Kinetics of Photoinhibition in Hydroxylamine-Extracted Photosystem II Membranes: Relevance to Photoactivation and Sites of Electron Donation†

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ABSTRACT: Kinetic analyses were made of the effects of weak-light photoinhibition on the capacity of NH_2OH-extracted photosystem II membranes to photooxidize the exogenous electron donors Mn^{2+}, diphenylcarbazide, and I^- or to assemble functional water-oxidizing complexes during photoactivation. The loss of capacity for photooxidation of the donors showed two first-order components (half-times of 2–3 min and 1–4 h) with relative amplitudes dependent on the donor, suggesting two photodamageable sites of electron donation (sites 1 and 2, respectively), a conclusion confirmed by analyses of velocity curves of electron donation by each donor. All of the donors appear to be oxidized preferentially by site 1 both at saturating and at limiting light intensity; however, the contribution by site 2 was nearly comparable in saturating light. Loss of photoactivation also exhibited biphasic kinetics, with components having half-times of approximately 0.8 and 3.2 min. The major component ($t_{1/2}$ = 3.2 min) corresponded to loss of site 1; essentially no photoactivation was observed after its loss. From these and other analyses, we conclude (1) the relative contributions of site 1 and site 2 to the photooxidation of various exogenous electron donors is determined largely by the rates of equilibration of the donors with the two sites, and (2) only site 1 contributes to photoactivation of the water-oxidizing complex. Site 1 is attributed to tyrosine Z of the reaction center's D_1 polypeptide. The molecular identity of site 2 is unknown but may be tyrosine D of the D_2 polypeptide.

It is generally believed [e.g., see Babcock and Sauer (1975a–c), Ghanotakis et al. (1982), Van Gorkom (1985), and Babcock (1987)] that artificial electron donors to photosystem II (PSII)[1] donate electrons to the endogenous redox component Z, known to be tyrosine-161 of the D_1 polypeptide of the PSII reaction center (Ikeuchi & Inoue, 1988; Debus et al., 1988b; Metz et al., 1989), when the normal flow of electrons from the water-oxidizing complex (WOC) to Z is disrupted. Under such conditions, oxidized Z (Z^+) is reduced with a half-time of ~20–800 ms, dependent on the exogenous donor (Babcock & Sauer, 1975a–c; Ghanotakis et al., 1982; Yerkes & Babcock, 1980), as opposed to 0.1–1 ms when the WOC is functional, dependent on the oxidation state of the WOC (Babcock et al., 1976; Dekker et al., 1984).

Another redox-active tyrosine, D, is known to exist at position 160 of the D_2 polypeptide of the PSII reaction center (Debus et al., 1988a; Vermaas et al., 1988). The half-time of D^+ reduction is normally on the order of hours in chloroplasts with a functional WOC, even in the presence of exogenous redox couples (Babcock & Sauer, 1973). However, in detergent-fractionated membrane fragments enriched in PSII (PSII membranes) (Babcock & Sauer, 1973), as well as in Tris-washed chloroplasts (Lozier & Butler, 1973), D^+ decay is significantly accelerated.

The function of D is poorly understood, although even in PSII membranes with active WOC's, D apparently competes efficiently with Z for the reduction of the oxidized primary electron donor chlorophyll (P_{680}^+) at moderately low temperatures (Nugent et al., 1987). At alkaline pH, the reduction of Z^+ by D has been reported in Tris-washed chloroplasts (Boussac & Etienne, 1982), and in chloroplasts at neutral pH, a slow reduction by D of the higher valency oxidation states S_2 and S_3 of the WOC (Babcock & Sauer, 1973), as well as a slow oxidation by D^+ of the S_0 state to S_1 (Styring & Rutherford, 1987), has been observed.

In PSII membranes in which WOC has been inactivated, the relative contribution of D^+ versus Z^+ to the oxidation of various exogenous donors is not clear. According to Babcock (1987), Z^+ is the principal site for electron donation in such membranes, based on the decay kinetics of EPR signal II; however, the very similar EPR spectra of Z^+ and D^+ may complicate such conclusions.

