# Superoxide Contributes to the Rapid Inactivation of Specific Secondary Donors of the Photosystem II Reaction Center during Photodamage of Manganese-Depleted Photosystem II Membranes†

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ABSTRACT: The role of superoxide in the mechanism of photoinactivation of the secondary donors of the reaction center of photosystem II membranes depleted of Mn by extraction with $NH_2OH$ plus EDTA ($NH_2OH$/EDTA-PSII) was assessed. EPR analyses ($g = 2$ region) in continuous light, optical kinetic spectrophotometric analyses of $P_{680}^+$ and $Car^+$, and $A_T$-band emission measurements were made after various durations of weak and strong light treatment of $NH_2OH$/EDTA-PSII in the presence and absence of superoxide dismutase, or of PSII electron acceptors to suppress superoxide formation. Additionally, flash-induced variable fluorescence of chlorophyll *a* and the capabilities of the membranes of photooxidize $Mn^{2+}$ (in the presence of $H_2O_2$) via a high-affinity site ($K_m$ ~180 nM) and to carry out the photoactivation of the Mn-cluster were determined. In the absence of any additions to the $NH_2OH$/EDTA-PSII membranes which were highly depleted of Mn, weak light treatment caused rapid ($t_{1/2}$ ~20 s) and parallel losses of (a) the ~10 μs phase of $P_{680}^+$ reduction, which reflects the TyrZ → $P_{680}^+$ reaction, (b) the amplitude of chlorophyll *a* variable fluorescence, (c) the capability to accumulate the $TyrZ^+$-radical in continuous light, and (d) the capability to photooxidize $Mn^{2+}/H_2O_2$ in continuous light. As reported previously [Blubaugh et al. (1991) *Biochemistry 30,* 7586−7597], a dark-stable 12-G-wide featureless EPR signal centered at $g = 2.004$ was formed rapidly during illumination. This signal previously was tentatively identified as a $Car^+$ radical and was suggested to contribute to the quenching of chlorophyll *a* variable fluorescence and to the slowing of the TyrZ → $P_{680}^+$ reaction. However, we failed to detect $Car^+$ formation by sensitive optical spectrophotometry and obtained no definable evidence for either a quencher of fluorescence other than $P_{680}^+$ itself or a slowing of the TyrZ → $P_{680}^+$ reaction. Addition of a saturating concentration (96 units/mL) of superoxide dismutase diminished the rate of photodamage(s) by ~30-fold, but did not abolish it completely. Superoxide dismutase similarly suppressed strong light-induced photodamages, causing the loss of capability to photooxidize $Mn^{2+}/H_2O_2$, to carry out photoactivation, and to generate the $A_T$-band emission as well as $TyrZ^+$ EPR signal. In contrast to others, we found no evidence that the initial target(s) of photodamage is (are) different in weak versus strong light treatment. The totality of the results suggests that the initial event in either weak light or strong light photodamage of $NH_2OH$/EDTA-PSII is a decoupling of the redox activity of TyrZ from $P_{680}$. This occurs slowly when mediated by $P_{680}^+/TyrZ^+$ but much more rapidly in the presence of superoxide.

Exposure of photosystem II (PSII)[1] to an excess intensity of visible light causes irreversible photodamage affecting one or more of its reaction center (RC) components. These so-called photoinhibitory events promote subsequent proteolysis and replacement of the homologous $D_1$ and $D_2$ polypeptides of the RC. The more rapid turnover of $D_1$ suggests that it is more susceptible to photodamage(s) than $D_2$. At least two distinct types of photodamage have been proposed with vastly different quantum yields, light intensity requirements, and apparent mechanisms [for reviews, see Critchley (1988), Krause (1988), Andersson and Styring (1991), Prasil et al. (1992), and Aro et al. (1993)].

One type of photodamage may be initiated by the overreduction of $Q_A$, the PSII primary electron acceptor, as well as a one-electron redox component having an $E'_o$ of +20 mV (Nedbal et al., 1992) by electrons from the oxygen-evolving complex. The resulting irreversible lesions are thought to be caused by the highly oxidizing and reactive singlet oxygen that is formed in a reaction between oxygen

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[1] Abbreviations: Car, carotenoid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chl, chlorophyll; cyt *b*-559, cytochrome *b*-559; $D_1$ and $D_2$, homologous 32-kDa polypeptides which, as a dimer, form the PSII RC core; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEPC, diethyl pyrocarbonate; DPC, diphenylcarbazide; EPR, electron paramagnetic resonance; FeCN, potassium ferricyanide; His, histidine residue; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; $NH_2OH$/EDTA-PSII, PSII membranes extracted with $NH_2OH$ and EDTA to completely remove the Mn cluster; P680, PSII primary electron donor chlorophyll; PBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; $Q_A$ and $Q_B$, primary and secondary plastoquinone electron acceptors of PSII, respectively; RC, reaction center; $S_n$ (state), oxidation state of the water-oxidizing complex; SOD, superoxide dismutase; TyrZ and TyrD, redox-active tyrosines-161 of the $D_1$ and $D_2$ RC polypeptides, respectively.

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and the triplet state of $P_{680}$, the latter being generated by charge recombination between reduced pheophytin and $P680^+$ after the dissociation of doubly reduced $Q_A$ (van Mieghem et al., 1989; Vass et al., 1992; Hideg et al., 1994; Telfer et al., 1994). Photodamaging events of a different kind presumably are initiated by one or more of the highly oxidizing PSII radicals ($TyrZ^+/Chl^+/P_{680}^+$) which accumulate in the light following perturbation/inhibition of the oxygen-evolving complex by depletion of its Mn, $Cl^-$, or $Ca^{2+}$ [reviewed by Debus (1992) and Rutherford et al. (1992)]. Photodamages initiated by an overreduction of the PSII acceptor side require much greater light intensities and occur at a much slower rate with a much greater quantum requirement than damages initiated by the highly oxidizing PSII radicals (Callahan et al., 1986; Eckert et al., 1991). Thus, under physiological conditions, the functional status of the oxygen-evolving complex and the light intensity appear to be major determinants of the extent of contribution of the two types of photodamage. According to Eckert et al. (1991), the two mechanisms can operate simultaneously [see also Prasil et al. (1992)].

While it is clear that the donor side of the RC becomes selectively inactivated during illumination of PSII preparation depleted of $Cl^-$ (Theg et al., 1986; Jegerschold et al., 1990) or Mn (Callahan et al., 1986; Klimov et al., 1990; Blubaugh & Cheniae, 1990; Blubaugh et al., 1991; Eckert et al., 1991; Ono & Inoue, 1991b; Chen et al., 1992), the details of the mechanism(s) of inactivation of redox-active PSII secondary donors and the specific reactions catalyzing their inactivation remain poorly understood. On the other hand, it is generally agreed that primary charge separation ($P_{680}^+/Q_A^-$ formation) is unaffected by photodamaging treatments in either weak light (Blubaugh et al., 1991) or strong light (Klimov et al., 1990; Eckert et al., 1991) that cause extensive loss of secondary donor formations and functions.

Four or perhaps five different redox-active amino acid residues function on the donor side of PSII [for reviews, see Debus (1992) and Barry (1993)], and all have been shown to suffer damage/inactivation during the photodamage of Mn-depleted PSII [see Ono and Inoue (1991b) and Chen et al. (1992) and references cited therein]. These residues are as follows: (1) TyrZ of $D_1$, which normally serves to transfer electrons from the oxygen-evolving complex to $P_{680}^+$, and to transfer most electrons from artificial electron donors to $P_{680}^+$ when the oxygen-evolving complex is inhibited (Babcock, 1987), at least at low rates of $P_{680}^+/Q_A^-$ charge separation (Blubaugh & Cheniae, 1990); (2) TyrD of $D_2$, which is oxidized sluggishly by the $S_2/S_3$ states, reduced very slowly in the $S_0$ to $S_1$ dark transition (Barry, 1993), and normally reacts poorly with virtually all exogenous electron donors (Buser et al., 1990); (3) another tyrosine radical ($M^+$) which has been observed in *Synechocystis* mutants lacking TyrZ (Noren & Barry, 1992), or TyrD (Boerner et al., 1993; Boerner & Barry, 1994), and which, in the reduced state, may serve as an alternate donor to $P_{680}^+$ or to $TyrZ^+$ (Buser et al., 1990); (4) a redox-active, EPR-detectable $His^+$ radical which is observed at the $S_3$ state in PSII preparations depleted of $Ca^{2+}$ (Boussac et al., 1989; Ono & Inoue, 1990; Sivaraja et al., 1989) or $Cl^-$ (Baumgarten et al., 1990); and (5) His-190 of $D_1$, which is oxidizable (Kramer et al., 1994) at least after depletion of Mn, and which may be required (Ono & Inoue, 1991b) in the photoactivation process leading to the assembly of the Mn-cluster of the oxygen-evolving complex. In its oxidized form, His-190 of $D_1$ (Kramer et al., 1994) may give rise to the $A_T$ thermoluminescence band by recombination with $Q_A^-$ (Koike et al., 1986; Ono & Inoue, 1990, 1991a), and it can presumably oxidize exogenous $Mn^{2+}$ via a site having a very low affinity for $Mn^{2+}$ ($K_m \approx 17$ $\mu$M) (Ono & Inoue, 1991a). According to Ono and Inoue (1991b), this His residue may be the same His residue that becomes oxidized during illumination of $Ca^{2+}$-depleted PSII preparations.

Measurements of rates of electron transport, along with EPR analyses ($g = 2.0$ region) and spectrophotometric analyses of $P_{680}^+$ amplitude/relaxation following weak-light photoinhibition of $NH_2OH$-PSII under aerobic conditions, revealed that the resulting photodamages could be described essentially by three first-order reactions having half-times of ~0.8 min, 2−3 min, and ≥1 h at a light intensity of 40 $\mu E\ m^{-2}\ s^{-1}$. Photoactivation (partial), photooxidation of exogenous donors through TyrZ (site 1), and the capability to form $TyrZ^+$ become rapidly inhibited in a nearly parallel fashion, (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991). Such results were confirmed by Ono and Inoue (1991b), who additionally showed that the capability of $A_T$-band emission also was lost rapidly.

On the other hand, corresponding analyses made following strong-light photodamage of $NH_2OH$-PSII showed that about half of the capability of $A_T$-band emission and photoactivation and a portion (22%) of the total $Mn^{2+}$ photooxidation capability in the presence of 100 $\mu$M $Mn^{2+}$ were lost rapidly ($t_{1/2}$ ~1−2 s) (Ono & Inoue, 1991b). The complete loss of $TyrZ^+$ formation and the loss of the remaining fraction of $Mn^{2+}$ photooxidation capability occurred more slowly with a half-time of 17−22 s. This rate of loss of $TyrZ^+$ formation was about 4-fold faster than that measured by the same authors under a weak light regime and was about 10-fold faster than reported by Blubaugh et al. (1991) in weak light-induced loss of $TyrZ^+$ formation capability. Such marked differences between the processes/components in their hierarchy of susceptibility to photodamage by weak versus strong light led to the suggestion that two different mechanisms of photoinactivation operate at high and at low light intensities (Eckert et al., 1991; Ono & Inoue, 1991b; Prasil et al., 1992). Alternatively, one might infer that one common process operates in both weak and strong light, but the photodamaging actions on the different components/processes exhibit different dependencies on light intensities.

More recently, Chen et al. (1992) gave indirect evidence suggesting that, minimally, two mechanisms underlie the photoinactivation of PSII donor side components by weak light: (1) a rapid process requiring both superoxide radicals, generated at the acceptor side of PSII, and PSII RC radical(s) ($Chl^+/P_{680}^+$) on the donor side of PSII and (2) a slower process driven only by the donor side PSII RC radicals. However, these conclusions were based only on measurements of the capability of $NH_2OH$/EDTA-PSII to photoreduce DCIP by the electron donor combination of 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ following aerobic or anaerobic weak light photodamaging regimes in the presence of PSII electron acceptors or scavengers of superoxide radicals. Here we report EPR and optical measurements of the abundances of $P_{680}^+$, $TyrZ^+$, and $TyrD^+$ and kinetic measurements of $P_{680}^+$ relaxation, as well as the capability of $A_T$ emission, after various durations of weak or strong light photodamage of $NH_2OH$/EDTA-PSII in the presence or absence of superoxide

dismutase. The data further support the hypothesis offered by Chen et al. (1992) for the rapid kinetic component of photodamage of $NH_2OH$/EDTA-PSII, namely, a bimolecular reaction between superoxide radicals and PSII donor side radicals at both low and high light conditions that possibly leads to the formation of highly reactive singlet oxygen.

