relative amplitude of the superfast component would be 100% if the NH_2OH/EDTA-PSII membranes had been completely free of Mn and estimate that only ~720 and 2190 nM Mn^{2+} are required for 50% and 100% conversion of the superfast component into the fast/slow components, respectively. Since there are 220 Chl/RC for our PSII membrane preparations, these Mn^{2+} concentrations equate to only 0.83 Mn/RC and 2.12 Mn/RC with correction for the unextracted residual Mn. We also estimate that ~1250 nM Mn^{2+} (equivalent to ~1 Mn/RC) is sufficient for giving maximum amplitude of the fast component. The curve describing the increase in amplitude of the fast component as a function of Mn^{2+} concentration yields a K_m value of ~400 nM for the ~1 Mn/RC. This K_m value for Mn^{2+} compares favorably with the K_m for Mn^{2+}/H_2O_2 PSII electron donation (Inoue & Wada, 1987; Boussac et al., 1986), in Mn^{2+}/DPC competition assays of DCIP photoreduction (Hsu et al., 1987; Preston & Seibert, 1991), for Mn^{2+} in the first step in the assembly of the Mn cluster (Blubaugh & Cheniae, 1990a), and for the reduction of Y_Z^+ by Mn^{2+} bound at D_1 Asp170 (Nixon & Diner, 1992).

In the experiments of Figure 3, membranes were preincubated before illumination with 1 μM Mn^{2+} to give ~1 Mn^{2+}/RC (●) and, additionally, either 30 μM NH_2OH (◆) or 100 μM NH_2NH_2 (▲). Similarly, Mn^{2+} was omitted, and either 30 μM NH_2OH (◇), 100 μM NH_2NH_2 (△), or 50 μM benzidine (■) was added. These additions were predicated on the following: (1) at these low concentrations, neither Mn^{2+}, NH_2OH, nor NH_2NH_2 alone is a highly effective PSII electron donor as determined from quantum yield measurements of DCIP photoreduction with NH_2OH-PSII; (2) together at the same low concentrations, Mn^{2+} plus NH_2OH or Mn^{2+} plus NH_2NH_2 effectively support the photoreduction of DCIP by Mn-depleted PSII with a quantum yield essentially equivalent

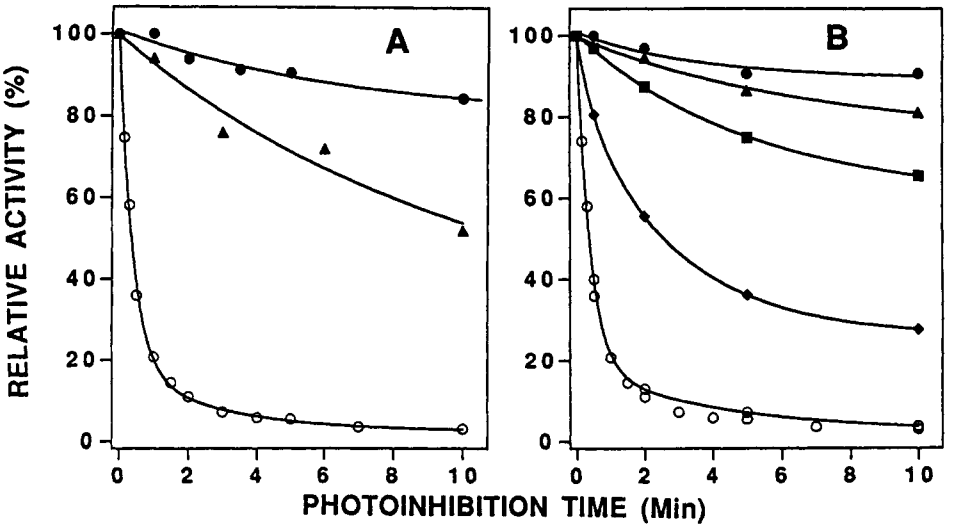

FIGURE 4: Strict anaerobiosis (A) and oxy-radical scavengers under aerobic conditions (B) protecting the donor side of NH_2OH/EDTA-PSII from weak light photoinactivation. (A) The closed circle (●) and closed triangle (▲) data were obtained by preillumination of NH_2OH/EDTA-PSII under strict anaerobic conditions (Materials and Methods) in the absence and presence of 1 mM FeCN, respectively. The open circle (○) data were obtained similarly except the illumination was made under aerobic conditions and without any additions to the membranes. (B) Aerobic conditions were employed (Materials and Methods) with additions of 2 mM Tiron (◆), epinephrine (▲), propyl gallate (●), or 1 mM butylcatechol (■) or with no addition (○).

to that using 1 mM DPC; and (3) the K_m for Mn^{2+} in the NH_2OH- or NH_2NH_2-supported electron donation is only ~400 nM (D. J. Blubaugh and G. M. Cheniae, unpublished results).

These requirements for sustained, high quantum yield electron donation by high-affinity bound Mn are reflected in the extents of protection from photoinhibition. In the absence of any additions (○) or the addition of 100 μM hydrazine (△), photoinhibition occurs rapidly. Though 1 μM Mn^{2+} (●) or 30 μM NH_2OH (◇) slows the photoinhibition process, the combination of Mn^{2+} plus NH_2OH (◆) or Mn^{2+} plus NH_2-NH_2 (▲) greatly inhibits the process even over prolonged illumination times. The extent of protection by Mn^{2+} plus NH_2NH_2 was about equivalent to that by benzidine (■), which has a high rate constant for the reduction of Y_Z^+ (Yerkes & Babcock, 1980).

These results (Figures 1–3) indicate that the kinetic components of weak light photoinhibition are modifiable by high-affinity Mn^{2+} and that sustained electron flow to Y_Z^+ via this Mn^{2+} or from benzidine effectively prevents photodamage of NH_2OH/EDTA-PSII. Photodamage of Tris-PSII by high light intensity also is inhibited by PSII-exogenous electron donors (Klimov et al., 1990; Eckert et al., 1991); thus, such observations generally support the idea that photodamages to the donor side RC components are of an oxidative nature and are promoted by the strongly oxidizing PSII cation radicals.