The ligation of Mn^{2+} as a tetra-Mn complex of WOC by the photoactivation process is kinetically remarkably similar in leaves (Inoue et al., 1975), variously grown/extracted algae (Cheniae & Martin, 1973), cyanobacteria (Cheniae & Martin, 1971, 1972), chloroplasts (Yamashita et al., 1978), and PSII membranes (Tamura & Cheniae, 1987a). The observed kinetics have been interpreted to reflect a series mechanism in which the product of the reaction of Mn^{2+} with the first PSII charge separation is unstable until processed by a second PSII charge separation (Radmer & Cheniae, 1971). Because Mn^{2+} is strictly required and the unstable intermediate is subject to increased rates of decay in the presence of added reductants of Mn^{3+} (Cheniae & Martin, 1972; Tamura & Cheniae, 1987b; Ono & Inoue, 1987), it has been hypothesized (Radmer & Cheniae, 1977; Tamura & Cheniae, 1987a) that the unstable intermediate is Mn^{3+} ligated with high affinity and

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[1] Abbreviations: Chl, chlorophyll; DPC, diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; EPR, electron paramagnetic resonance; NH_2OH-PSII, PSII membranes extracted with NH_2OH to inactivate WOC; P_{680}, PSII reaction center chlorophyll *a* dimer; PSII, photosystem II; Q_A and Q_B, primary and secondary plastoquinone electron acceptors of PSII, respectively; WOC, water-oxidizing complex; Z and D, redox-active tyrosines-160 and -161, respectively, of the D_1 and D_2 reaction center polypeptides.

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stabilized by ligands supplied by WOC polypeptides. According to this hypothesis, photooxidation of a second Mn^{2+} to Mn^{3+}, followed by ligation of two more Mn^{2+} atoms, results in formation of a stable $Mn(II)_2$–$Mn(III)_2$ complex capable of water oxidation. The functions of D^+ and Z^+, or cytochrome *b*-559, in the photoactivation mechanism are not resolved. Hypotheses implicating specific functions of both D^+ (Nugent et al., 1987; Styring & Rutherford, 1987) and cytochrome *b*-559 (Cramer et al., 1986) have been advanced.

Illumination by even weak light of leaves or chloroplasts containing functional PSII reaction centers but inactive WOC's causes a loss of photoactivation capacity and a decrease in the quantum yield of PSII photooxidation of some exogenous electron donors (Callahan & Cheniae, 1985; Theg et al., 1986; Callahan et al., 1986). Apparently, the intrinsic donors to P_{680}^+ are photodamaged, a consequence of light absorption by PSII in the absence of electron donation from either the WOC or an exogenous electron donor. Resynthesis and reassembly of the D_1 and D_2 reaction center polypeptides are then required for recovery of PSII activities (Callahan et al., 1986). This photodamage contrasts to that which is suggested to occur to the electron-acceptor quinones (Kyle et al., 1984; Cleland et al., 1986; Kyle, 1987), and to the light-harvesting chlorophyll *a*/*b* protein complex (Hayden et al., 1986) under 40–400 times higher light intensities.

Here we report on the kinetics of photoinhibition affecting the secondary donors to P_{680}^+ and its consequences on Mn^{2+} photooxidation and photoactivation, as well as the photooxidation of diphenylcarbazide (DPC) and I^-, commonly used electron donors to PSII. These analyses were made by using NH_2OH-extracted PSII membranes (NH_2OH-PSII) in attempts to identify factors which prohibit complete photoactivation, to determine if D^+ is specifically required in this process, and to gain some insights into the specificity of Z^+ and D^+ for the photooxidation of exogenous PSII electron donors.

Materials and Methods

Membrane Preparation. PSII membranes, with rates of O_2 evolution (VO_2) in excess of 700 μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}, were prepared, as described by Tamura and Cheniae (1985), from 9-day-old wheat seedlings and stored at −80 °C. NH_2OH extraction of the thawed and washed membranes was done as described elsewhere (Tamura & Cheniae, 1987a), with the following exceptions: (1) the extraction and subsequent washings were done in strict darkness; (2) four repetitive washings were done to ensure removal of NH_2OH; and (3) the extracted/washed membranes, with residual $VO_2 \leq 25$ μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}, were resuspended (≥2 mg of Chl/mL) in 0.8 M sucrose, 15 mM NaCl, and 50 mM Mes·NaOH, pH 6.5 (buffer A), before use or storage at −80 °C. Determinations of [Chl] were made as described by MacKinney (1941).