## MATERIALS AND METHODS

*Sample Preparation.* The preparation of spinach $NH_2OH$/EDTA-PSII membranes, highly depleted of their Mn (0.19 ± 0.05 Mn/RC), and their subsequent photodamage in 15-mm-diameter glass vials by weak light (50−60 $\mu$E $m^{-2}$ $s^{-1}$ from cool white diffused fluorescent lamps) under conditions to avoid contamination by $Mn^{2+}$ have been described (Chen et al., 1992). These membranes contain normal abundance of the 33-kDa extrinsic protein but are partially depleted of the 17/23-kDa extrinsic proteins. The same light intensity and Chl concentration (250 $\mu$g of Chl/mL) with equivalent optical geometry were used for the preparation (in 1-L beakers) of photodamaged samples to be analyzed by EPR. Several such batches of membranes photodamaged for the same duration at 23 °C were pooled, concentrated by centrifugation at 4 °C, and then resuspended/homogenized in Chelex-100-treated 0.8 M sucrose/50 mM Mes−NaOH/15 mM NaCl, pH 6.5 (buffer A), to give 3−4 mg of Chl/mL. Subsequently, they were incubated at 4 °C for ≥6 h to permit decay of $TyrD^+$ (Buser et al., 1990). The samples generally were stored several weeks at −80 °C before making EPR measurements.

Strong light (5100 $\mu$E $m^{-2}$ $s^{-1}$) illumination of samples (250 $\mu$g of Chl/mL in buffer A) was carried out at 23 °C in a 0.3 cm light path cuvette which was uniformly illuminated by a light beam filtered through a hot mirror, two Schott S116 filters, and one Corning 3−68 filter. Light intensity was varied using neutral density filters. A UniBlitz shutter facilitated the timing of the exposure of the sample to the light beam. Following illumination, the membranes were transferred to microfuge tubes at 4 °C in darkness, pelleted, and then resuspended for assays. When superoxide formation was measured using the cyt-*c* based procedure (McCord & Fridovich, 1968), 80 $\mu$M cyt-*c* was present during illumination. After the membranes were pelleted as described previously, the absorbance (550 nm) of the supernatants from illuminated versus dark-incubated samples was determined directly.

Photoactivation was carried out essentially as described by Tamura and Cheniae (1987) using a sample mixture (0.3 mL) containing 250 $\mu$g of Chl/mL, 50 $\mu$M DCIP, 1 mM $MnCl_2$, and 25 mM $CaCl_2$ in buffer A. Following 10 min preincubation in darkness at 23 °C, the samples were illuminated at 40 $\mu$E $m^{-2}$ $s^{-1}$ for 30 min to give maximal photoactivation. Under these conditions, different photoactivated spinach $NH_2OH$/EDTA-PSII preparations routinely gave $\Delta V_{O_2}$ values of 300−350 $\mu$mol of $O_2$·(mg of Chl)$^{-1}$·$h^{-1}$. All handling of membranes prior to and following the photoinhibition regimes was done in strict darkness.

Chemical modification of $NH_2OH$/EDTA-PSII membranes with DEPC was done essentially as described by Tamura et al. (1989) with the exception that all buffers had been treated with Chelex-100 resin.

*EPR Measurements.* Immediately prior to the EPR measurements ($g = 2$ region), the thawed samples were supplemented (from a 100× stock solution) with 81 units/mL SOD (unless stated otherwise) and then loaded in standard quartz flat cells. The SOD markedly slowed the otherwise rapid decrease in the amplitude of $TyrZ^+$ during illumination of the samples in the EPR cavity. The measurements were made at room temperature with a Varian Model E109B spectrometer operating near 9.5 GHz with a modulation amplitude of 5.0 G, a microwave power of 20 mW, a time constant of 0.3 s, and a sweep width of 100 G. Illumination by 16.4 mE $m^{-2}$ $s^{-1}$ of white light at the slotted face of the EPR cavity was provided by a projector lamp. The first-derivative spectra were recorded simultaneously by a microcomputer and a chart-recorder. The spectra were normalized with respect to Chl concentration.

*$P_{680}^+$ and $Car^+$ Formation and Decay.* The formation and decay of $P_{680}^+$ were measured at room temperature essentially as described by Blubaugh et al. (1991) with the following exceptions: (1) a CW-titanium sapphire laser provided the monochromatic 820 nm measuring light; and (2) the sample (80 $\mu$g of Chl/mL in buffer A) contained 1 mM FeCN, 4 mM EDTA, and 40 $\mu$M benzidine, an effective reductant of $TyrZ^+$ (Yerkes & Babcock, 1980). The amplitude of the fast decaying ($t_{1/2} \leq 10$ $\mu$s) component of $P_{680}^+$ relaxation was about 2-fold greater in the presence than in the absence of benzidine. Signal-averaging of 16 flashes (1 Hz frequency) was used on all samples. The formation and decay of $Car^+$ (Schenck et al., 1982) were measured similarly, but at 920 nm in the presence or absence of 5 $\mu$M CCCP and with omission of the benzidine.

*Activity Assays and Thermoluminescence Measurements.* DCIP photoreduction at room temperature was measured essentially as described previously (Blubaugh & Cheniae, 1990) at 600 nm in buffer A supplemented with 50 $\mu$M DCIP and with either 1 mM DPC, 100 $\mu$M $MnCl_2$ or 2 $\mu$M $MnCl_2$/3 mM $H_2O_2$ as electron donor unless otherwise noted. Assays at low light intensity (48 $\mu$E $m^{-2}$ $s^{-1}$) were made using 25 $\mu$g of Chl/mL while those at saturating light intensity (4800 $\mu$E $m^{-2}$ $s^{-1}$) were made using 5 $\mu$g of Chl/mL. At the quantum yield intensity, a rate of 20−25 $\mu$mol of DCIP reduced·(mg of Chl)$^{-1}$·$h^{-1}$ routinely was observed with either 1 mM DPC or 2 $\mu$M $MnCl_2$/3 mM $H_2O_2$ as the electron donor. At the saturating light intensity, the typically observed rates of DCIP photoreduction, expressed as micromoles per milligram of chlorophyll per hour, were 327−380 for 1 mM DPC or 2 $\mu$M $MnCl_2$/3 mM $H_2O_2$ and 90−119 for 100 $\mu$M $MnCl_2$.

$O_2$-evolution activity was measured polarographically at room temperature in saturating light. The assay mixture contained 0.8 M sucrose/50 mM Mes−NaOH (pH 6.2)/1 mM FeCN/300 $\mu$M PBQ/15 mM $CaCl_2$ and photoactivated $NH_2$-OH/EDTA-PSII membranes equivalent to 10 $\mu$g of Chl/mL. Flash-induced measurements of Chl *a* variable fluorescence yield by control and photodamaged samples were made essentially as described previously (Blubaugh & Cheniae, 1990) with the exception that the samples (250 $\mu$g of Chl/mL) in buffer A contained 10 $\mu$M DCMU either in the absence or in the presence of 10 mM $NH_2OH$, as indicated in the figure legends and text.

Thermoluminescence was measured with a setup similar to the one described by Ichikawa et al. (1975). Following the light treatments, the membranes were diluted (~50 $\mu$g of Chl/mL) with buffer B [0.4 M sucrose/50 mM Mes−NaOH (pH 6.5)/15 mM NaCl], pelleted, and then washed

twice (~50 μg of Chl/mL) and resuspended (>1 mg of Chl/mL) in buffer B. For measurements of the $A_T$ band (Koike et al., 1986), the dark-adapted samples were frozen in liquid $N_2$; then during heating (approximately 1 °C $s^{-1}$), they were illuminated between −40 and −20 °C with a 15 W incandescent lamp and then immediately cooled in liquid $N_2$. Subsequently, light emission was recorded against the sample temperature.

Determinations of [Chl] were made as described by MacKinney (1941). The Cu, Zn-type SOD (EC 1.15.1.1) was purchased from Sigma Chemical Co., St. Louis, MO, and had a specific activity of 3250 units/mg of protein.

## RESULTS

*Characterization of the Susceptibility of the Kinetic Constants of $Mn^{2+}$ in the $Mn^{2+}/H_2O_2$-PSII Donor System to Weak Light Photodamage.* It is generally accepted that exogenous $Mn^{2+}$ is oxidized almost exclusively by $TyrZ^+$ under either weak continuous light or repetitive flash conditions (Hoganson et al., 1989; Blubaugh et al., 1991; Nixon & Diner, 1992). However, in strong continuous light, in addition to $TyrZ^+$ (site 1), another unidentified redox-active PSII component (site 2) contributes to the oxidation of $Mn^{2+}$ (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991), the $K_m$ values for $Mn^{2+}$ at sites 1 and 2 being 10 and 200 μM, respectively. The 10 μM $K_m$ value agrees with the value reported by Hoganson et al. (1989), who measured $TyrZ^+$ reduction using repetitive measurements (200−500 experiments at 0.25 Hz), and is close to the 17 μM $K_m$ value reported by Ono and Inoue (1991a) for $Mn^{2+}$-dependent inhibition of the $A_T$ thermoluminescence. Weak-light photodamage of wheat $NH_2OH$-PSII membranes has been shown to decrease the 10 μM $K_m$ value for $Mn^{2+}$ at site 1 to a 2.5 μM $K_m$ value at this same site (Blubaugh & Cheniae, 1990).

On the other hand, high-affinity ($K_m \leq 1$ μM) $Mn^{2+}$ binding site(s) can be observed under continuous light conditions by addition of low concentration(s) of $H_2O_2$ to assays of $Mn^{2+}$ photooxidation (Boussac et al., 1986; Inoue & Wada, 1987; Inoue et al., 1987). Presumably, the limiting step of $Mn^{2+}$ photooxidation in continuous light is the exchange of $Mn^{2+}$ with the $Mn^{3+}$ formed and bound at the Mn binding site (Boussac et al., 1986). $H_2O_2$, which reduces $Mn^{3+}$, overcomes this limitation and permits high rates of $Mn^{2+}$ photooxidation in strong light (Boussac et al., 1986) with high quantum yield in weak light (Chen et al., 1992; Blubaugh & Cheniae, 1992). The high-affinity $Mn^{2+}$ site observed in the presence of $H_2O_2$ possibly is identifiable with (1) the $D_1$-Asp-170 high-affinity $Mn^{2+}$ binding site ($K_m \sim 1$ μM), determined from measurements of the $Mn^{2+}$ concentration dependence for inhibiting charge recombination between $Q_A^-$ and the PSII donor side following a single actinic flash (Nixon & Diner, 1992; Diner & Nixon, 1992), and (2) the $Mn^{2+}$ binding site in the first step of the light-driven assembly of the $O_2$-evolving Mn-cluster (Blubaugh & Cheniae, 1992). This so-called photoactivation process has been shown to be one of the most rapidly inhibited reactions during photodamage by either weak (Blubaugh & Cheniae, 1990; Chen et al., 1992; Ono & Inoue, 1991b) or strong light (Ono & Inoue, 1991b; Klimov et al., 1990).

Figure 1 summarizes experiments analyzing the effects of weak light treatment of spinach $NH_2OH$/EDTA-PSII membranes on the $K_m$ value of the high-affinity $Mn^{2+}$ binding site measured in the presence of 3 mM $H_2O_2$. An Eadie−Scatchard plot of the $Mn^{2+}$ concentration dependence for the photoreduction of DCIP at a weak light intensity by control, nonphotoinhibited membranes was linear, indicating only one $Mn^{2+}$ binding site having a $K_m$ value of 174 nM [see also Blubaugh and Cheniae (1992)]. The same conclusion was reached when strong saturating light was used in the DCIP photoreduction assays; thus, in our hands [see, however, Inoue et al. (1987)] with $Mn^{2+}/H_2O_2$ as the exogenous electron donor, the two independent sites of $Mn^{2+}$ photooxidation observed in studies of $Mn^{2+}$ photooxidation in the absence of added $H_2O_2$ (Blubaugh & Cheniae, 1990; Preston & Seibert, 1991) were not observed.

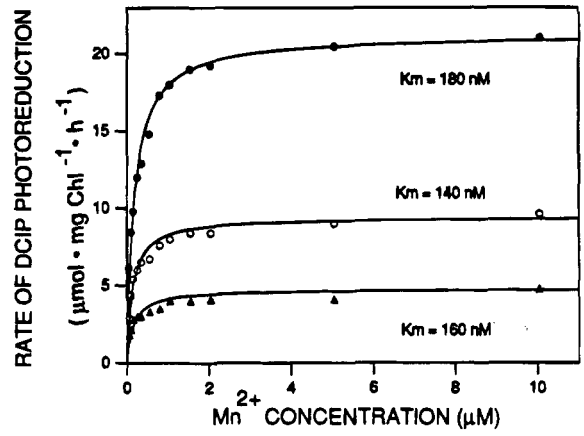


FIGURE 1: Velocity curves of the $Mn^{2+}$ concentration dependence for photoreduction of DCIP in the presence of 3 mM $H_2O_2$ by control nonphotodamaged membranes (closed circles) and membranes photodamaged for 30 s (open circles) and for 60 s (closed triangles). The $Mn^{2+}$ concentration included the Mn bound to the membranes (0.19 Mn/RC). The light intensity used was 48 μE $m^{-2}$ $s^{-1}$. [Chl] was 25 μg $mL^{-1}$. The solid line velocity curves shown were calculated (IGOR Software, Lake Oswego, OR) from $K_m$ and $V_{max}$ values: solid circles, $K_m$ = 180 nM, $V_{max}$ = 21; open circles, $K_m$ = 140 nM, $V_{max}$ = 9.3; and closed triangles, $K_m$ = 160 nM, $V_{max}$ = 4.6 where $V_{max}$ is expressed as μmol·(mg of Chl)$^{-1}$·$h^{-1}$ measured at quantum yield intensity.