Effects of Oxygen and Oxygen-Radical Scavengers. Jegerschold and Styring (1991) have shown that high light (2900 μE m^{-2} s^{-1}) illumination of Cl^--depleted or Tris-treated thylakoids under either aerobic or strict anaerobic conditions leads to rather rapid ($t_{1/2}$ ~ 25 min) degradation of the D_1 polypeptide of the PSII RC. Likewise, their results indicated that a photoinactivation ($t_{1/2}$ ~ 2.5 min) of the water oxidation capability of Cl^--depleted thylakoids was independent of oxygen provided that electrons could flow through the PSII acceptor side. They therefore concluded that the D_1 protein was damaged by accumulated PSII cation radicals and that oxy radicals were not involved.

In the experiments of Figure 4A, NH_2OH/EDTA-PSII were photoinhibited for various durations under either aerobic conditions (○) or strict anaerobic conditions either in the presence of 1 mM FeCN (▲) or no additions (●). Bicarbonate (20 mM) was included in the suspensions to eliminate the possibility of anaerobic-induced inhibition of electron transfers through Q_A/Q_B (Sundby, 1990), and FeCN was added to maintain Q_A/Q_B in their oxidized forms. As shown, strict anaerobiosis in the absence of FeCN addition essentially abolished the photoinhibition observed at aerobic conditions. The large protective effect from strict anaerobiosis conceivably might reflect photoreduction of the limited electron acceptor pool in these types of membranes which would promote charge recombination and thereby decrease the population of the potentially damaging $P_{680}^+/Chl^+/Y_Z^+$ cation radicals.

On the other hand, photoinhibition was also greatly decreased by strict anaerobiosis when Q_A/Q_B was maintained oxidized by FeCN. Under such conditions, the PSII cation radicals should accumulate; nevertheless, the observed rate of photoinhibition was slow compared to that observed aerobically. Apparently, oxygen radicals, in addition to the PSII cation radicals, contribute to the rapid damages of PSII RC components during weak light illumination of NH_2OH/EDTA-PSII.

This supposition was examined in the experiments of Figure 4B where the effects of additions of various *o*-diphenols on aerobic photoinhibition of NH_2OH/EDTA-PSII were measured. These types of compounds have a high reactivity with oxygen radicals, including superoxide (Elstner, 1982). For example, the reduction of superoxide by propyl gallate has a reaction constant of 2.6×10^6 M^{-1} s^{-1} (Bors et al., 1989); moreover, this compound has been shown to significantly inhibit the light-dependent degradation of the D_1 polypeptide of oxygenic *Spirodela* cells under aerobic conditions (Sopory et al., 1990).

As shown in Figure 4B, all of the different *o*-diphenols added (closed symbols) inhibited the aerobic photoinhibition process relative to the control (○). The hierarchy of their inhibitory effectiveness was propyl gallate ≥ epinephrine > butylcatechol > Tiron, with propyl gallate and epinephrine giving essentially complete protection. The near complete protection by propyl gallate or epinephrine might suggest all damages are caused by oxygen radicals generated during

illumination of NH_2OH/EDTA-PSII. However, some of these *o*-diphenols also act as exogenous electron donors to PSII. In saturating light with NH_2OH/EDTA-PSII, 2 mM epinephrine and butylcatechol gave DCIP photoreduction rates of 320 and 90 μmol/(mg of Chl·h), respectively. (Rapid chemical reduction of DCIP by 2 mM propyl gallate precluded DCIP photoreduction measurements with NH_2OH/EDTA-PSII.) In contrast, no photoreduction of DCIP was obtained using 4 mM Tiron which, at a 2 mM concentration, increased the half-time of photoinhibition about 7–8-fold relative to that of the control. We interpret this protective effect from Tiron to be due to a scavenging of oxygen radicals formed during illumination of NH_2OH/EDTA-PSII. The virtually complete protection given by epinephrine/propyl gallate most likely reflects their capacity to (1) scavenge oxygen radicals and (2) donate electrons to PSII, thereby preventing the accumulation of the strongly oxidizing PSII cation radicals.

Evidences That Superoxide Contributes to Photodamages of NH_2OH/EDTA-PSII. The experiments of Figure 4 gave suggestive evidence that reactive oxygen species contributed to damages affecting the donor side components of NH_2OH/EDTA-PSII during weak light illumination. However, they gave no information as to the identity of the presumed damaging oxygen species or the reaction mechanisms leading to their formations. In photosynthetic systems, highly reactive and damaging singlet oxygen can be formed in a reaction between molecular oxygen and Chl triplet states. The double-reduction of Q_A or an empty Q_A site facilitates the light-induced formation of a Chl triplet due to charge recombination of P_{680}^+/$Pheo^-$ (van Mieghem et al., 1989; Vass et al., 1992). Such Chl triplet state formation has been shown to occur during anaerobic photoinhibition in strong light (Vass et al., 1992). Moreover, the photochemistry of PSII centers lacking Q_A is rapidly inhibited presumably by singlet oxygen formed during the illumination of such preparations (Takahashi et al., 1987).

It is unlikely that singlet oxygen is generated in this way and contributes to photoinhibition of NH_2OH/EDTA-PSII, since Q_A is present and functional in these membranes before and after photoinhibitory weak light regimes (Blubaugh & Cheniae, 1990b; Blubaugh et al., 1991; Ono & Inoue, 1991b). If reactive oxygen species are actually involved in photoinhibition of NH_2OH/EDTA-PSII, then they are likely to be generated by reactions between reduced Q_A/Q_B with molecular oxygen to give H_2O_2 and/or the superoxide radical, which together can react to form the highly oxidizing hydroxyl radical via the Haber–Weiss reaction (Elstner, 1982; Halliwell & Gutteridge, 1989). Illumination of oxygen-evolving PSII membranes in the absence of electron acceptors has been shown to produce H_2O_2 (Schröder & Akerlund, 1990).

Under aerobic conditions, the addition of PSII electron acceptors such as FeCN (■) or DCIP (◆) markedly decreased the initial rate and extent of weak light induced photoinhibition relative to that observed in the absence of any additions (Figure 5A, ○). Very similar protective effects from FeCN and DCIP were obtained even when a high light intensity (5100 μE m^{-2} s^{-1}; 0.3-cm light path) was used to promote rapid ($t_{1/2}$ ~ 5 s) photoinactivation (data not shown). Such results contrast sharply to those indicating a 2–3-fold acceleration of high light induced photoinhibition of Tris-treated PSII membranes by the addition of DCIP or FeCN (Klimov et al., 1990). They also contrast to those of Eckert et al. (1991), who showed that 40 μM DCIP had no effect on photoinactivation of Tris-PSII by a high intensity of light.