Photoinhibition. NH_2OH-PSII were diluted to 250 μg of Chl/mL in buffer A. Aliquots of 500 μL were distributed to glass vials (sample thickness ~2 mm), covered with clear plastic wrap, and shaken gently at room temperature under fluorescent white light of 40 μE m^{-2} s^{-1} for varying durations. The light intensity varied less than 1% from the mean over the area occupied by the samples, as determined with a LI-COR Model LI-185B quantum radiometer. In any given experiment, all samples were kept at room temperature for the same length of time, regardless of the duration of illumination, after which all were put back on ice.

Photoactivation. NH_2OH-PSII were treated exactly as for photoinhibition except (1) they were illuminated in 300-μL aliquots (sample thickness ~1.2 mm) and (2) DCIP, $CaCl_2$, and $MnCl_2$ were added (from a combined 20× stock) to give final concentrations, respectively, of 45 μM, 25 mM, and 1 mM (except where stated otherwise), 5–10 min prior to a 30-min illumination.

Activity Assays. O_2 evolution of photoactivated samples in buffer A (15 μg of Chl/mL) was measured polarographically in saturating light at room temperature, with 1 mM ferricyanide and 300 μM phenyl-*p*-benzoquinone as electron acceptors (Tamura & Cheniae, 1985). The light was filtered by two Schott KG-1, one Corion FR-400-S, and one Corning 2-63 filters. Electron transport, in nonphotoactivated samples (5 μg of Chl/mL in buffer A), was measured spectrophotometrically using, unless stated otherwise, 1 mM DPC, 100 μM Mn^{2+}, or 10 mM I^- as artificial donors, and 50 μM DCIP as electron acceptor. The rates were determined by following the disappearance of absorbance at 600 nm, essentially as described (Tamura & Cheniae, 1985), except the photomultiplier tube was shielded with a 600-nm interference filter and a Corion FR-400-S infrared filter. The absorptivity of DCIP at the pH of the experiment was calculated according to Armstrong (1964). All activities were corrected for the small residual rates observed in the absence of photoactivation or of any added donors, attributed to a small percentage of centers unaffected by the NH_2OH treatment.

Decay of Chlorophyll a Fluorescence. The Chl *a* fluorescence yield of photoinhibited and control samples in buffer A (250 μg of Chl/mL) was measured with a pulse-modulated fluorometer (Walz Co., Effeltrich, Germany), which has been described in detail (Schreiber, 1986). The weak, noninductive measuring light (λ = 660 nm) was modulated at a frequency of 100 kHz for 30 ms, beginning 2 ms before the actinic flash, provided by a Walz XST-103 xenon flash lamp (8-μs flash width). Fluorescence detection began 120 μs after the flash, and the signal was captured on a digital oscilloscope and then transferred to an electronic plotter.

Results

Time Course of Photoinhibition: Effects on Photoactivation and Photooxidation of Mn^{2+}, DPC, and I^-. In the experiments of Figure 1, NH_2OH-PSII were prepared with precautions to exclude all light during the treatment and washes and then exposed to weak light for varying durations. Subsequently, the capacity of the membranes to photooxidize some commonly used exogenous donors to PSII and to photoactivate O_2 evolution was determined. Figure 1 shows representative time courses for the loss of capacity to photooxidize DPC (closed squares), I^- (open circles), and Mn^{2+} (open squares), measured in saturating light, or to undergo photoactivation (closed circles). The data suggest a hierarchy of susceptibility to photoinhibition (photoactivation > Mn^{2+} photooxidation > DPC/I^- photooxidations), which confirms and extends observations made by Tamura and Cheniae (1987a). Comparable results were also obtained at pH 5.5 (data not shown).