The three velocity curves of DCIP photoreduction as a function of $Mn^{2+}$ concentration in the presence of 3 mM $H_2O_2$ which are shown in Figure 1 were obtained using nonphotodamaged membranes (closed circles) and membranes subjected to weak light treatment for either 30 s (open circles) or 60 s (closed triangles) to give 55.7% of $V_{max}$ and 78.1% photodamage of $V_{max}$, respectively. The solid line for each set of data points represents a "best-fit" analysis (IGOR Software, Lake Oswego, OR) to a single site of $Mn^{2+}$ photooxidation following Michaelis−Menten kinetics with various $K_m$ and $V_{max}$ values. Best fits were obtained using the $K_m$ and $V_{max}$ values indicated in the legend and the $K_m$ values shown by each of the velocity curves. These analyses show that the $K_m$ for $Mn^{2+}$ at its high-affinity site of photooxidation is only slightly affected during light treatments causing ≤78.1% loss of DCIP photoreduction by electrons from $Mn^{2+}/H_2O_2$. These data were obtained using thoroughly Mn-depleted membranes which previously have been shown to suffer loss of $Mn^{2+}/H_2O_2$ photooxidation capacity in weak light via a mechanism exhibiting a simple exponential with a half-time of ~0.3 min and an amplitude of 80−90% in the absence of addition of SOD or PSII electron acceptors (Chen et al., 1992). Such rapid decay kinetics are typical of the photodamages affecting TyrZ (site 1) and are atypical for those affecting site 2 (Blubaugh &

Cheniae, 1990; Blubaugh et al., 1991). Thus, such "Mn-free" PSII membranes and the presumed strictly TyrZ-dependent $Mn^{2+}/H_2O_2$ photooxidation assay were used along with direct measurements of $TyrZ^+$ formation in efforts to directly assess the contribution(s) of superoxide to damages of PSII donor side components during weak and strong light treatment regimes.

*Effects of Weak Light Photodamage on the Fast Phase of $P_{680}^+$ Reduction: Response to Addition of SOD.* Two main phases of $P_{680}^+$ reduction are observed in non-$O_2$-evolving PSII membranes: a fast reduction by TyrZ with a pH-dependent half-time of 2−40 $\mu$s (Conjeaud & Mathis, 1980) and a slower reduction (100−200 $\mu$s half-time) by charge recombination between $P_{680}^+/Q_A^-$ (Gläser et al., 1976; Conjeaud & Mathis, 1980). Either weak light exposure of wheat $NH_2OH$-PSII (Blubaugh et al., 1991) or strong light exposure of spinach Tris-PSII (Eckert et al., 1991) has been shown to decrease rapidly the amplitude of the fast phase of $P_{680}^+$ reduction (TyrZ → $P_{680}^+$ electron transfer) without diminishing the initial amplitude of the flash-induced absorbance change reflecting $P_{680}^+/Q_A^-$ charge separation. Though prolonged strong light treatment of Tris-PSII diminishes the amplitude of $P_{680}^+$, this decrease is >60 times slower than the loss of the fast phase of $P_{680}^+$ reduction (Eckert et al., 1991).

A previous analysis of weak light photodamage to donor-side components of $NH_2OH$-PSII (Blubaugh et al., 1991) gave evidence that the loss of the fast phase component of $P_{680}^+$ reduction (equivalent to $TyrZ^+$ formation) occurred with the same half-time (0.8 min) as the appearance of a 12-G-wide featureless EPR signal (centered at $g = 2.004$) which was tentatively identified as a carotenoid radical ($Car^+$) [see also van Wijk et al. (1992)]. On the other hand, the loss of the capability to photooxidize exogenous donors via $TyrZ^+$ or to generate $TyrZ^+$, as viewed by EPR measurements, occurred much more slowly ($t_{1/2}$ ~2 min). The disparity between the rate of loss of the capacity to form $TyrZ^+$ as seen in flash-optical measurements versus EPR measurements in continuous light was attributed to a drastic slowing of electron transfer from TyrZ to $P_{680}^+$, possibly due to an electrostatic interaction between TyrZ and the presumed $Car^+$. Such impairment was suggested to diminish the quantum yield of $TyrZ^+$ formation in the flash regime used for optical measurements; however, complete conversion of TyrZ to $TyrZ^+$ occurred in continuous light in the EPR measurements. In the measurements of the abundance of $P_{680}^+$ and its reduction by optical analyses, no exogenous electron donor to $TyrZ^+$ was used; thus, the full amplitude of the fast phase component of $P_{680}^+$ reduction possibly may not have been observed. Accordingly, in the measurement of $P_{680}^+$ abundance and its subsequent reduction by TyrZ reported here, benzidine was added to serve as an electron donor to $TyrZ^+$ (Yerkes & Babcock, 1980) while the presence of FeCN maintained $Q_A/Q_B$ in their oxidized states.

Figure 2A shows the effects of increasing durations (0−300 s) of weak light treatment of "Mn-free" spinach $NH_2$-OH/EDTA-PSII in the absence of any additions on the amplitude and decay of the absorbance change at 820 nm. Precautions were taken to exclude $Mn^{2+}$ contamination from buffers and labware, since even nanomolar $Mn^{2+}$ concentrations (equivalent to ≤1 Mn/RC) significantly slow the kinetics of photodamage (Chen et al., 1992). With control nonphotodamaged membranes ($t = 0$), ≥90% of the $\Delta A_{820}$

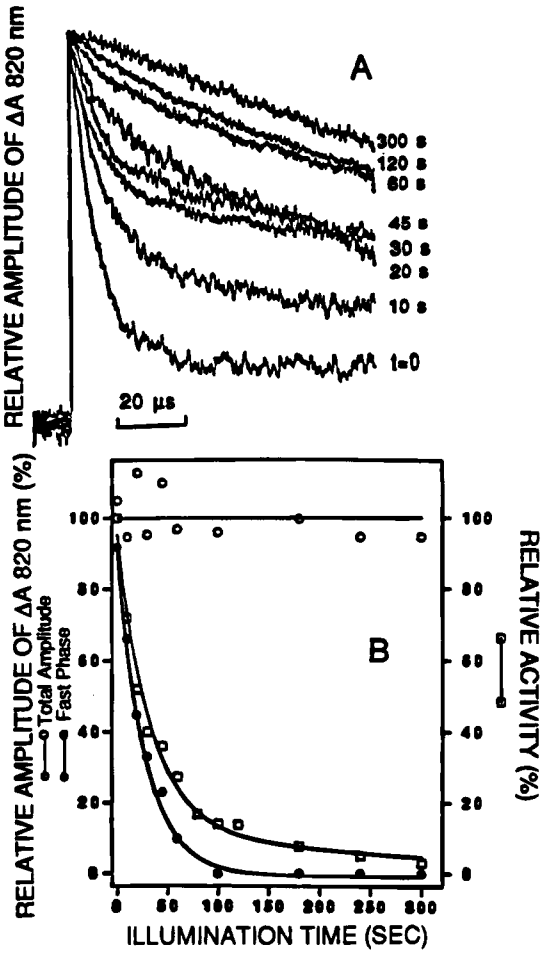


FIGURE 2: (A) Effects of weak light photodamage of spinach $NH_2$-OH/EDTA-PSII in the absence of SOD on the formation and reduction of $P_{680}^+(\Delta A_{820\ nm})$ following an actinic laser flash. The number to the right of each trace indicates the duration (seconds) of weak light treatment. (B) Effects of increasing time of weak light photodamage on the photoreduction of DCIP by 2 mM $Mn^{2+}$/3 mM $H_2O_2$ at a quantum yield intensity (open squares) versus the total relative amplitude of $P_{680}^+$ (open circles) and the relative amplitude of the fast (10 $\mu$s) phase of $P_{680}^+$ reduction (closed circles). The relative amplitudes of the fast phase of $P_{680}^+$ reduction were obtained by best-fit analyses (IGOR Software, Lake Oswego, OR) of the traces of panel A to biphasic or monophasic exponential decays.

absorbance change decayed with a half-time of ~10 $\mu$s in the determinations employing benzidine and FeCN. This amplitude of the fast phase of $P_{680}^+$ reduction is about 2-fold greater than the amplitude observed by Blubaugh et al. (1991) with nonphotodamaged wheat $NH_2OH$-PSII and no added electron donor. As shown, increasing durations of photodamage gave increasing inhibition of the rapid $\Delta A_{820}$ decay, but did not diminish the total amplitude of the $\Delta A_{820\ nm}$ absorption change reflecting $P_{680}^+/Q_A^-$ charge separation capability. Qualitatively, these results are consistent with those reported by Eckert et al. (1991) from studies of high light treatment of spinach Tris-PSII and by Blubaugh et al. (1991) from weak light treatment of wheat $NH_2OH$-PSII.

In Figure 2B, we have replotted from Figure 2A the relative total amplitude of the $\Delta A_{820\ nm}$ absorbance change (open circles) and its 10 $\mu$s decay component (closed circles) versus the time of photodamage treatment. Also shown is the same membranes' capability to photooxidize the electron donor combination of 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ (open squares). Loss of the 10 $\mu$s component of $P_{680}^+$ reduction

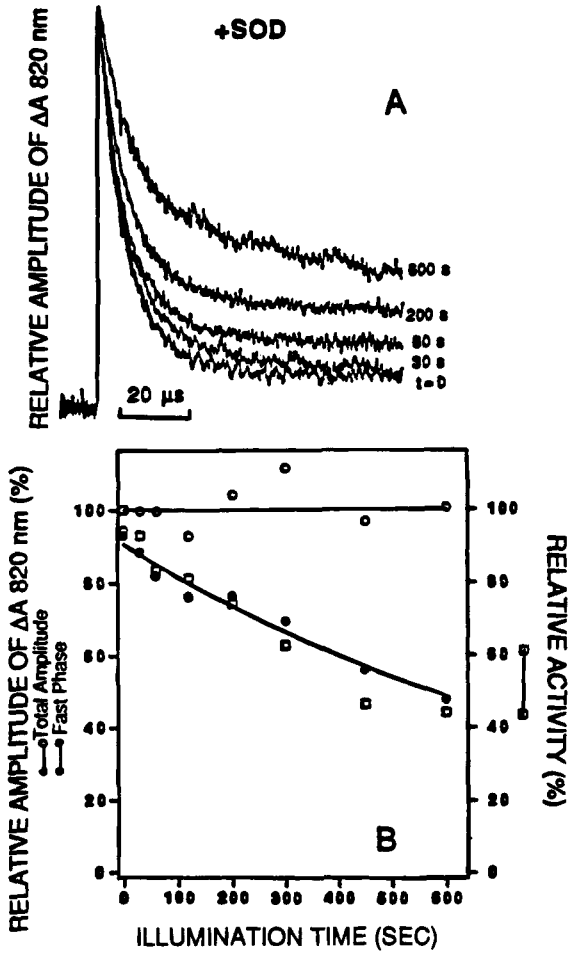


FIGURE 3: (A) Effect of weak light photodamage of spinach $NH_2$-OH/EDTA-PSII in the presence of SOD on the formation and reduction of $P_{680}^+$ ($\Delta A_{820\ nm}$) following an actinic flash. The number to the right of each trace is the duration (seconds) of photodamaging treatment. For purpose of clarity, data for 100, 120, 300, and 450 s have been omitted. The SOD concentration during illumination was 81 units/mL. (B) Plot of the duration of photodamage in the presence of SOD versus the relative rate of DCIP photoreduction by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ (open squares) and the total relative amplitude (open circles) and the fast phase reduction of $P_{680}^+$ (closed circles). For other details, see Figure 2 legend.

occurred with a half-time of 21 s and was monophasic, whereas the loss of $Mn^{2+}/H_2O_2$ photooxidation capability was biphasic: one component having a half-time and amplitude of 21 s and 75%, respectively, and the other component having a half-time and amplitude of 107 s and 25%, respectively. The weak light-induced parallel loss of $P_{680}^+$ reduction by TyrZ and the rapid phase of loss of $Mn^{2+}/H_2O_2$ photooxidation capability (Figure 2B) are ~6−9-fold more rapid than the loss of $Mn^{2+}$ photooxidation capability previously reported from studies of weak light photodamage of wheat $NH_2OH$-PSII membranes (Blubaugh et al., 1991); however, those membranes had not been rigorously treated to remove adventitious $Mn^{2+}$ which affords protection from photodamages (Chen et al., 1992).