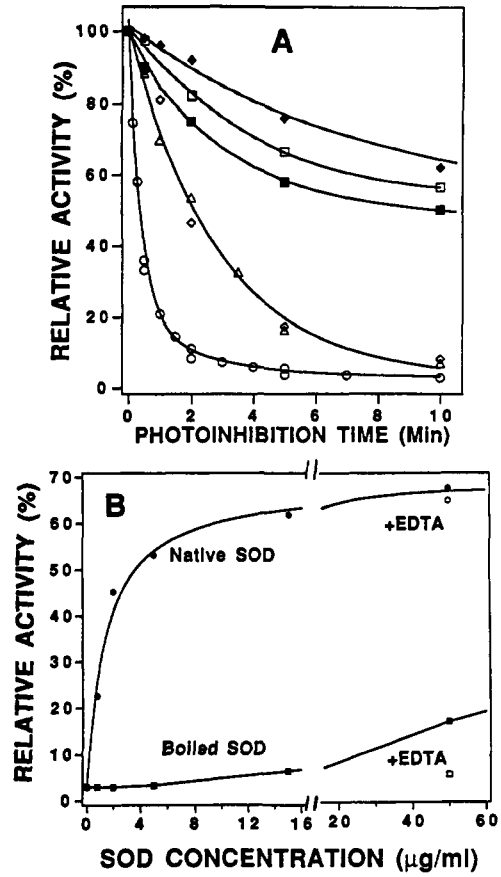

FIGURE 5: Evidences indicating that superoxide radicals are required in the photoinactivation mechanism. (A) The additions to NH_2-OH/EDTA-PSII during preillumination (Materials and Methods) were the following: none (○); 80 μM Cyt-*c* alone (△) plus either 5 μg/mL boiled SOD (◇) or 5 μg/mL native SOD (□); 50 μM DCIP (◆); or 1 mM FeCN (■). (B) SOD concentration dependence for inhibition of photoinactivation of NH_2OH/EDTA-PSII. Membranes were preilluminated for 5 min in the presence of the indicated concentrations of native (●) or boiled SOD (■). The open symbols indicate supplementation of the suspensions with 100 μM EDTA.

Cytochrome *c* reacts slowly, if at all, with reduced Q_A/Q_B, but it reacts rapidly with superoxide radicals and is frequently used to scavenge these radicals (McCord & Fridovich, 1968, 1969). Illumination of NH_2OH/EDTA-PSII in the presence of 80 μM Cyt-*c* resulted in the photoinhibition time course shown by the open triangles (△) of Figure 5A. Compared to the control (○), the initial rate of photoinhibition is 6-fold slower; nevertheless, virtually complete loss of activity occurred after about 10 min of illumination. Both the initial rate and the final extent of photoinhibition were further decreased when Cyt-*c* and 5 μg/mL native SOD were added before illumination of the membranes (Figure 5A, □). With these additions, the time course of photoinhibition was similar to those obtained with additions of either FeCN or DCIP; as little as 30–50% loss of activity occurred in 10 min while only approximately 3 min sufficed to nearly completely abolish the activity of control membranes. Nevertheless, prolonged illumination (>10 min) of the 5 μg/mL SOD plus Cyt-*c* containing membrane suspension caused further photoinactivation.

We asked if more complete protection from photodamage of NH_2OH/EDTA-PSII could be obtained simply by increasing the concentration of SOD during illumination. The concentration dependence of SOD for protection of NH_2-OH/EDTA-PSII from photodamage is shown in Figure 5B. In the absence of SOD addition, almost complete loss of activity occurred during a 3-min illumination; however, this loss was diminished maximally to ~32% with addition of 50–125 μg/

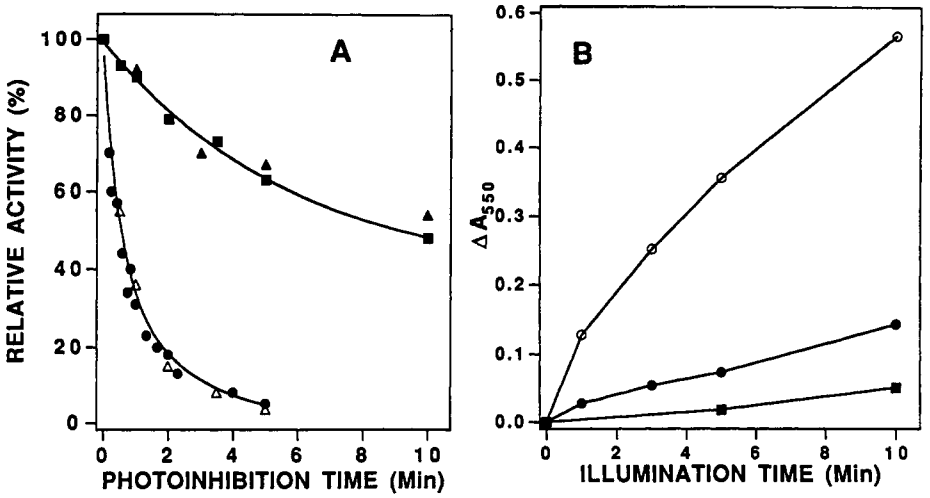

FIGURE 6: (A) Effects of additions of catalase and/or SOD on the time course of aerobic photoinhibition of NH_2OH/EDTA-PSII. The membranes were photoinhibited (Materials and Methods) either with no additions (●) or with the addition of 125 μg/mL (1.1×10^4 IU/mL) catalase (△), 20 μg/mL SOD (■), and 125 μg/mL catalase and 20 μg/mL SOD (▲). (B) Evidence for superoxide production during weak light illumination of NH_2OH/EDTA-PSII. Superoxide production was assayed by measurement of Cyt-*c* reduction (ΔA_{550}) by membranes in the presence of 80 μM Cyt-*c* alone (○) or, additionally, 10 μM atrazine (■), or 20 μg/mL SOD (●). For details, see Materials and Methods.

mL native SOD. Half-maximal protection required only ~1.2 μg (or 3.9 units/mL) of native SOD/mL, and no protection was obtained with boiled, denatured SOD solutions when EDTA was used to complex the Cu^{2+} released from the Cu/Zn-type SOD. The small extent of protection seen by boiled SOD in the absence of EDTA addition is attributed to free Cu^{2+}, which catalyzes the disproportionation of superoxide radicals but at rates far less than an equivalent amount of Cu^{2+} as Cu/Zn-superoxide dismutase. Indeed, addition of 10 μM $CuCl_2$ gave 30% protection from a 10-min photoinhibitory regime.