The loss of electron donor photooxidation is biphasic for each of the donors, but the relative amplitude of the faster component is dramatically greater for Mn^{2+} than it is for either DPC or I^-. Best fits to the data of Figure 1 (solid lines, see Figure 1 legend) indicate half-times of 2–3 min and 1–4 h for the two components. The loss of photoactivation exhibits an additional fast component ($t_{1/2} \lesssim 1$ min), with a relative amplitude >40%; the slow 1–4-h component, however, is conspicuously absent.

The behavior shown in Figure 1 was highly reproducible in multiple replicate experiments. We conclude (1) weak light causes photodamage to three kinetically distinguishable sites

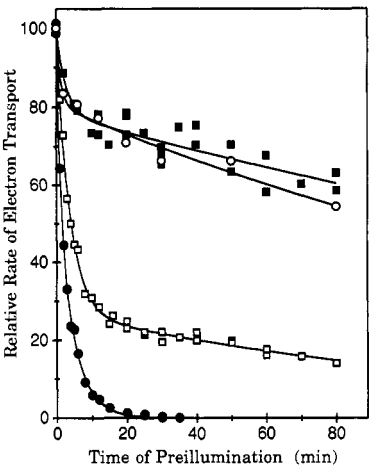

FIGURE 1: Time courses of photoinhibition in wheat NH_2OH-PSII. Shown are relative rates of electron transport, in saturating light, from various electron donors [(■) 1 mM DPC; (○) 10 mM I^-; (□) 100 μM Mn^{2+}] to DCIP or, after photoactivation, from H_2O to ferricyanide plus phenyl-*p*-benzoquinone (●). The lines are best fits by the Levenborg–Marquardt algorithm (Press et al., 1988) to decay curves having multiple first-order components. The following half-times and amplitudes were obtained: DPC, 1.9 min and 20.8%, 210 min and 79.2%; I^-, 0.8 min and 19.2%, 140 min and 80.8%; Mn^{2+}, 2.6 min and 72.9%, 90 min and 27.1%; photoactivation, 0.8 min and 42.7%, 3.2 min and 57.3%. Samples were photoinhibited for varying durations and then divided into aliquots for subsequent assays of DPC, Mn^{2+}, or I^- photooxidation or for photoactivation. The data are from two to three experiments (except for I^-) done on separate days with the same parent membranes and combined without any normalization. The reproducibility of the results is apparent from the fit of the combined data to each curve. Other experimental details are described under Materials and Methods. The rates corresponding to 100 on the relative scale, in microequivalents per milligram of chlorophyll per hour, were (■) 310, (○) 38, (□) 102, and (●) 1250.

in the PSII reaction center, (2) photodamage to two sites differentially affects the magnitudes of the loss of DPC/I^- and Mn^{2+} photooxidations, and (3) loss of the more rapidly photodamaged sites ($t_{1/2} \simeq 1$ and 3 min) abolishes photoactivation even though Mn^{2+} photooxidation, considered to be an essential reaction in photoactivation, can still occur after ≥30-min photoinhibition.

We observed no loss of the activities of Figure 1 during 120 min of illumination at 77 K, at which only a single turnover of the reaction center occurs. Similarly, 1 mM Mn^{2+} had a large (>80%) protective effect on all of the activities during 40 min of illumination at ~23 °C. These results, and the insensitivity of membranes containing active WOC's to weak light photoinhibition, suggest that photodamage occurs only when the secondary donors to P_{680} are oxidized at the time of reaction center turnover.

Evidence for Two Sites of Electron Donation: Effects of Photoinhibition on the Kinetic Constants of Mn^{2+}, DPC, and I^- Electron Donation. In order to clarify and extend the conclusions reached from the data of Figure 1, the velocity of Mn^{2+} photooxidation by nonphotoinhibited NH_2OH-PSII was determined as a function of Mn^{2+} concentration (Figure 2A) and analyzed by an Eadie–Scatchard plot (Figure 2B). Two distinct linear regions were obtained, indicating two independent sites of Mn^{2+} photooxidation [see pp 64–71 of Segel (1975)]. For each site, K_m and V_{max} values were calculated (Spears et al., 1971), and then the velocity curves 1 and 2 shown in Figure 2A were calculated, according to Michaelis–Menten kinetics. Summation of curves 1 and 2 yielded the curve shown through the data.