The data of Figure 3 record the effects of addition of SOD (81 units/mL) to $NH_2OH$/EDTA-PSII membranes on their susceptibility to weak light photodamage affecting the TyrZ → $P_{680}^+$ reaction and the $Mn^{2+}/H_2O_2$ electron donor photooxidation capability through $TyrZ^+$. With the exception of the addition of SOD, these experiments were made exactly as those in Figure 2. Comparison of the traces of Figure 3A with those of Figure 2A shows that the loss of the 10 $\mu$s TyrZ → $P_{680}^+$ reaction was markedly slowed by the presence of SOD during the photodamage regime. This protective effect by SOD is particularly evident when the amplitude of the fast phase (10 $\mu$s) of $P_{680}^+$ reduction and the relative electron donor photooxidation capability are plotted as a function of the duration of light treatment (Figure 3B). This analysis reveals that both the TyrZ → $P_{680}^+$ reaction and $Mn^{2+}/H_2O_2$ electron donor photooxidation capability are lost with a half-time of ~600 s, i.e., more slowly by a factor of 30 than in the absence of SOD (Figure 2B). Nevertheless, the suppression of photodamages by such a saturating SOD concentration was incomplete. The remaining slow photodamages without loss of $P_{680}^+/Q_A^-$ charge separation capability presumably reflect direct oxidations by $TyrZ^+/P_{680}^+/Chl^+$ of nearby amino acid residue(s) and/or pigments, thereby causing inhibition of electron transfer transfer from TyrZ to $P_{680}^+$.

*Interrelationship between Weak Light-Induced Loss of $TyrZ^+$ Formation and $P_{680}^+$ Reduction by TyrZ: Protection by SOD against Loss of TyrZ.* The capability of $NH_2OH$-PSII to carry out photoactivation and to form $TyrZ^+$ is rapidly lost during weak light illumination (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991). Similarly, the capability to form the $A_T$ thermoluminescence band is lost rapidly as well (Ono & Inoue, 1991b). In contrast, the capability to form $A_T$ and to carry out photoactivation are lost more rapidly than the capability to form $TyrZ^+$ during strong light treatment (Ono & Inoue, 1991b). Such observations have led to the suggestion that a histidine residue(s) essential for photoactivation (Tamura et al., 1989) and for formation of $A_T$ (Ono & Inoue, 1991a) may be the initial target of photodamage in Mn-depleted PSII (Ono & Inoue, 1991b). In *Chlamydomonas*, mutation of His-190 of the $D_1$ polypeptide to phenylalanine completely abolishes the $A_T$ band and $O_2$ evolution as well as severely slowing (100−1000-fold) electron transfer from both TyrZ and TyrD to $P_{680}^+$ (Kramer et al., 1994). A similar slowing of TyrZ to $P_{680}^+$ is observed with mutation of $D_1$-His-190 to glutamine or aspartate in *Synochocystis* 6803 (Diner et al., 1991). Nevertheless, nearly full amplitude of TyrZ without modification of its EPR line shape can be observed with continuous illumination of an isolated RC preparation from such mutants [B. Diner, personal communication; see, however, Roffey et al. (1994b)]. If we postulate that $D_1$-His-190 is the initial and only target of photodamage during illumination of $NH_2OH$/EDTA-PSII and that this event would cause effects similar to those from mutation of this His residue, then no close kinetic correlation should be observed between the loss of the 10 $\mu$s component of $P_{680}^+$ reduction (TyrZ → $P_{680}^+$) measured in a 1 Hz repetitive flash regime and the loss of the $TyrZ^+$ EPR signal measured in continuous light. Alternatively, we cannot rule out the possibility that $TyrZ^+$ is shorter lived in our photodamaged samples than in the $D_1$-His-190 mutant (Roffey et al., 1994a), particularly in the absence of an electron acceptor to oxidize $Q_A^-$, and that a slowing of $TyrZ^+$ formation might cause an apparent loss of the $TyrZ^+$ EPR signal in continuous light.

Accordingly, for the experiments of Figure 4, $NH_2OH$/EDTA-PSII membranes were treated with weak light for various durations under the exact conditions used in the experiments of Figure 2. After concentrating the suspension to (>3−4 mg of Chl/mL), they were incubated in darkness

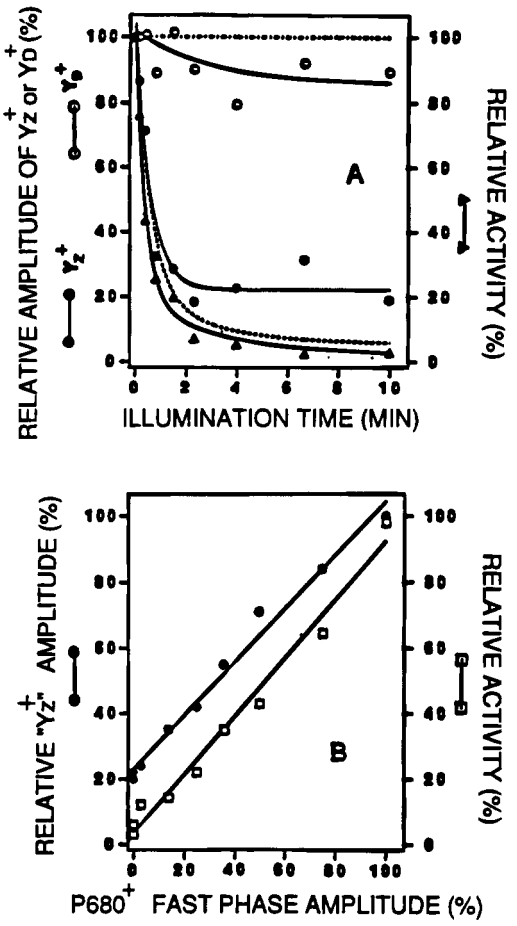


FIGURE 4: (A) Effects of weak light photodamage of spinach $NH_2$-OH/EDTA-PSII in the absence of SOD on the relative amplitudes of EPR signals $TyrZ^+$ and $TyrD^+$ and the relative activity of DCIP photoreduction by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$. The amplitudes of $TyrZ^+$ and $TyrD^+$ were estimated from peak heights at $g = 2.0104$ from derivative spectra recorded first with dark-adapted membranes and then during illumination of the membranes, and again in darkness following the illumination (spectra not shown). All signal amplitudes were normalized on a Chl basis. The solid lines represent "best-fits" to the experimental data points. The "best-fit" for relative activity was obtained with two components having half-times and amplitudes of 0.3 min (82%) and 2 min (18%). For loss of $TyrZ^+$ formation, the "best-fit" was obtained with two components, one having a "stable constant" amplitude (20%) and the other decaying with a half-time and amplitude of 0.4 min and 80% amplitude. The dashed line represents the loss of $TyrZ^+$ formation when corrected for the difference between the dot−dash line (constant 100%) and the curve for "loss" of $TyrD^+$ formation. See text for details. (B) Comparison between the loss of DCIP photoreduction by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ (open squares) and the loss of $TyrZ^+$ formation (closed circles) versus decrease of the fast phase of $P_{680}^+$ reduction ($TyrZ \rightarrow P_{680}^+$) caused by weak light photodamage.

(≥6 h/4 °C) to permit decay of $TyrD^+$ (Buser et al., 1990) and then stored at −80 °C in darkness for several weeks prior to EPR analyses. EPR spectra were recorded at room temperature under an illumination regime permitting observation of $TyrZ^+$, $TyrD^+$, and other EPR signals in the $g =$ 2.0 region. Generally, the so-obtained first-derivative spectra (data not shown) were entirely similar to those reported previously for weak light photoinhibited wheat $NH_2OH$-PSII (Blubaugh et al., 1991): (1) all of the photodamaged samples contained the featureless 12-G-wide signal (centered at $g =$ 2.004) even without reillumination; and (2) whereas the amplitude of the low-field maximum under continuous illumination was relatively constant, irrespective of the duration of photodamage treatment, the amplitude of the low-field shoulder ($g = 2.0104$; $TyrZ^+/TyrD^+$) progressively diminished, principally as a result of loss of the $TyrZ^+$ signal.

In Figure 4A, we have plotted the relative amplitudes of $TyrZ^+$ (closed circles) and $TyrD^+$ (open circles) as well as the relative $Mn^{2+}/H_2O_2$ donor photooxidation capability of the membranes (closed triangles) versus illumination time. Samples which had been photodamaged for >50 s gave a small "constant" signal amplitude (measured at the low-field shoulder, $g = 2.0104$) during illumination which decayed in ~1 min in subsequent darkness to an amplitude appreciably greater than the 12-G-wide signal present prior to illumination of the samples [see also Blubaugh et al. (1991)]. This behavior is typical for $TyrZ^+$ formation and decay. Though this light-induced signal with "constant" small amplitude is plotted in Figure 4A as $TyrZ^+$, we note the following: (1) the relative donor photooxidation capability measured in weak light continues to decrease at >50 s duration of photodamage; (2) in weak light, virtually all exogenous donors are photooxidized by $TyrZ^+$ (Babcock, 1987; Blubaugh & Cheniae, 1990); and (3) during weak light treatment of $NH_2OH$-PSII, any loss of capability to form $TyrD^+$ occurs very slowly ($t_{1/2}$ ~3−4 h) (Blubaugh et al., 1991; Ono & Inoue, 1991b). Accordingly, we assumed that this "constant" signal in photodamaged samples reflected a small amount (≤20%) of the total $TyrD^+$ which decayed rapidly in darkness following illumination, and that no actual loss of $TyrD^+$ occurred during ≤10 min weak light treatment. Such partial modification of the slow to a rapid decaying $TyrD^+$ signal has been observed with PSII preparations from mutants in which $D_1$-Tyr-161 was replaced by phenylalanine (Metz et al., 1989). With this assumption, we corrected the curve describing $TyrZ^+$ loss at >50 s by the difference between the assumed $TyrD^+$ amplitude (dash−dot line) and the measured $TyrD^+$ amplitude (open circles). This thus-obtained corrected curve for the relative amplitude of $TyrZ^+$, shown by the dashed line of Figure 4A, closely parallels the loss of capability to photooxidize the $Mn^{2+}/H_2O_2$.

In Figure 4B, we plotted the uncorrected relative amplitude of $TyrZ^+$ (closed circles) from the EPR analyses and the relative $Mn^{2+}/H_2O_2$ donor photooxidation capability (open squares) data of Figure 4A versus the relative amplitude of the 10 $\mu$s fast phase of $P_{680}^+$ reduction from the experiments of Figure 2. Although the uncorrected relative $TyrZ^+$ amplitude is displaced from zero because of the "constant" signal amplitude discussed above, this plot shows there are virtually proportionate decreases in $Mn^{2+}/H_2O_2$ donor photooxidation capability and $TyrZ^+$ amplitude measured with continuous light with the decrease in the 10 $\mu$s fast phase of $P_{680}^+$ reduction ($TyrZ \rightarrow P_{680}^+$) measured in a 1 Hz repetitive flash regime. If we assume the loss of the $TyrZ^+$ EPR amplitude reflects actual loss of capability to generate $TyrZ^+$, then the analysis shown in Figure 4B does not reveal a large slowing of electron transfer from $TyrZ \rightarrow P_{680}^+$ as it does after mutation of $D_1$-His-190.

The experiments summarized in Figure 5 were made to confirm conclusions reached from the data of Figure 3—namely, that dismutation of superoxide made during illumination of $NH_2OH$/EDTA-PSII diminished the rate of photodamage affecting electron donation from $TyrZ \rightarrow P_{680}^+$, measured by loss of the 10 $\mu$s component of $P_{680}^+$ reduction. Although SOD is known to scavenge reactive radicals besides superoxide, we consider the results with SOD to be indicative

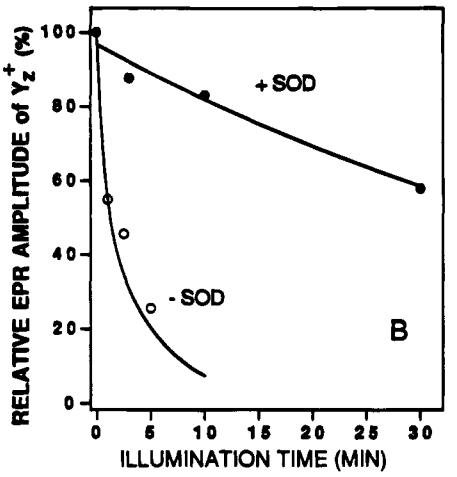


FIGURE 5: Effect of addition of SOD on the rate of loss of $TyrZ^+$ formation caused by weak light photodamage of spinach $NH_2OH$/EDTA-PSII. Batch photodamage treatment of membranes for subsequent EPR analyses was done as described under Materials and Methods. The SOD concentration was 81 units/mL.

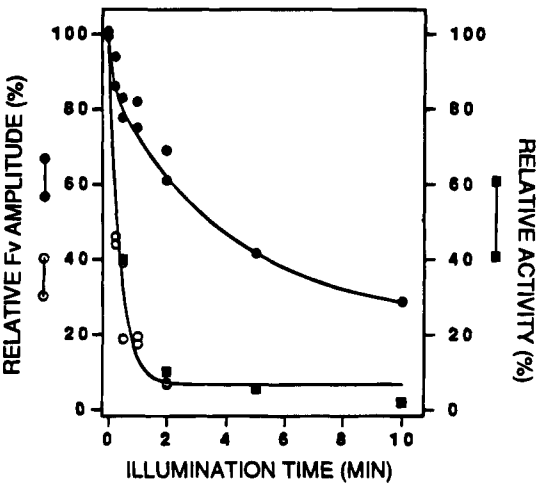


FIGURE 6: Relative amplitude of variable $Chl_a$ fluorescence induced by a flash (open and closed circles) and relative rate of DCIP photoreduction by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ at a quantum yield light intensity (closed squares). $NH_2OH$/EDTA-PSII membranes were photodamaged for the times indicated and then dark-adapted for >30 min before measurements of flash-induced $Chl_a$ fluorescence in the absence (open circles) and the presence of 10 mM $NH_2OH$ (closed circles). For other details, see Materials and Methods.

of an involvement of superoxide based on results previously reported (Chen et al., 1992). In those experiments, the rate of photodamage under strictly anaerobic conditions was essentially equivalent to the rate of photodamage under aerobic conditions in the presence of SOD or other oxygen radical scavengers. Here, direct EPR measurements of $TyrZ^+$ formation capability were measured with membranes previously subjected to weak light illumination in the absence and presence (81 units/mL) of SOD. As shown, the initial rate of loss of $TyrZ^+$ amplitude was very rapid in the absence of SOD but was slowed ~30-fold when SOD was present to dismutate the superoxide; thus, the data of both Figures 3 and 5, in combination with the results of Chen et al. (1992), clearly indicate that superoxide markedly enhances the rate of weak light-induced photodamage affecting TyrZ to $P_{680}^+$ and $TyrZ^+$ formation, respectively.