Such protective effects observed from Cyt-*c* or SOD could indicate a direct contribution of superoxide radicals to the damages of the donor side components of NH_2OH/EDTA-PSII. Alternatively, they might suggest that the damages are partly due to extremely reactive hydroxyl radicals formed in reactions between superoxide and H_2O_2 (Elstner, 1982; Halliwell & Gutteridge, 1989; Fridovich, 1985). The latter supposition predicts that addition of either SOD or catalase would give protection, and the combination of SOD and catalase might provide even greater protection than the single enzymes. Such differential protective effects by SOD and catalase have been reported in studies of photoinhibition of O_2-evolving broken spinach thylakoids (Barenyi & Krause, 1985). A partial protective effect of SOD/catalase was also reported by Richter et al. (1990) in their studies of photoinhibition of O_2-evolving thylakoids at a high light intensity. However, as shown in Figure 6A, catalase addition (△) to NH_2OH/EDTA-PSII did not modify the time course of photoinhibition observed with control membranes (●). Moreover, the extent of protection by SOD alone (■) was as great as that from the addition of both SOD and catalase (▲). A 10-fold greater amount of catalase did not modify the results shown; thus, these results further implicate superoxide in the photoinactivation mechanism. They give no evidence that hydroxyl radicals are formed and contribute to the mechanism.

A search of the literature, however, gave no evidence for superoxide production by illuminated NH_2OH-PSII (or PSII) membranes. Therefore, in the experiments of Figure 6B, the Cyt-*c*-based assay system (McCord & Fridovich, 1968, 1969) for superoxide was used to determine the capacity of NH_2OH/EDTA-PSII for superoxide production. This assay system somewhat underestimates the actual rate of superoxide formation, since the rate constant for Cyt-*c* reduction by superoxide (5×10^5 M^{-1} s^{-1}; Kono et al., 1976) is only ~5-fold greater than the rate constant for the spontaneous dismutation of superoxide to H_2O_2 and molecular oxygen (Elstner, 1982; Fridovich, 1985) at the pH of our experiments.

The open circles describe the time course of reduction of Cyt-*c* (ΔA_{550}) on illumination of NH_2OH/EDTA-PSII at a condition equivalent to that used for weak light photoinhibition. As shown, the initial slope (≤1 min) is steep but then progressively bends off with time. We note here that the loss of capacity of Cyt-*c* reduction (superoxide production) is slower than the loss of capacity of photooxidation of Mn^{2+}/H_2O_2 during photoinhibition of NH_2OH/EDTA-PSII in the presence of Cyt-*c* (Figure 5A, ◇). The increase of ΔA_{550} was markedly inhibited by only 10 μg/mL SOD (Figure 6B, ●) and essentially abolished by 10 μM atrazine (Figure 6B, ■), an inhibitor of electron transfer from Q_A^- to Q_B. Thus, the data of Figure 6B show that superoxide is generated during illumination of NH_2OH/EDTA-PSII by reactions requiring Q_A/Q_B electron transfers. The rate of superoxide production, calculated from the initial slope of the open circle data of Figure 6B, was only 1.2 μmol/(mg of Chl·h) (5 nmol/min) and varied little from experiment to experiment with many different NH_2OH/EDTA-PSII preparations. The actual rate of superoxide production must be greater than this value on the basis of the data of Figure 5A. In those experiments, relatively rapid photoinactivation was shown even in the presence of Cyt-*c* and was greatly decreased by addition of SOD. Apparently, the "target" in the photoinactivation mechanism is more reactive or accessible to the photoproduced superoxide than the added Cyt-*c*.

Effect(s) of Increased Superoxide Concentration on the Rate of Photoinactivation. Thus far, the data suggest that light generated cation radicals ($Y_Z^+/P_{680}^+/Chl^+$) of the RC and the superoxide radical somehow contribute to photoinactivation of NH_2OH/EDTA-PSII but they give no clue whether one or both type radicals may be rate limiting. With addition to NH_2OH/EDTA-PSII of sufficient xanthine/xanthine oxidase (McCord & Fridovich, 1968) to give 50 nmol of superoxide (Cyt-*c* reduced)/min under the same conditions used for photoinhibition, we observed the following (data not shown): (1) no decrease in the capability of NH_2OH/EDTA-PSII to photooxidize Mn^{2+}/H_2O_2 even with

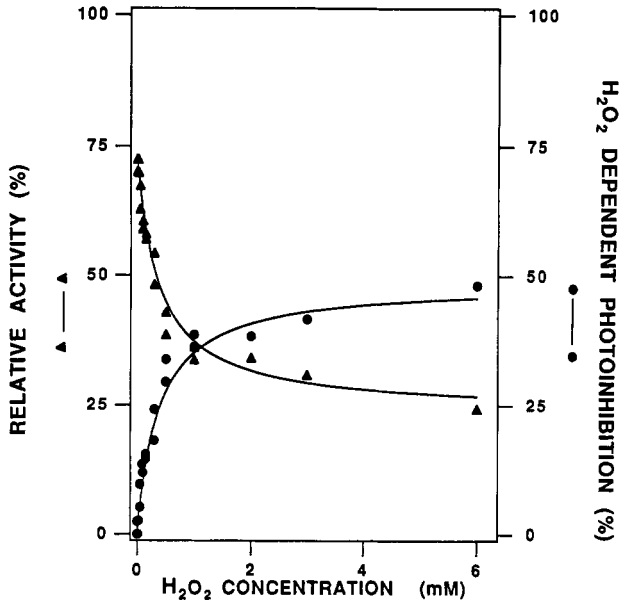

FIGURE 7: Hydrogen peroxide concentration dependence for increasing the extent of photoinactivation of NH_2OH/EDTA-PSII. Membranes were preilluminated with weak light for 10 s in the presence of the indicated concentrations of H_2O_2 and then pelleted, washed, and resuspended in buffer A before the assay of DCIP photoreduction (Materials and Methods).

prolonged (≥10 min) preincubation in darkness and (2) an approximately 2-fold increase in the initial rate of loss of capability to photooxidize Mn^{2+}/H_2O_2 relative to illuminated control membranes producing only ~5 nmol of superoxide/min.

The absence of inactivation of PSII photochemical activity by superoxide in darkness is not particularly surprising. Only a few proteins including those containing catalytic 4Fe-4S clusters have been shown to be irreversibly denatured by superoxide directly (Fridovich, 1986; Gardner & Fridovich, 1991, and references therein). The 2-fold increase in the initial rate of photoinactivation by the addition of the xanthine/xanthine oxidase-superoxide generating system may seem minimal; however, the dismutation of superoxide is a rapid, spontaneous process with the reaction second order in superoxide; thus, for a 10-fold increase in rate of superoxide production with addition of the xanthine/xanthine-oxidase system, we would have expected a 3.16-fold increase in the initial rate of photoinactivation, if the rate were linearly proportionate to superoxide concentration. The observed ~2-fold increase in the rate of photoinactivation is less than predicted; nevertheless, these results suggested that the rate of superoxide production by NH_2OH/EDTA-PSII during illumination did limit the rate of photoinactivation of the secondary donors to P_{680}^+.

According to Mano et al. (1987), the photooxidation of low concentrations of hydrogen peroxide to molecular oxygen by Tris-PSII membranes proceeds via superoxide as an intermediate. If the mechanism of photoinactivation of the secondary donors involves reaction(s) between cation radicals ($Y_Z^+/P_{680}^+/Chl^+$) and superoxide, or products formed therefrom, then the rate of photoinactivation should be increased by addition of low concentrations of hydrogen peroxide.

The closed triangles (▲) of Figure 7 record the effect of addition of increasing hydrogen peroxide concentrations on the extent of photoinhibition produced by a 10-s illumination of NH_2OH/EDTA-PSII. In the absence of any additions, the relative activity remaining was 73% of the dark controls; however, as the hydrogen peroxide concentration was increased, the relative activity sharply decreased and ultimately approached a value of about 24% at 6 mM H_2O_2. We plotted the increased extent of photoinactivation caused by H_2O_2 addition versus H_2O_2 concentration and estimated a K_m of 0.38 mM for H_2O_2 from the resulting curve (●, Figure 7). This hydrogen peroxide concentration dependence for promotion of photoinactivation compares favorably with the hydrogen peroxide concentration dependence for superoxide production by Tris-PSII in flashing light (Mano et al., 1987). On the other hand, the contribution of superoxide to photoinactivation shown earlier cannot be directly related to the hydrogen peroxide effects shown in Figure 7: (1) even if we assume all superoxide produced (Figure 6B) disproportionated to give hydrogen peroxide, the concentration (≤6 μM) after 2 min of illumination is far less than the K_m value (360 μM) for hydrogen peroxide obtained in the experiments of Figure 7; and (2) the addition of catalase to illuminated control members (Figure 6A) to decompose any hydrogen peroxide formed had no effect on the course of photoinactivation.

The open (○) and closed (●) circles of Figure 8A show the time course of the loss of Mn^{2+}/H_2O_2 photooxidation capability resulting from weak light illumination of NH_2OH/EDTA-PSII in the absence and presence of 3 mM hydrogen peroxide, respectively. As shown, the loss is greatly accelerated by the addition of hydrogen peroxide with only 5 and 45 s being required to give 50% and nearly total inactivation, respectively. Similarly, as shown in Figure 8B, the loss of capability for assembly of the Mn cluster of the oxygen-evolving enzyme (photoactivation) is markedly accelerated by weak light illumination of the membranes in the presence (●) relative to the absence (▲) of hydrogen peroxide. No loss of Mn^{2+}/H_2O_2 photooxidation or photoactivation capability occurred during dark incubations (≤5 min at room temperature) with 3 mM hydrogen peroxide; thus, we believe the acceleration of photoinactivation to be due to superoxide formed from univalent photooxidations of hydrogen peroxide by the PSII RC.

This conclusion is supported by the data shown by the closed squares (■) of Figure 8A. These results were obtained with membranes also preilluminated in 3 mM hydrogen peroxide; however, in this experiment SOD was added to rapidly disproportionate any superoxide formed during the illumination of NH_2OH/EDTA-PSII. Under these conditions, the accelerative effect from hydrogen peroxide was essentially abolished such that only ~13% photoinactivation occurred after a 10-min preillumination. Moreover, the time course of photoinactivation in the presence of SOD and hydrogen peroxide was appreciably slower than that observed with the addition of only SOD to the membranes (□). This protective effect from hydrogen peroxide seen in the presence of SOD is attributed to a low rate of donation of electrons from the hydrogen peroxide to the PSII RC. This would diminish the population of the highly oxidizing cation radicals. Apparently, the rate of SOD-catalyzed disproportionation of superoxide formed from hydrogen peroxide is much greater than the rate of superoxide, or a product formed therefrom, with its "target" in the PSII RC.

Effects of Scavengers of Hydroxyl Radicals and Singlet Oxygen. In aqueous solutions at physiological pH's, both hydrogen peroxide and superoxide have moderate reactivity; thus, in biological systems suffering inactivation/damage from their presence, the damage is generally believed to be due to a conversion of hydrogen peroxide/superoxide into more reactive species such as hydroxyl or hydroperoxyl radicals or singlet oxygen (Elstner, 1982; Halliwell & Gutteridge, 1989).