*Effects of Weak Light Photodamage on Flash-Induced $Chl_a$ Variable Fluorescence: Evidences against Formation of a Quencher Other than $P_{680}^+$.* The yield of flash-induced variable $Chl_a$ fluoresence is maximum in the state $P_{680}/Q_A^-$. Rapid $P_{680}^+/Q_A^-$ charge recombination or an impaired reduction of $P_{680}^+$ leading to the presence of the quencher, $P_{680}^+$, acts to diminish the fluorescence yield [for a review, see van Gorkom (1986)]. Since the quantum yield of $P_{680}^+$ reduction by TyrD or cyt *b*-559 is low (Buser et al., 1990), the amplitude of flash-induced variable $Chl_a$ fluorescence, in the absence of nonspecific quenchers, is a measure of the capability of TyrZ to reduce $P_{680}^+$. Previous analyses (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991) of the effects of weak light photodamage on the yield of flash-induced $Chl_a$ variable fluorescence, using wheat $NH_2OH$-PSII membranes less rigorously depleted of adventitious Mn than the membranes used here, gave evidence for a rapid ($t_{1/2}$ ~0.6 min) and large loss in the amplitude of the variable $Chl_a$ fluorescence, which kinetically correlated with the appearance of the 12-G-wide EPR signal and some partial slowing of $P_{680}^+$ reduction by TyrZ. As mentioned earlier, the actual loss of capability to form $TyrZ^+$ occurred 3–4 times more slowly. The explanation offered for this disparity was that the 12-G EPR oxidized species, tentatively identified as a carotenoid radical, was a quencher of $Chl_a$ variable fluorescence. This hypothesis found some apparent support from observations showing that postaddition of chemical reductants ($NH_2OH$, $NH_2NH_2$, DPC) abolished the presumed quencher. Yet, even with addition of reductant, the loss of amplitude of flash-induced $Chl_a$ variable fluorescence was ≥3–4 times slower than the loss of capability to form $TyrZ^+$. No cogent explanation was offered for this behavior. Moreover, subsequent efforts to optically detect formation of $Car^+$ radicals during a repetitive 1 Hz flash illumination of control and photodamaged $NH_2OH$/EDTA-PSII were uniformly negative (data not shown). Addition of a lipophilic anion did permit detection of $Car^+$, as previously reported by Schenck et al. (1982).

Accordingly, we reinvestigated the effects of weak light treatment on flash-induced variable $Chl_a$ fluorescence using "Mn-free" spinach $NH_2OH$/EDTA-PSII membranes. In the experiments summarized in Figure 6, membranes were illuminated for the times shown and then dark-incubated for >30 min at 4 °C before measurements of flash-induced $Chl_a$ variable fluorescence in the absence (open circles) or the presence of 10 mM $NH_2OH$ (closed circles). Additionally, rates of DCIP photoreduction by $Mn^{2+}/H_2O_2$ (closed squares) were determined since, as shown in previous sections, the loss of this activity closely parallels the loss of capability to form $TyrZ^+$ or to reduce $P_{680}^+$ by TyrZ. In the absence of $NH_2OH$ addition, both the variable $Chl_a$ fluorescence amplitude and the $Mn^{2+}/H_2O_2$ photooxidation capability declined rapidly and in parallel with increasing duration of photoinhibition. This result suggests that loss of functional TyrZ alone is sufficient to explain the loss of flash-induced $Chl_a$ variable fluorescence without having to invoke formation of a quencher other than $P_{680}^+$ itself. From these observations, as well as the apparent insensitivity of the $F_o$ level of $Chl_a$ fluorescence (Blubaugh & Cheniae, 1990), we therefore question the validity of the suggestion that a quencher is formed during weak light treatment of $NH_2OH$-PSII (Blubaugh et al., 1991). The loss of flash-induced $Chl_a$ variable fluorescence and also the loss of $Mn^{2+}/H_2O_2$ photooxidation capability were greatly and equally suppressed but not abolished by the presence of SOD (65 units/

mL) or DCIP (50 $\mu$M) during the weak light photodamaging regime (data not shown). Thus, these data and those given in previous sections give direct evidence that superoxide contributes significantly to the rapid mechanism of weak light photodamage.

As shown by the closed circles of Figure 6, addition of 10 mM $NH_2OH$ to the membranes restored much of the diminished fluorescence, even to those membranes illuminated for times causing virtually complete loss of TyrZ functions (Figures 2A and 4A). Restoration of variable fluorescence nearly equivalent to that shown by the closed circle data of Figure 6 could be obtained with photodamaged membranes which had been preincubated in darkness with 1−2 mM $NH_2OH$ or $NH_2NH_2$ and then washed repetitively in strict darkness to remove the $NH_2OH$ or $NH_2NH_2$ before measurements of flash-induced $Chl_a$ fluorescence (data not shown). The effect from these exogenous electron donors was abolished by one to two actinic flashes given to the preincubated and subsequently washed membranes prior to the fluorescence measurements. It could be restored by a new addition of $NH_2OH$ or $NH_2NH_2$ or by adding the high redox potential electron donor benzidine. These results suggest the occurrence of an intrinsic PSII component having the properties of the following: (1) during photodamage of $NH_2OH$/EDTA-PSII, this component in the abundance of 1−2 per RC becomes oxidized and remains oxidized; (2) it is slowly reducible by exogenous PSII electron donors; and (3) in the reduced state, it reduces $P_{680}^+$ more rapidly than $P_{680}^+/Q_A^-$ charge recombination when TyrZ donation to $P_{680}^+$ is lost. Although we have no evidence for the identity of this presumed component, it seems likely that its oxidation is correlated with the appearance of the 12-G-wide EPR signal, which was reported to be dark-stable (Blubaugh & Cheniae, 1991). Since the kinetics with which this 12 G signal appeared were not correlated with loss of the $TyrZ^+$ EPR signal under nonstringent conditions of Mn depletion, it is unlikely to be merely a modified TyrZ. The curve described by the solid circles of Figure 6 therefore shows the time course of photodamage to this presumed intrinsic PSII component that renders it unable to serve as an "alternate" electron donor to $P_{680}^+$.

*Dependence of the Rate of Photoinactivation of $Mn^{2+}/H_2O_2$ Photooxidation and the Rate of Superoxide Formation on Light Intensity.* Previous results (Blubaugh et al., 1991; Ono & Inoue, 1991b; Chen et al., 1992) and those in previous sections have shown that the photodamage(s) abolishing TyrZ-redox activity can be described essentially by two kinetic processes. Under a weak light regime, data in previous sections indicate that the faster process (process 1) is dependent on both superoxide radicals and a PSII donor side radical(s) ($TyrZ^+/P_{680}^+$) while the slower process (process 2) appears to be driven only by the PSII donor side radical(s). It is clear that the relative rate of photodamage to PSII donor side components of $NH_2OH$-PSII (Ono & Inoue, 1991b) and Tris-PSII (Eckert et al., 1991) is accelerated by the increase of light intensity; moreover, it has been suggested that the mechanism and the initial target of photodamage are different in weak versus strong light conditions. Such reported differences from weak versus strong light regimes conceivably could relate to a difference in the dependence of process 1 versus process 2 on light intensity. Accordingly, we measured the rate of photoinactivation of the $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation

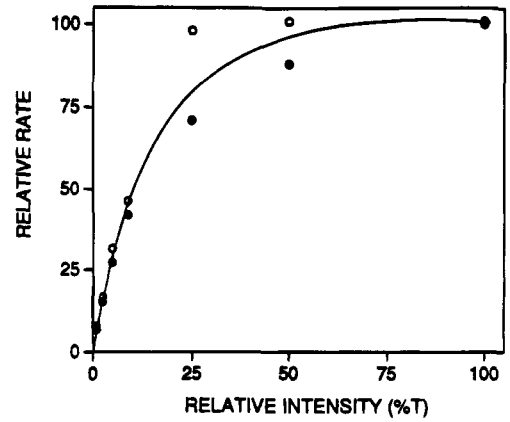


FIGURE 7: Light intensity dependence of the rate of loss of $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation capability and the rate of formation of superoxide. Rates of loss of $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation were determined at various light intensities using $NH_2OH$/EDTA-PSII membranes at 250 $\mu$g of Chl/mL in buffer A in a 0.3 cm light path cuvette (Materials and Methods). Rates of superoxide formation were similarly determined with the exception that the suspension contained 80 $\mu$M cytochrome *c*. Both sets of data were normalized to 100% T and then plotted versus relative light intensity. The 100%T intensity corresponds to 3.5 mE $m^{-2}$ $s^{-1}$. The open and closed circles correspond to the rate of loss of $Mn^{2+}/H_2O_2$ photooxidation capability and to the rate of formation of superoxide, respectively.

as well as the rate of formation of superoxide over a 100-fold range of light intensities. These experiments were done with the same preparation of $NH_2OH$/EDTA-PSII membranes so as to avoid any possible small differences in behavior between different preparations.

At the lowest light intensity employed (35 $\mu$E $m^{-2}$ $s^{-1}$), photoinactivation was slow ($t_{1/2}$ ~143 s), but it became more rapid with increasing light intensity, reaching a near-maximum rate ($t_{1/2}$ ~11 s) at a light intensity of 875 $\mu$E $m^{-2}$ $s^{-1}$ (data not shown). This maximum rate is slower than the rate ($t_{1/2}$ ~3 s) usually observed (e.g., Figure 8) in strong light. Apparently, the $NH_2OH$/EDTA-PSII preparation used in these experiments contained some adventitious $Mn^{2+}$ which is known to slow photoinactivation (Chen et al., 1992). Similarly, the same range of increasing light intensity caused significant increases in the rate of superoxide formation, as determined by cyt-*c* reduction measured spectrophotometrically at 550 nm (not shown). The rate of superoxide production at any light intensity was constant with time (zero order), despite the exponential (first order) and near total loss of the capability for $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation. The calculated rate constants, normalized at the maximum light intensity used, are plotted in Figure 7.

It is apparent that the rate of photoinactivation of $Mn^{2+}/H_2O_2$ photooxidation capability (open circles) and the rate of superoxide formation (closed circles) had a very similar dependence on light intensity with the possible exception of intensities greater than 25% T. This result indicates that the superoxide-mediated mechanism of photoinactivation (process 1) operates over the entire light range and is correlated with the loss of activities. This finding suggests that the mechanism by which PSII donor side intrinsic components are photodamaged may be the same in both high and weak light regimes.

*Is the Mechanism of Photodamage to PSII Donor Side Components Different in Weak versus Strong Light?* Ac-

cording to Chen et al. (1992), PSII electron acceptors markedly suppress the photodamages resulting from weak light treatment of $NH_2OH$/EDTA-PSII or Tris-PSII, but during photoinhibitory treatments in strong light they were found either to have no effect (Eckert et al., 1991) or to accelerate them by 2−4-fold (Klimov et al., 1990). Accordingly, we tested experimentally whether the presence of PSII electron acceptors or SOD during strong light illumination of $NH_2OH$/EDTA-PSII offered protection against photodamage similar to that observed using weak light. The light intensity for these strong light exposures was ~125-fold greater than the weak light used in Figures 1−6 and in previous studies (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991; Chen et al., 1992) and was comparable to the high intensity employed by Ono and Inoue (1991b); moreover, the intensity was ~4-fold in excess of that required to give the maximal rate of loss of the capability to photoreduce DCIP by electrons from $Mn^{2+}/H_2O_2$ (Figure 7).

As shown in Figure 8A (open circles), in the absence of any additions to the membranes, the loss of $Mn^{2+}/H_2O_2$ capability was biphasic and rapid. The kinetic component exhibiting the more rapid loss of activity had a half-inhibition time of only 3 s, a half-time comparable to the half-time ($t_{1/2} \approx 1.4-1.7$ s) of the "fast" component loss of capability of $Mn^{2+}$ photooxidation, $A_T$-band emission, and photoactivation reported by Ono and Inoue (1991b). On the other hand, the ~3 s half-time was ~30-fold less than the half-inhibition time reported by Klimov et al. (1990) for loss of $Mn^{2+}$ photooxidation capability during strong light treatment of Tris-PSII in the absence of any additions.