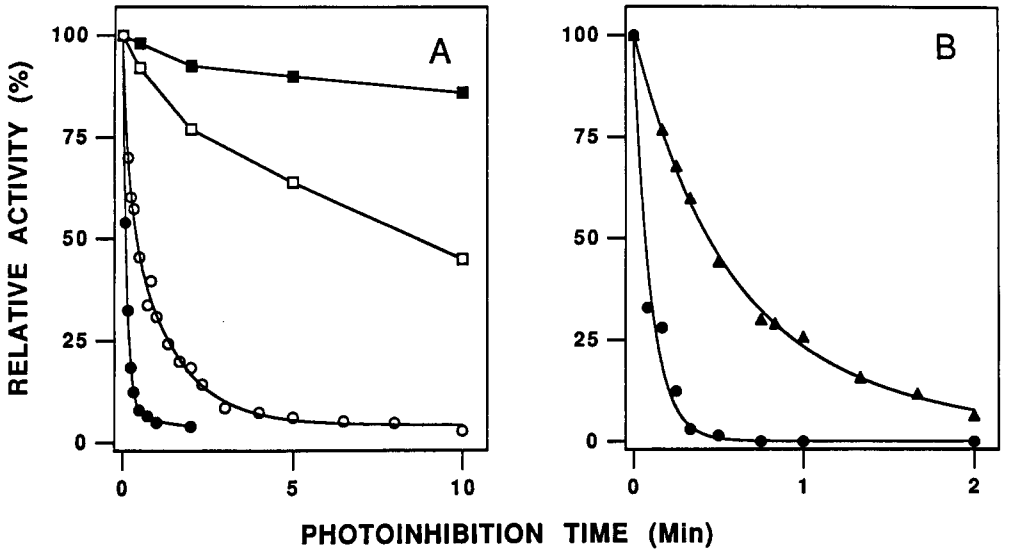

FIGURE 8: Effect of addition of hydrogen peroxide on the time course of aerobic photoinhibition of DCIP photoreduction by Mn^{2+}/H_2O_2 (A) and the photoactivation of the O_2-evolving enzyme (B) by NH_2OH/EDTA-PSII. (A) Membranes were photoinhibited in either the absence (open symbols) or the presence of 3 mM hydrogen peroxide (closed symbols). The open (□) and closed (■) squares show the effect of addition of 20 μg/mL SOD to membrane suspensions containing either no or 3 mM hydrogen peroxide, respectively. Subsequently, the membranes were pelleted and washed before determination of DCIP photoreduction capability (Materials and Methods). (B) Membranes were photoinhibited in either the absence (▲) or presence of 3 mM hydrogen peroxide (●) then pelleted and washed. Following photoactivation, rates of O_2 evolution were determined (Materials and Methods).

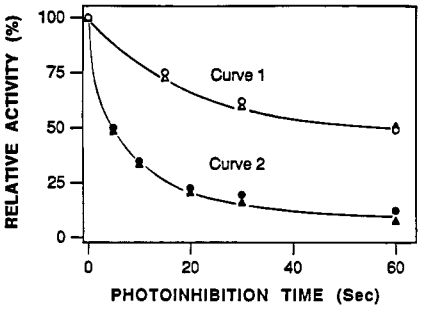

FIGURE 9: Mannitol, a scavenger of singlet oxygen and hydroxyl radicals, does not protect NH_2OH/EDTA-PSII from photoinactivation. Membranes were washed (80 μg of Chl/mL) and resuspended (250 μg/mL) in Chelexed 0.35 M NaCl/20 mM Mes, pH 6.5, containing no mannitol (○, ●) or 1 M mannitol (△, ▲) and then photoinhibited (Materials and Methods) in the presence (closed symbols) or absence (open symbols) of 3 mM H_2O_2. Following washes with buffer A, DCIP photoreduction assays were made.

The possible involvement of Haber–Weiss cycle generated hydroxyl radicals in the photoinactivation of NH_2OH/EDTA-PSII in the absence of any additions was considered earlier (Figure 6A); however, this possibility was disfavored on the basis of the insensitivity of photoinactivation to inhibition by catalase.

The experiments of Figure 9 were directed toward ascertaining whether the more rapid photoinactivation seen in the presence of hydrogen peroxide possibly was due to hydroxyl radicals formed via Haber–Weiss cycle reactions between the hydrogen peroxide added and the superoxide formed during illumination of the membranes. The rate constant for the reaction between the hydroxyl radical and mannitol has a reasonably high value (2.7×10^9 M^{-1} s^{-1}; Halliwell & Gutteridge, 1989); thus, mannitol was used as a scavenger. For these experiments, NH_2OH/EDTA-PSII membranes were washed and resuspended in 0.35 M NaCl/20 mM Mes, pH 6.5 buffer to eliminate sucrose from the system and thereby maximize any effects from addition of the mannitol.

The open circles (○) and triangles (△) of curve 1 of Figure 9 describe the course of photoinactivation of NH_2OH/EDTA-PSII in the absence and presence of 1 M mannitol, respectively, without addition of hydrogen peroxide. The closed circles (●) and triangles (▲) of curve 2 were obtained similarly except for the addition of 3 mM hydrogen peroxide to the membranes during illumination. In neither case did the high mannitol concentration offer any protection against photoinactivation. Since the rates of reaction of mannitol with hydroxyl radicals and singlet oxygen are similar (Elstner, 1982), the results of Figure 9 suggest that neither hydroxyl radicals nor singlet oxygen is involved in the rapid photoinactivation of NH_2-OH/EDTA-PSII membranes in either the presence or absence of hydrogen peroxide. We note that the half-time for photoinactivation in the presence of the 0.35 M NaCl buffer (Figure 9, ○) is about 2–3 times slower than shown in earlier figures with membranes in sucrose containing buffers. Possibly this reflects an effect of ionic strength on the reactivity of superoxide in the photoinactivation process. The reactivity of superoxide in its self-disproportionation is known to be diminished by ionic strength (Bray et al., 1977).

DISCUSSION

Previous studies made with Cl^-- and Mn-depleted thylakoids/PSII preparations have established that some of the redox-active components on the donor side of the PSII RC are highly susceptible to photoinactivation (photoinhibition) even in very weak light. Water oxidation is blocked in such preparations, and it is generally thought that the photodamage(s) to PSII RC donor side components/reactions is(are) a consequence of oxidation/modification by P_{680}^+/Chl^+ of nearby amino acid residues of $D_1 > D_2$ proteins and of nearby Chl/Car accessory pigments (Thompson & Brudvig, 1988; Blubaugh & Cheniae, 1990b, 1991; Jegerschold et al., 1990; Klimov et al., 1990). According to this thinking, the photoinactivation of Cl^-/Mn-depleted PSII should be inhibited by the presence of exogenous electron donors and be independent of molecular oxygen and reactions yielding highly reactive singlet oxygen and/or oxy radicals. On the other hand, reactive oxygen species are thought to contribute to the irreversible inactivation of PSII during high light illumination of oxygen-evolving preparations (Andersson & Styring, 1991; Vass et al., 1992).

In this study, we used spinach PSII membranes depleted of the water-oxidizing Mn cluster by NH_2OH/EDTA extraction with precautions to eliminate adventitious Mn^{2+}. We focused on two questions relevant to the mechanism of photoinhibition of these membranes by weak light—namely, the following: (1) Were photodamages suppressed by physiological concentrations of Mn^{2+} serving as an electron donor to PSII, and, if so, how many Mn^{2+} binding sites were required and how tightly did they bind Mn^{2+}?; (2) Is the inactivation mechanism for Mn-depleted PSII independent of molecular oxygen, as has been shown for Cl^--depleted thylakoids (Jergerschold & Styring, 1991)?

The analyses of the effects of added Mn^{2+} concentrations on the kinetics of photoinhibition of spinach NH_2OH/EDTA-PSII (Figures 1–2) gave evidence for a high-affinity ($K_m \sim$ 400 nM) Mn^{2+} site with an abundance of ~1 site/RC. Increasing occupancy of this site by Mn^{2+} increasingly diminished the most rapid ($t_{1/2} \sim 0.3$ min) component and increased the amplitude of a slower component ($t_{1/2} \sim 2$ min). Addition of Mn^{2+} (2 μM) completely eliminated the most rapid component and further increased the amplitude of the slowest component ($t_{1/2} \sim 10$ min). Though the additions of ≤ 2 μM Mn^{2+} thus greatly modified the kinetics and suppressed weak light photoinhibition, the protection was far from complete. Even Mn^{2+} concentrations of 5, 10, and 50 μM gave only 20%, 65%, and 85% protection from a 10-min weak light illumination regime (data not shown). On the other hand, addition of only 1 μM Mn^{2+} (~1 Mn/RC) gave approximately 85% protection when supplemented with 100 μM NH_2NH_2, which, by itself, gave no protection (Figure 3). This magnitude of protection was equivalent to that offered by benzidine, an effective reductant of Y_Z^+ (Yerkes & Babcock, 1980).

Taken together, such results strongly support the generally held view that an accumulation of P_{680}^+/Chl^+ can lead to an inactivation of the PSII donor side components. Additionally, they indicate (1) electron donation by Mn^{2+} bound at 1 high-affinity site/RC effectively suppresses photoinactivation and (2) under continuous weak light illumination, the protection by exogenous Mn^{2+} is limited either by the rate of displacement by Mn^{2+} of the Mn^{3+} photochemically formed at the high-affinity Mn^{2+}-binding site or by the reduction of the Mn^{3+} at the site by NH_2NH_2 (or NH_2OH). These interpretations are equivalent to those previously made by Inoue and Wada (1987) and Boussac et al. (1986) for electron donation by hydrogen peroxide to PSII.

Other data presented here lead us to continue that the donor side components of Mn-depleted PSII are subject to damage by another mechanism when the PSII cation radicals accumulate in the presence of oxygen. We suggest that this more rapid process involves a bimolecular reaction(s) between superoxide radicals with the PSII cation radical, Y_Z^+. Several observations, all made without addition of exogenous electron donors, support this conclusion. First, photoinhibition of NH_2-OH/EDTA-PSII is strongly suppressed in the absence of oxygen (Figure 4) even when FeCN (or PBQ) is supplied to maintain Q_A/Q_B oxidized and HCO_3^- is added to eliminate any inhibition of Q_A/Q_B electron transfers induced by anaerobic conditions (Sundby, 1990). This observation contrasts to those of Jegerschold and Styring (1991) indicating that the rate of photoinactivation of electron transfer from water or DPC to DCIP with Cl^--depleted thylakoids and the light-dependent degradation of D_1 with Cl^--depleted or Tris-treated thylakoids are not inhibited by strict anaerobiosis. This observation also contrasts to these by Wang et al. (1992) indicating that anaerobic conditions do not protect against high light photoinactivation of H_2O to DCIP activity of PSII membranes or DPC to DCIP activity of PSII membranes pretreated to inactivate water oxidation. However, they used a much higher light intensity than used in our work, and, at least with NH_2OH-PSII, the redox components on the donor side of PSII are differently photoinactivated under weak versus strong light (Ono & Inoue, 1991b). Moreover, Y_Z^+ does accumulate in Mn-depleted PSII but not in Cl^--depleted PSII (Boussac et al., 1992). Apparently, the mechanism of high light induced photoinactivation of donor side components of Cl^--depleted thylakoids is different from the mechanism of weak light induced photoinactivation of NH_2OH/EDTA-PSII.

Second, under aerobic conditions, PSII electron acceptors (FeCN, DCIP) strongly suppress the reaction(s) causing rapid loss of Mn^{2+}/H_2O_2 (or DPC) photooxidation capability. This effect by electron acceptors was observed under either weak or high light photoinhibitory regimes, and with either NH_2-OH/EDTA-PSII or Tris-PSII membranes. This protective effect contrasts to the stimulative effect the electron acceptors have on the photobleaching of carotenoids of Tris-PSII and interpreted to indicate that the high light induced photodamages of PSII donor side redox-active components are strictly oxidative (Klimov et al., 1990). This disparity possibly may indicate that carotenoid photobleaching is not necessarily closely correlated with photodamage of the donor side redox-active components of Mn-depleted PSII (Klimov et al., 1990; Blubaugh & Cheniae, 1990b; Blubaugh et al., 1991; Ono & Inoue, 1991b). According to our thinking, the suppression of photoinhibition by exogenous electron acceptors is a consequence of their oxidation of reduced PSII acceptors which otherwise are oxidized by oxygen to produce superoxide radicals. Atrazine, an inhibitor of electron transfer from Q_A^- to Q_B, inhibits the photoproduction of superoxide (Figure 6B); thus, the oxidation of Q_A^- by oxygen does not contribute significantly to superoxide formation.