The closed circles, closed squares, and open squares of Figure 8A record how the time course of loss of capability of $Mn^{2+}/H_2O_2$ photooxidation during strong light treatment of $NH_2OH$/EDTA-PSII was affected by the presence of DCIP, FeCN, and DCIP/FeCN, respectively. In all cases, the presence of a PSII electron acceptor markedly slowed the course of strong light photodamage similar to their effect on weak light photodamage (Chen et al., 1992). A major protective effect of the PSII electron acceptors is attributed to inhibition of formation of superoxide, which otherwise could contribute to the mechanism of strong as well as weak light photodamage (Chen et al., 1992). The observed greater protective effectiveness of DCIP alone than in a mixture with FeCN is attributed to a reduction of potentially damaging PSII radicals ($P_{680}^+/TyrZ^+/Chl^+$) by their back-reaction with any reduced DCIP formed in the light. The presence of FeCN along with DCIP would maintain DCIP oxidized and would eliminate an additional protective effect observed with DCIP alone.

The supposition that the major protective effect of PSII electron acceptors against strong light photodamage is a consequence of their inhibition of superoxide formation is supported by the results obtained when 81 units/mL SOD was present during strong light treatment of $NH_2OH$/EDTA-PSII membranes and their subsequent capability to carry out photoactivation or to photooxidize $Mn^{2+}/H_2O_2$ (Figure 8B). The open and closed circles of Figure 8B record the loss of photoactivation capability during strong light treatment of the membranes in the absence and presence of SOD, respectively, while the open and closed squares show the loss of $Mn^{2+}/H_2O_2$ capability also in the absence and presence of SOD, respectively. We note the following: (1) in the absence of SOD, the initial rates of loss of capability

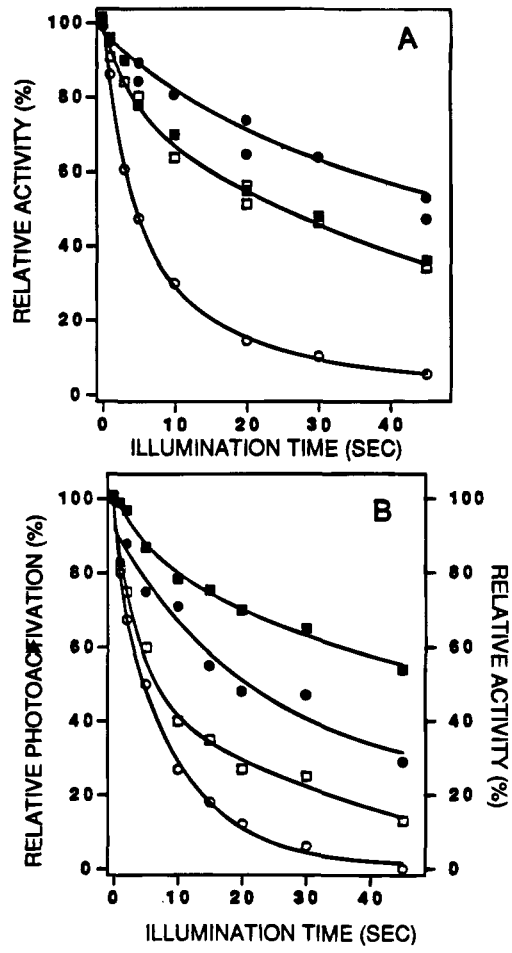


FIGURE 8: (A): Addition of DCIP or FeCN partially protects $NH_2$-OH/EDTA-PSII membranes from strong light photodamage. Membranes (250 $\mu$g of Chl/mL) were treated with strong light (Materials and Methods) in the absence of any additions (open circles) or in the presence of 50 $\mu$M DCIP (closed circles), 1 mM FeCN (open squares), and 50 $\mu$M DCIP/1 mM FeCN (closed squares) for the durations shown. Subsequently, the membranes were pelleted and washed twice (~250 $\mu$g of Chl/mL) with buffer A before assay of photoreduction of DCIP by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ at quantum yield light intensity. (B) Addition of SOD partially protects $NH_2$-OH/EDTA-PSII membranes from strong light-induced loss of photoactivation and DCIP photoreduction capacity. The open and closed circles record the loss of photoactivation capacity from strong light treatment of membranes in the absence and presence of 81 units/mL SOD, respectively. The open and closed squares show the loss of DCIP photoreduction by 2 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ in the absence and presence of 81 units/mL SOD, respectively. One hundred percent relative photoactivation and relative DCIP photoreduction activity correspond to 1208 and 50 $\mu$equiv·(mg of Chl)$^{-1}$·h$^{-1}$, respectively.

to carry out photoactivation and to photooxidize $Mn^{2+}/H_2O_2$ were nearly equivalent as reported earlier (Chen et al., 1992); and (2) these initial rates of loss were markedly suppressed (~5−8-fold) by the presence of SOD during the strong light treatment. If we accept arguments made in preceding sections that the loss of capability to photooxidize $Mn^{2+}/H_2O_2$ strictly parallels the loss of capability to generate $TyrZ^+$, then the first observation above does not support the conclusions reached by Klimov et al. (1990) and Ono and Inoue (1991b) that the capability to carry out photoactivation is lost more rapidly during strong light photodamage of Mn-depleted membranes than their ability to generate $TyrZ^+$, at least when the membranes are thoroughly depleted of Mn (see Discussion). With respect to the observed marked

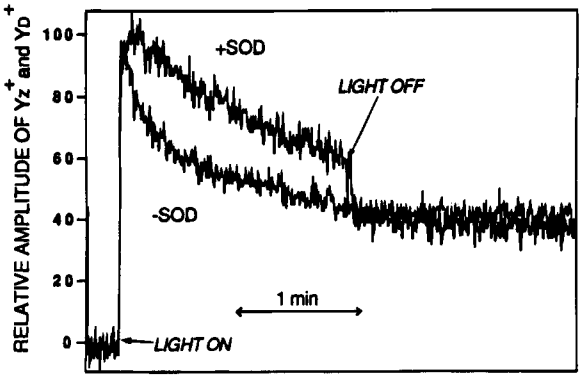


FIGURE 9: Real time, *in situ* EPR measurements ($g = 2.0104$) of the effects of strong light illumination of $NH_2OH$/EDTA-PSII on the amplitudes of $TyrZ^+$ and $TyrD^+$. Membranes (4 mg of Chl/mL) were illuminated directly in the cavity with strong light (16.4 $mE{\cdot}m^{-2}{\cdot}s^{-1}$) either in the absence or in the presence of SOD (81 units/mL) as indicated. The receiver gain was $2.5 \times 10^4$. For other details, see Materials and Methods and text.

suppressions of strong light photodamages to photoactivation and $Mn^{2+}/H_2O_2$ photooxidation by SOD (Figure 8B) or to $Mn^{2+}/H_2O_2$ photooxidation by PSII electron acceptors (Figure 8A), we recognize a considerable similarity to the situation encountered in weak photodamaging light (Chen et al., 1992; see also previous sections). This tends to suggest that a common mechanism contributes to the photodamages of the donor side of PSII irrespective of the photodamaging light intensity.

This idea finds further support from the EPR measurements shown in Figure 9. In these experiments, $NH_2OH$/EDTA-PSII membranes were illuminated directly in the EPR cavity with strong continuous light while recording the amplitude of the $TyrZ^+$ and $TyrD^+$ signals at the low-field shoulder ($g = 2.0104$). The lower trace, measured in the absence of addition of SOD, shows a rapid decay of about half the maximum amplitude during continuous illumination. Curve-fitting of the rapidly decaying curve revealed two components having half-times and amplitudes of 6.6 s (81%) and ~63 s (19%). No significant decrease of the EPR signal amplitude occurred on cessation of illumination following a 2 min strong light exposure. Thus, in the absence of SOD, all the $TyrZ^+$ signal was lost during the 2 min strong light treatment without significantly diminishing the amplitude of the dark-stable $TyrD^+$ signal remaining after the illumination.

A decidedly different response was seen in the presence of 81 units/mL SOD. As seen in the upper trace of Figure 9, the decrease in signal amplitude during illumination exhibited simple exponential kinetics with a half-time of ~68 s, a value very similar to the slower decaying minor kinetic component seen in the absence of SOD. Due to the slow decay of the EPR signal amplitude in the presence of SOD, appreciable signal amplitude remained at the time the light was turned off, but this signal decayed rapidly ($\leq 1$ s) in the following darkness to the level of $TyrD^+$. Reillumination of the membranes after a few minutes darkness caused the signal to recover to the level observed at the time of cessation of illumination (trace not shown). Such rapid decay in darkness and rapid formation in the light is typical for TyrZ; thus, the data of Figure 9 offer direct evidence that the capability to generate $TyrZ^+$ is lost rapidly in strong light and that SOD greatly suppress the photodamage(s) causing this effect. At least qualitatively, such results are entirely

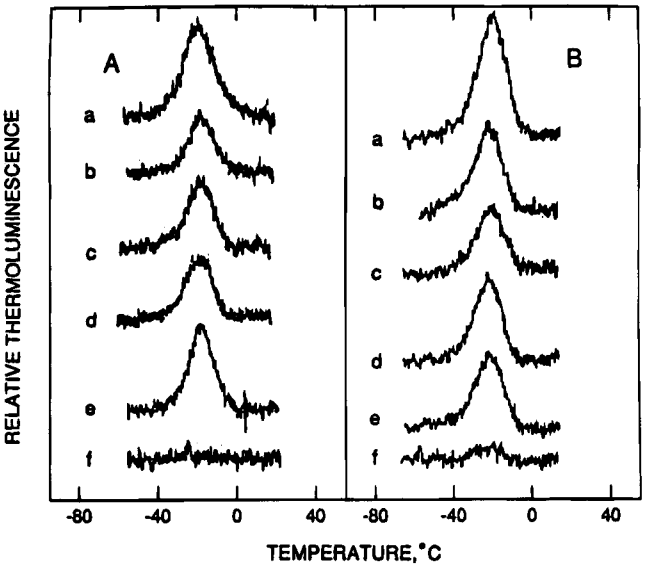


FIGURE 10: Effects of the presence of SOD or PSII electron acceptors during weak and strong light treatment on the $A_T$ thermoluminescence band. (A) Membranes were illuminated at 23 °C with weak light for 5 min in the presence of 50 $\mu$M DCIP plus 32.5 units/mL SOD (trace b), 32.5 units/mL SOD (trace c), 10 mM FeCN (trace d), or 50 $\mu$M DCIP (trace e) or without any additions (trace f). Trace a was obtained with membranes incubated at 23 °C in darkness for 5 min. (B) Membranes were illuminated at 23 °C with strong light for 20 s in the presence of the same additions as noted above or without any additions (trace f). Trace a was obtained with dark-incubated membranes. See Materials and Methods for details.

similar to those obtained under a weak light illumination regime (Figures 2–4).

*Weak versus Strong Light Photodamage of $A_T$-Band Emission Capability: Effects of PSII Electron Acceptors and SOD.* Ono and Inoue's (1991b) studies of photodamage of $NH_2OH$-PSII showed that the capability to generate the $A_T$-band emission was a sensitive indicator of photodamage from either weak or strong light. In efforts to obtain additional evidence supporting our conclusion that the mechanism of photodamage of $NH_2OH$/EDTA-PSII is essentially the same in either weak or strong light, we examined the effects of weak and strong light treatment of $NH_2OH$/EDTA-PSII membranes in the absence and presence of PSII electron acceptors or SOD on their capability to generate the $A_T$-band emission.

Figure 10 shows measurements of the $A_T$-band emission following 5 min weak light (panel A) and 20 s strong light (panel B) treatment of $NH_2OH$/EDTA-PSII in the absence and presence of various additions. Trace a of both panels was obtained with control dark-incubated membranes and trace f with membranes illuminated in the absence of any additions. As shown, in the absence of any additions, both the weak and strong light treatments essentially abolished $A_T$-band formation capability. This result confirms those of Ono and Inoue's (1991b) showing that the capability to generate the $A_T$ band is highly susceptible to photodamage. Traces b, c, d, and e record the effects during illumination in the presence of 50 $\mu$M DCIP plus 33 units/mL SOD, 33 units/mL SOD, 10 mM FeCN, and 50 $\mu$M DCIP, respectively, on the subsequent capability of $NH_2OH$/EDTA-PSII to generate the $A_T$-band emission. Comparison of traces b–e with traces of f of panel A or panel B shows that the addition of SOD (trace c) to dismutate superoxide formed during

Table 1: Effects of Addition of SOD and PSII Electron Acceptors on the Extent of Loss of Photoreduction of DCIP Capacity and the $A_T$ Thermoluminescence Band Caused by Weak and Strong Light Treatments

| | relative rate of DCIP photoreduction | | | | | |
|---|---|---|---|---|---|---|
| | 5 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ | | 100 $\mu$M $Mn^{2+}$ | | $A_T$ band[a] relative peak area | |
| additions during illumination | weak light | strong light | weak light | strong light | weak light | strong light |
| (1) none | 0.04 | 0.13 | 0.09 | 0.17 | 0.00 | 0.14 |
| (2) 50 $\mu$M DCIP | 0.89 | 0.83 | 0.91 | 0.76 | 0.87 | 0.50 |
| (3) 10 mM FeCN | 0.75 | 0.78 | 0.77 | 0.68 | 0.66 | 0.56 |
| (4) 32.5 units/mL SOD | 0.60 | 0.72 | 0.62 | 0.78 | 0.61 | 0.55 |
| (5) 50 $\mu$M DCIP plus 32.5 units/mL SOD | 0.88 | 0.84 | 0.92 | 0.76 | 0.55 | 0.61 |
| (6) control (dark) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

[a] The relative values tabulated below were obtained from the traces shown in Figure 9.

illumination, or the addition of FeCN (trace d) or DCIP (trace e) to inhibit formation of superoxide, gives appreciable protection from either weak or strong light photodamage.