The production by NH_2OH/EDTA-PSII of superoxide and its contribution to the inactivation of PSII donor side components appears improbable for a number of reasons: (1) the redox potential of the O_2/O_2^- couple [$E^1{}_o \sim 160$ mV (Rao & Hayon, 1973)] is not greatly different than the redox potentials of Q_B/Q_B^- ($E^1{}_o \sim +40$ mV), $Q_B/Q_B H_2$ ($E^1{}_o \sim -40$ mV), and LP Cyt-*b*-559 ($E^1{}_o \sim +50$ mV) (Cramer & Knaff, 1991); (2) the superoxide produced by NH_2OH/EDTA-PSII from the oxidation of Q_B^- or Q_BH_2 would be on the face of the membrane opposite the face containing the secondary donors to P_{680} and opposite to the bulk solution phase; (3) at the pH of our experiments, superoxide ($pK_a \sim$ 4.8) would exist mostly as a polar anion and thus would not freely diffuse across the membrane to the secondary donor reaction site(s); and (4) the source of electrons for its formation is limited to the photooxidations of Chl/Car (Klimov et al., 1990; Blubaugh et al., 1991) and any adventitious Mn^{2+}.

Nevertheless, scavenging of superoxide by Cyt-*c* or by *o*-diphenols, particularly Tiron, or the dismutation of superoxide by added SOD all greatly diminish the rate but do not abolish weak light photoinactivation of NH_2OH/EDTA-PSII. This strong suppression by SOD of weak light photoinactivation also is observed under high light conditions with either NH_2OH/EDTA-PSII or Tris-PSII membranes (data not shown). Thus, superoxide radicals contribute to the mechanism of strong light photoinhibition causing inactivation of a putative redox-active histidine while leaving Y_Z unaffected (Ono & Incoue, 1991b) as well as to the mechanism in weak

light causing parallel inactivations of Y_Z and the putative redox-active histidine (Ono & Inoue, 1991b; Blubaugh et al., 1991).

Third, increases of superoxide concentration above the low concentration produced during illumination of NH_2OH/EDTA-PSII increase the rate of weak light photoinhibition, measured by loss of capability of either exogenous donor photooxidation or photoactivation of the Mn cluster of the water-oxidizing enzyme. This is seen most notably (Figure 8) when the superoxide is produced near its presumed "target(s)" by photooxidation of hydrogen peroxide by Y_Z^+ (Mano et al., 1987), thereby minimizing any rate limitations imposed by diffusion of superoxide produced at the Q_B^- site to its "target(s)" or by a decrease in its concentration from disproportionation reactions.

On the other hand, prolonged preincubation (≥10 min) of NH_2OH/EDTA-PSII in strict darkness with enzymatically generated superoxide caused no detectable damages even when the enzymatic rate was 10 times the rate of superoxide production by illuminated membranes. The absence of an effect on PSII by superoxide anions in darkness tends to reflect the physicochemical properties of this radical. Namely, in aqueous solutions, the unprotonated superoxide anion is extensively hydrated and is not a very reactive radical either as a reducing agent or a weak oxidizing agent (Fridovich, 1986; Gardner & Fridovich, 1991, and references therein; Elstner, 1982; Halliwell & Gutteridge, 1989). In many instances, superoxide causes damages to molecules only indirectly by giving rise to more highly oxidizing and reactive species such as hydroxyl radicals or singlet oxygen.

However, efforts to find evidence that these more reactive species were formed and contributed to photoinhibition were uniformly negative. For example, the addition of catalase alone to eliminate possible hydroxyl radical formation from hydrogen peroxide/superoxide via the Haber–Weiss cycle had no effect on the time course of photoinhibition; moreover, its addition along with SOD gave no greater suppression than that by SOD alone (Figure 6A). Conversely, the addition of SOD essentially eliminated the very rapid photoinhibition promoted by hydrogen peroxide (Figure 8A); therefore, we strongly disfavor the possibility that hydrogen peroxide directly or indirectly contributes to the inactivation mechanism. Additionally, compounds such as 1 M mannitol (Figure 9), 5 mM azide, and histidine, all of which rapidly react chemically with hydroxyl radicals and/or singlet oxygen or which effectively quench singlet oxygen (Elstner, 1982; Halliwell & Gutteridge, 1989), had no discernible effect on the time course of photoinactivation in either the absence or presence of hydrogen peroxide (Figure 9). Additionally, a very high concentration (300 mM) of DABCO, a quencher of singlet oxygen (Halliwell & Gutteridge, 1989), had no effect on hydrogen peroxide promoted rapid photoinactivation (data not shown). The singlet oxygen-dependent slower lipid peroxidation by illuminated thylakoids is virtually completely inhibited by 100 mM DABCO (Takahama & Nishimura, 1975). Our results therefore contrast to those showing partial suppression of photoinhibition of oxygen-evolving thylakoids by various scavengers of hydroxyl radicals/singlet oxygen or enzymes preventing their formation (Barenyi & Krause, 1985; Richter et al., 1990; Setlik et al., 1990). If singlet oxygen is involved in the photoinhibition of NH_2OH/EDTA-PSII and is formed from the photooxidation of superoxide, we must postulate that this site is completely inaccessible to all of the various scavengers we have used.

As a working hypothesis to explain the rapid oxygen-/superoxide-dependent photoinactivation of NH_2OH/EDTA-(or Tris) PSII, we postulate that a bimolecular reaction between the superoxide radical and the Y_Z^+ radical leads to the formation of a redox-inactive peroxy derivative of Y_Z. We believe that this mechanism of damage to Y_Z during aerobic photoinhibition of spinach NH_2OH/EDTA-PSII best explains our unpublished observations showing the following: (1) strictly parallel and rapid losses of the Y_Z^+-EPR signal, the rapid $Y_Z \rightarrow P_{680}^+$ reaction, Chl *a* variable fluorescence amplitude, photoactivation capability, and Y_Z^+-dependent exogenous donor photooxidation which we observe in the absence of SOD addition; (2) an equivalent suppression of loss of each of these activities with addition of SOD; and (3) the marked slowing of loss of the Y_Z^+-EPR signal by SOD during in situ EPR measurements made even under strong light conditions. Whether the proposed mechanism is correct and also operative at the physiological level of thylakoids, intact chloroplasts, or cells remains to be established.

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