To quantify the differences in intensity of the $A_T$-band traces shown in Figure 10, the area under each trace was determined. Additionally, the same membranes used to generate the data of Figure 10 were assayed for their capability to photoreduce DCIP by 5 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ to permit a comparison between loss of capability of $A_T$-band formation with loss of the capability of this exogenous electron donor mixture to reduce $TyrZ^+$. Assays also were made of the capability of the membranes to photoreduce DCIP by 100 $\mu$M $Mn^{2+}$, a $Mn^{2+}$ concentration sufficient to saturate the low-affinity $Mn^{2+}$ binding site ($K_m \sim 10$ $\mu$M) contributing to $TyrZ^+$ reduction (Hoganson et al., 1989; Blubaugh & Cheniae, 1990) as well as the low-affinity $Mn^{2+}$ binding site ($K_m \sim 17$ $\mu$M) causing suppression of the $A_T$-band emission (Ono & Inoue, 1991a).

The relative values of such measurements are summarized in Table 1. In the absence of any additions to membranes exposed to weak light (5 min), very similar extents of loss of capability to generate the $A_T$-band and to photooxidize $Mn^{2+}$ via its high- and low-affinity sites were observed (row 1). This result also was observed with membranes subjected to strong light treatment (20 s) in the absence of any additions (row 1). Rows 2−5 of Table 1 show the effectiveness of PSII electron acceptors (DCIP, FeCN) and SOD as protectants against weak and strong light-induced loss of capability to form the $A_T$ band and to photooxidize 5 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ or 100 $\mu$M $Mn^{2+}$ in the presence of DCIP. We observed the following: (1) the presence of DCIP, FeCN, or SOD during weak light treatment gave ~60−90% protection in either case, the extent depending on the specific addition; (2) for any given addition under the weak light condition, the extent of protection of each of these three activities was nearly equivalent with the exception of the comparatively low $A_T$-band emission observed after illumination of the membranes in the presence of 50 $\mu$M DCIP plus 33 units/mL SOD (row 5); and (3) the presence of PSII electron acceptors or SOD during strong light treatment (20 s) of the membranes offered significant protection against loss of capability to form the $A_T$ band and to photooxidize $Mn^{2+}/H_2O_2$ or 100 $\mu$M $Mn^{2+}$, but photooxidation activities were, in this case, 12−33% better retained than $A_T$-band formation capability. The relatively small differences between the extent of protection of the capability to generate the $A_T$-band emission and to photooxidize either $Mn^{2+}/H_2O_2$ or 100 $\mu$M $Mn^{2+}$ via $TyrZ^+$ tend to support the conclusions reached in previous sections—namely, (a) there may be a slight quantitative but no qualitative difference between TyrZ-dependent and His-dependent (photoactivation and $A_T$-band emission) PSII activities to weak or strong light photodamage during illumination of $NH_2OH$/EDTA-PSII membranes; and (b) superoxide contributes significantly to the mechanism of both weak and strong light photodamage of the TyrZ-dependent and the His-dependent PSII activities.

*Effects of DEPC Modification of $NH_2OH$/EDTA-PSII on Photoinhibition.* Chen et al. (1992) have suggested that the superoxide-dependent rapid phase of photodamage of $NH_2$-OH/EDTA-PSII was a consequence of a bimolecular reaction between a superoxide radical and the tyrosine radical, $TyrZ^+$, leading to a loss of the redox activity of TyrZ. However, the alternate possibility of a reaction between superoxide radicals and PSII donor-side $His^+$ residue(s) radicals resulting in the loss of redox activity of both the His and the $D_1$-Tyr-161 residues could not excluded. Consequently, we asked if abolishment of redox activity of His residues by DEPC modification would alter the susceptibility of $NH_2OH$/EDTA-PSII to weak light photodamage affecting $Mn^{2+}/H_2O_2$ electron donation to $TyrZ^+$ and whether it might eliminate the suppression of the photodamage(s) by SOD addition. The DEPC modification procedure used was one previously shown to prevent photoactivation (Tamura et al., 1989) and $A_T$-band emission (Ono & Inoue, 1991a). Figure 11A confirms that the DEPC-modified membranes (open circles) showed little capability of photoactivation relative to the control $NH_2OH$/EDTA-PSII membranes (closed circles) which had been carried through the DEPC modification procedure (1 h at room temperature) except for omission of DEPC. The relatively low degree of photoactivation of the control is explained by the necessary 1 h incubation at room temperature which has been shown previously to diminish the extent of photoactivation and the abundance of the high-potential form of cyt $b_{559}$ (Tamura & Cheniae, 1987). Nevertheless, in results not shown, the capability of the control membranes to photooxidize $Mn^{2+}/H_2O_2$ or DPC in either weak or strong light proved equivalent to that with the $NH_2OH$/EDTA-PSII membranes, whereas the capability of the membranes to photooxidize $Mn^{2+}/H_2O_2$ was diminished in weak and strong light by ~15 and ~40%, respectively, by DEPC modification. This observation indicates that the modification of His residues by DEPC imposes a significant rate constraint on $Mn^{2+}/H_2O_2$ photooxidation in saturating light without greatly diminishing the quantum yield. A similar extent of loss of capability to photooxidize DPC and 50 $\mu$M $Mn^{2+}$ in strong light was reported previously by Tamura et al. (1989) following DEPC treatment of $NH_2$-OH-PSII. Such modification, however, does not diminish

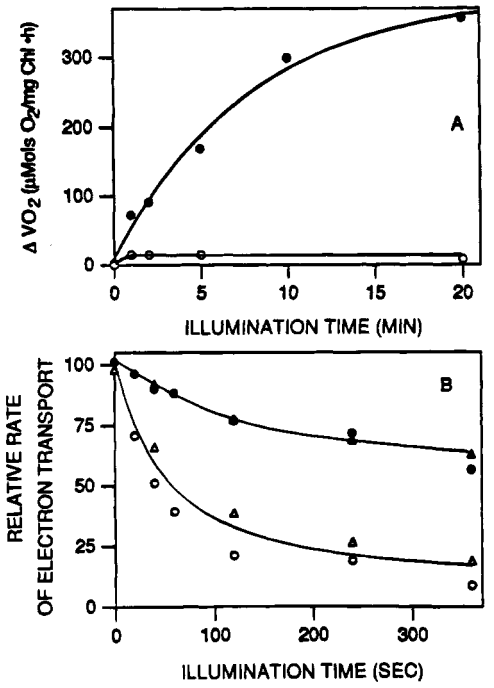


FIGURE 11: Effect of DEPC treatment of $NH_2OH$/EDTA-PSII on (A) photoactivation capability and (B) susceptibility to photodamage by weak light. (A) Control (closed circles) and DEPC-treated membranes (open circles) were photoactivated for the times shown, and then rates of $O_2$ evolution ($V_{O_2}$) were determined. See Materials and Methods and text for details. (B) Control membranes were illuminated with weak light in the absence (open circles) or presence of 97.5 units/mL SOD (closed circles) for the times shown. The open and closed triangle data were obtained with DEPC-modified membranes which were illuminated in the absence and presence of 97.5 units/mL SOD, respectively. Following illumination (Materials and Methods), the membranes were pelleted, washed once (250 $\mu$g of Chl/mL) with buffer A, and then resuspended in the same buffer for assay of DCIP photoreduction with 5 $\mu$M $Mn^{2+}$/3 mM $H_2O_2$ as the electron donor. Assays were made at a quantum yield light intensity. The relative rates of 100 for control and DEPC-treated membranes at time zero illumination were 45.8 and 39.4 $\mu$equiv·(mg of Chl)$^{-1}$·h$^{-1}$, respectively.

the affinity of Mn at its carboxylate-dependent high-affinity site ($K_m \leq 1$ $\mu$M) in PSII [Blubaugh & Cheniae, 1992; see, however, Tamura et al. (1989)].

In the experiments summarized in Figure 11B, the susceptibility of such control and DEPC-modified $NH_2OH$/EDTA-PSII membranes to weak light photodamage was measured either in the absence of any additions or in the presence of 98 units/mL SOD. In the absence of SOD, the control membranes (open circles) exhibited rapid ($t_{1/2}$ ~40 s) loss of capability to photooxidize $Mn^{2+}/H_2O_2$ through its $TyrZ^+$-dependent site. With DEPC-modified membranes in the absence of SOD (open triangles), the loss was essentially equivalent. However, in the presence of SOD, the photodamage of both control (closed circles) and DEPC-modified $NH_2OH$/EDTA-PSII membranes (closed triangles) was markedly slowed such that about 40% loss of $Mn^{2+}/H_2O_2$ photooxidation capability occurred during a light treatment that would have caused ≥80% loss in the absence of SOD. Since DEPC modification of $NH_2OH$-PSII abolishes the capability of $A_T$-band formation, presumably by elimination of the redox activity of the specific His residue involved in the $A_T$ emission (Ono & Inoue, 1990), we are led to conclude from the data of Figure 11 that the weak light photodamage causing loss of redox activity of TyrZ does not require that this His residue is redox-active during weak light photodamaging treatment.

## DISCUSSION

Studies of weak light photodamage (40 $\mu$E·m$^{-2}$·s$^{-1}$) of wheat $NH_2OH$-PSII causing irreversible damage of electron donor side components of PSII without damage to charge separation ($P_{680}^+Q_A^-$ formation) have identified four different kinetic components having vastly different half-times (Blubaugh & Cheniae, 1990; Blubaugh et al., 1991). In order of occurrence, these are as follows: (1) a partial loss of the 4−10 $\mu$s component of $P_{680}^+$ decay ($TyrZ \rightarrow P_{680}^+$) which correlated with partial loss of the capacity to photoassemble the Mn cluster, partial loss of variable $Chl_a$ fluorescence, and an increased quantum yield of formation of two as yet unidentified EPR-detectable radicals; (2) complete loss of TyrZ redox activity, analyzed by EPR in continuous light, partial loss of $Mn^{2+}$ (100 $\mu$M) photooxidation capability, and loss of the remaining capability to photoassemble the Mn-cluster; (3) a slower bleaching of 10% of the absorbance from $Chl_a$ (A. Bergonia and D. Blubaugh, unpublished results); and (4) a very slow loss of TyrD redox activity, correlated with irreversible bleaching of carotenoids (A. Bergonia and D. Blubaugh, unpublished results).

The first and second components had half-times and amplitudes of 0.8 min (43%) and 2−3 min (57%). Measurements similar to those above, along with measurements of the His-dependent $A_T$-band capability of spinach $NH_2OH$-PSII, gave half-times and amplitudes of 0.6 min (63%) and 3.5 min (37%) for the first and second components, respectively [Ono & Inoue, 1991b (approximate values determined from Figure 6)].

On the other hand, when the photodamage was done at a ~450-fold increase in light intensity (~15 mE·m$^{-2}$·s$^{-1}$), Ono and Inoue (1991b) observed a drastic separation in the half-times for the kinetic components: the loss of the capability to assemble the Mn-cluster and to generate the $A_T$ band were both lost biphasically, the two components having equal amplitudes with half-times of only 1.5 and 6−8 s, while the loss of TyrZ redox activity measured by EPR in continuous light was lost 10−20 times more slowly with a half-time of ~22 s. They concluded that a different mechanism of photodamage prevails under high versus low-light conditions. Loss of capability to assemble the Mn-cluster without loss of TyrZ/TyrD redox activities also has been observed by Klimov et al. (1990) in studies of strong light treatment of spinach Tris-PSII. The mechanism by which strong, but not weak light treatment selectively inactivates the His residue(s) contributing to photoassembly of the Mn-cluster and to the $A_T$-band emission while leaving TyrZ redox activity unaffected is not easily explained by the generally held hypothesis for photodamage to the PSII donor-side redox-active components. The hypothesis that these photodamages are simply the consequence of irreversible oxidations/modifications of amino acid residues in the near vicinity of the highly oxidizing PSII radicals, $TyrZ^+/P_{680}^+/Chl^+$, has been questioned on the basis of somewhat indirect arguments (Chen et al., 1992).

This study focused on the first two previously identified kinetic components. First, we directly examined the validity of the hypothesis of Chen et al. (1992), namely, that the photodamages to PSII donor-side redox-active components

are caused by two different mechanisms: (a) a rapid mechanism dependent on both superoxide radicals and the PSII radicals, $TyrZ^+/P_{680}^+/Chl^+$ and possibly $His^+$; and (b) a much slower mechanism driven only by $TyrZ^+/P_{680}^+/Chl^+$. Second, we asked if these presumed different mechanisms might contribute differently at weak versus strong light conditions and thus offer an explanation for the differing hierarchy of photodamaging events observed by Ono and Inoue (1991b) under weak versus strong light conditions.

In this study, we used $NH_2OH$/EDTA-treated spinach PSII membranes which had been exhaustively depleted of Mn. With these membranes, the faster event reported by Ono and Inoue (1991b) and Blubaugh et al. (1991) is accelerated to a half-time of only 21 s at the same photoinhibitory weak light intensity (40 $\mu E\ m^{-2}\ s^{-1}$); futhermore, $\geq 80\%$ of the activity, measured as the capability to assemble the Mn-cluster and to photooxidize $Mn^{2+}/H_2O_2$, was lost with this fast half-time when membranes were illuminated in the absence of PSII electron acceptors or SOD either to prevent formation of or to dismutate superoxide radicals. In the view of Chen et al. (1992), the slower half-time of 2−3 min reported by Blubaugh et al. (1991) and Ono and Inoue (1991b) is reflective of photodamage to PSII centers which still had a high-affinity Mn bound. This assumption is based on observations showing that addition of $Mn^{2+}$ ($K_m \sim 720$ nM) suppressed the rapid 21 s component of photodamages. According to this view, the first kinetic components of weak light-induced photodamages reported by Blubaugh et al. (1991) and Ono and Inoue (1991b) reflect only a single site of photodamage which abolishes the redox activity of TyrZ, with the two apparent half-times being a consequence of the heterogeneity of reaction centers with respect to the binding status of a high-affinity Mn. On the other hand, the two kinetic components of Blubaugh et al. (1991) and Ono and Inoue (1991b) could reflect (a) photodamage of $D_1$-His-190, possibly causing a slowing of the reduction of $P_{680}^+$ by TyrZ without loss of $TyrZ^+$ formation capability similar to the effect observed from site-directed mutation of this residue (Diner et al., 1991; Diner, personal communication), and (b) actual inability to generate $TyrZ^+$ at any measuring light intensity.

In the studies made here with "Mn-free" $NH_2OH$/EDTA-PSII membranes and with no additions during weak light exposure, we observed that the following events all occurred with a half-time of 21 s and an amplitude of $\geq 80\%$: (1) loss of the 4−10 $\mu$s component of $P_{680}^+$ decay (Figure 2); loss of $TyrZ^+$ formation capability measured by EPR in continuous light (Figure 4A); (3) loss of flash-induced $Chl_a$ variable fluorescence (Figure 6); and (4) loss of $Mn/H_2O_2$ photooxidation capability, measured either in a strong light-saturating or in a very weak light-limiting condition, via a high-affinity binding site for $Mn^{2+}$ ($K_m \sim 180$ nM). Previously, the capability to carry out photoactivation and to photooxidize $Mn^{2+}/H_2O_2$ was shown to be photoinactivated essentially in parallel (Chen et al., 1992). All of the above PSII activities are dependent on the redox activity of TyrZ. Thus, the loss of its redox activity, measured in a flash regime (Figure 2) or in strong continuous light (Figure 4A) without modification of the high-affinity $Mn^{2+}$ site (Figure 1), or the loss of the capability to generate $TyrD^+$ and $P_{680}^+/Q_A^-$, lead us to conclude that the initial sites of weak light-induced photodamage to $NH_2OH$/EDTA PSII are few and rather specifically targeted.

When SOD was present to dismutate any superoxide formed during illumination, the loss of all of the above-mentioned activities occurred nearly 30 times slower (Figures 3, 5, and 6); however, the loss was not completely suppressed. Moreover, in the presence of SOD or PSII electron acceptors, the loss of the capability to generate the His-dependent $A_T$-band emission and to photooxidize $Mn^{2+}/H_2O_2$ via $TyrZ^+$ were suppressed essentially equivalently (Figure 10A; Table 1). Taken together with previous observations (Chen et al., 1992) showing that incubation of $NH_2OH$/EDTA-PSII with superoxide in darkness causes no deleterious effects on PSII redox components and that strict anaerobiosis in the absence of SOD slowed the loss of $Mn^{2+}/H_2O_2$ photooxidation activity as effectively as the presence of SOD under aerobic conditions, the totality of our results lends direct and strong support to the idea that the weak light-induced rapid inactivation process of TyrZ and His redox activities requires both superoxide radicals and donor-side PSII radicals whereas the slower photoinactivation process is caused by donor-side PSII radicals only.

We believe these same two processes contribute in much the same way to the photodamages induced by high light treatment of $NH_2OH$/EDTA-PSII. This conclusion is based on the following observations: (1) the formation of superoxide occurs with essentially the same light intensity dependence as the loss of the $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation over a 100-fold range of photodamaging light intensity (Figure 7); moreover, the light intensity dependence curve proved monophasic, suggesting that the same two photoinactivation processes contributed equivalently over this range of light intensity; (2) at a light intensity 4-fold in excess of that yielding a maximum initial rate ($t_{1/2} \sim 3$ s) of loss of the $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation activity, this activity and the capability to carry out photoactivation were both lost biphasically and nearly in parallel (Figure 8B); additionally, the loss of these activities was greatly inhibited with suppression of superoxide formation and accumulation by addition of PSII electron acceptors and SOD, respectively; (3) in the absence of SOD, rapid biphasic loss of $TyrZ^+$ could be observed directly in the *in situ* measurements of this radical with the two components having half-times and amplitudes of 6 s (81%) and 63 s (19%), whereas, in the presence of SOD, the loss of $TyrZ^+$ was monophasic with a half-time of 68 s (Figure 9); and (4) at a light intensity giving a maximal rate of loss of $Mn^{2+}/H_2O_2$ photooxidation activity, the presence of PSII electron acceptors or SOD strongly suppressed the loss of the capability to generate the $A_T$ band (Figure 10B or Table 1) or to photooxidize $Mn^{2+}$ (100 $\mu$M) even through its low-affinity site (Table 1).

Several previous reports (Barenyi & Krause, 1985; Richter et al., 1990; Setlik et al., 1990; Tschiersch & Ohman, 1993; Mishra et al., 1993) have implicated superoxide in the mechanism of photodamage of various types of $O_2$-evolving preparations. In these reports, however, the contribution of superoxide along with $H_2O_2$ to the inactivation(s) appears to be via Fenton chemistry to yield very oxidizing OH radicals. We dismiss the idea that the rapid superoxide-dependent photoinactivation of $NH_2OH$/EDTA-PSII reflects OH radical production and damage based on the following: (a) addition of catalase to eliminate $H_2O_2$ and thereby abolish OH radical formation via superoxide/$H_2O_2$-supported Fenton chemistry does not diminish the rate of weak light-induced loss of $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation activity;

and (b) mannitol (1 M), an effective scavenger of OH radicals, gives no protection against the loss of $Mn^{2+}/H_2O_2$ photooxidation activity during weak light photoinhibition (Chen et al., 1992).

We speculate that the rapid superoxide-dependent photoinactivation of $NH_2OH$/EDTA-PSII by weak or strong light is due to formation of highly reactive singlet oxygen formed from the oxidation of superoxide. Though dismutation of superoxide does not lead to a significant yield of singlet oxygen (Foote et al., 1980; Ferradini et al., 1981), the annihilation reaction between superoxide radicals and appropriate cation radicals can produce an appreciable yield of singlet oxygen (Mayeda & Bard, 1973). We suggest that both $D_1$-$TyrZ^+$-160 and $D_1$-$His^+$-190 are accessible and reactive with superoxide and that the reduction of these two cation radicals by superoxide results in formation of singlet oxygen which then oxidizes and/or cross-links these amino acid residues, resulting in loss of their redox activities. Histidyl and aromatic amino acid residues are known to be particularly reactive with singlet oxygen (Michaeli & Feitelson, 1994), with histidine being more reactive than tyrosine. Since exogenous singlet oxygen scavengers such as DABCO and histidine offer no protection (Chen et al., 1992), we must conclude they are inaccessible to the singlet oxygen.

Singlet oxygen production by PSII−RC complexes (Macpherson et al., 1993; Telfer et al., 1994) and oxygen-evolving thylakoids (Hideg et al., 1994) under high light photodamaging conditions has recently been shown and correlated with photodamage and photobleaching of pigment events in these type PSII preparations. In contrast to the above postulated mechanism of singlet oxygen production via the $TyrZ^+$ and/or $His^+$ annihilation reactions with superoxide, the singlet oxygen produced during strong light treatment of PSII RC complexes and oxygen-evolving thylakoids is generally believed to be formed by reaction of the triplet state of $P_{680}$($^3P_{680}$) with the triplet ground state of molecular oxygen ($^3O_2$). With oxygen-evolving thylakoids, the formation of singlet oxygen by reactions between $^3P_{680}$/$^3O_2$ requires very strong light to doubly reduce and dissociate $Q_A^{-2}$ from the PSII RC which then permits $^3P_{680}$ formation (Andersson & Styring, 1991; Prasil et al., 1992; Aro et al., 1993). On the other hand, the inactivation of $NH_2OH$/EDTA-PSII reactions by singlet oxygen formed in reactions of superoxide with $TyrZ^+$ and/or $His^+$ would occur even at very weak light intensities, as is observed.

If we accept our hypothesis that the photoinactivations of the redox activities of TyrZ and His of Mn-depleted PSII also are caused by singlet oxygen but generated from reaction of superoxide with $TyrZ^+/His^+$ sites, what cogent explanations can be offered for the somewhat contrasting results obtained here with "Mn-free" membranes versus those obtained by other workers using PSII membranes apparently less rigorously depleted of the Mn? We favor the idea of a branched model for electron transfer reactions on the donor side of Mn-depleted PSII in which the residue which contributes to the $A_T$ emission, possibly $D_1$-His-190, is a direct donor to $P_{680}^+$ in somewhat poor competition with TyrZ (Kramer et al., 1994). This branched model contrasts to a proposed linear model in which the His residue of the $A_T$ band is in series between TyrZ and the Mn-cluster (Ono & Inoue, 1991b). According to the above hypothesis of singlet oxygen formation by reactions of superoxide with $TyrZ^+$ and $His^+$, the relative rate of singlet oxygen production at these two sites will be governed by the abundances of these radicals, the available concentration of superoxide, and the relative reactivities of these amino acid residues with singlet oxygen. Under a given low intensity of continuous illumination and in the absence of exogenous electron donors to either of these two sites, we suggest that the relative abundances of $TyrZ^+$ and $His^+$ and the relative reactivities of TyrZ and His with singlet oxygen are such that the rates of inactivation of $TyrZ^+$ and $His^+$ redox activities would be nearly equivalent. Increases of light intensity up to light saturation would increase at least the rate of superoxide production and thereby increase the rate of singlet oxygen inactivation of $TyrZ^+$ and $His^+$ redox activities without altering the hierarchy of the damages. We believe this scenario best explains all our data presented here.

On the other hand, a low abundance of $Mn^{2+}$ (~1 $Mn^{2+}$/RC) has been shown to markedly slow weak light-induced loss of the $TyrZ^+$-dependent $Mn^{2+}/H_2O_2$ photooxidation activity (Chen et al., 1992). We suggest such protection by the ~1 $Mn^{2+}$/RC is due to two actions of this high-affinity $Mn^{2+}$ ($K_m$ < 1 $\mu$M) presumably bound at the $D_1$-Asp-170 site (Nixon & Diner, 1992; Diner & Nixon, 1992): (a) donation of an electron to $TyrZ^+$ to yield $Mn^{3+}$; (b) univalent oxidation of superoxide by the $Mn^{3+}$ to give ground state triplet $O_2$ and $Mn^{2+}$. In short, such bound $Mn^{2+}$ may mimic the activity of a SOD at the site of TyrZ. Under continuous illumination conditions, the abundances of both $TyrZ^+$ and $His^+$ would be diminished due to some electron transfers to $TyrZ^+$ from the $Mn^{2+}$ and to $P_{680}^+$ from TyrZ, respectively. Though the superoxide concentration at the $His^+$ site would be unchanged, its local concentration at $TyrZ^+$ would be diminished; thus, the rate of singlet oxygen production and inactivation of the $His^+$ site would occur more rapidly than $TyrZ^+$, a result reported by Ono and Inoue (1991b) in studies of strong light treatment of $NH_2OH$-PSII. At their weak light conditions yielding essentially equivalent rates of loss of $His^+/TyrZ^+$ redox activities, we further speculate that the abundances of $His^+/TyrZ^+$ available for reaction with superoxide were nearly equivalent as a consequence of some adventitious $Mn^{2+}$ and differing quantum yields of formations of $His^+/TyrZ^+$ as well as differing rates of recombination reactions. It remains to be proven, however, that reactions of $TyrZ^+$ and $His^+$ with superoxide generate singlet oxygen and that such produced singlet oxygen actually causes the rapid inactivation of these PSII secondary donors during illumination of Mn-depleted PSII membranes. If one assigns the $A_T$ band to $TyrZ^+/Q_A^-$ charge recombination (Debus, 1992), then all of our results obtained at either weak or strong light conditions can be explained by postulating that the initial photodamage is the abolishment of the redox activity of TyrZ.

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