Similarly, Figure 2C,D shows the velocity data and Eadie–Scatchard plot for Mn^{2+} photooxidation after 30 min of photoinhibition, which according to Figure 1 eliminates virtually all but the slowly damaged component. As before, velocity curves were calculated from the K_m and V_{max} values for each site. A comparison of curves 1 and 2 of Figure 2C with those of Figure 2A reveals that 30-min photoinhibition diminishes the V_{max} of site 1 (curve 1) ~4-fold more than the V_{max} of site 2 (curve 2). We therefore attribute site 1 to the

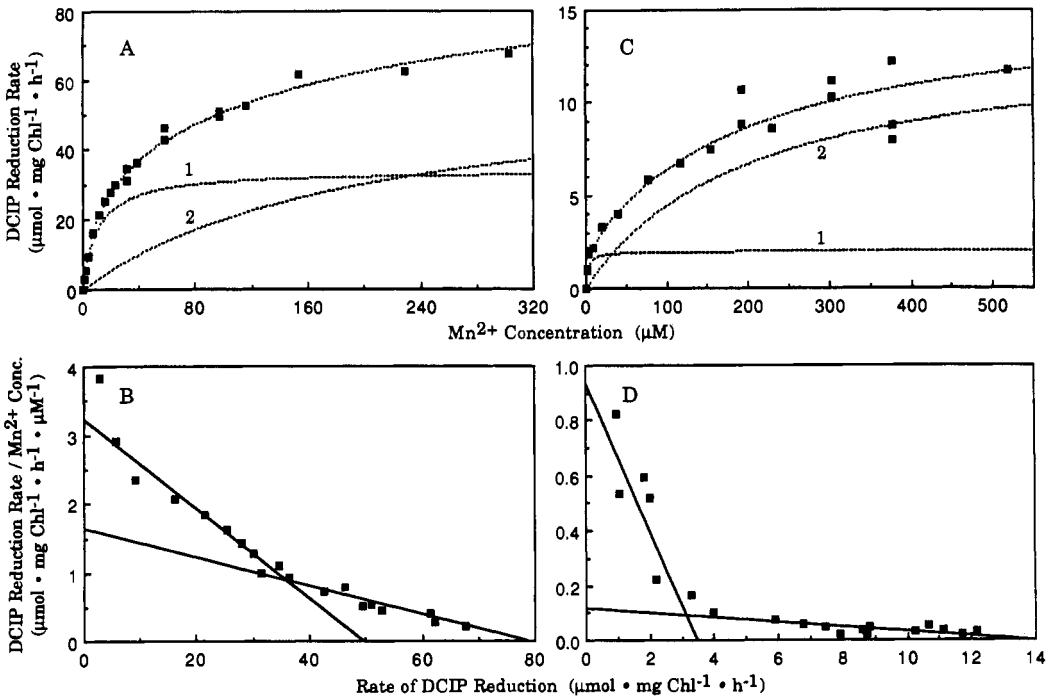

FIGURE 2: Mn^{2+} concentration dependence of Mn^{2+} photooxidation in wheat NH_2OH-PSII, expressed as velocity curves (A, C) and as Eadie–Scatchard plots (B, D). The membranes were photoinhibited, as described under Materials and Methods, for (A, B) 0 min and (C, D) 30 min. The activity was assayed in saturating light as photoreduction of DCIP (Materials and Methods), with various concentrations of Mn^{2+} as electron donor. The solid lines labeled 1 and 2 in (A, C) are velocity curves calculated from K_m and V_{max} values, determined from the Eadie–Scatchard plots by successive approximations (Spears et al., 1971; Segel, 1975, pp 64–71), for two independent sites (site 1 and site 2, respectively). Curves 1 and 2, in each panel, were summed to produce the velocity curve which fits the data.

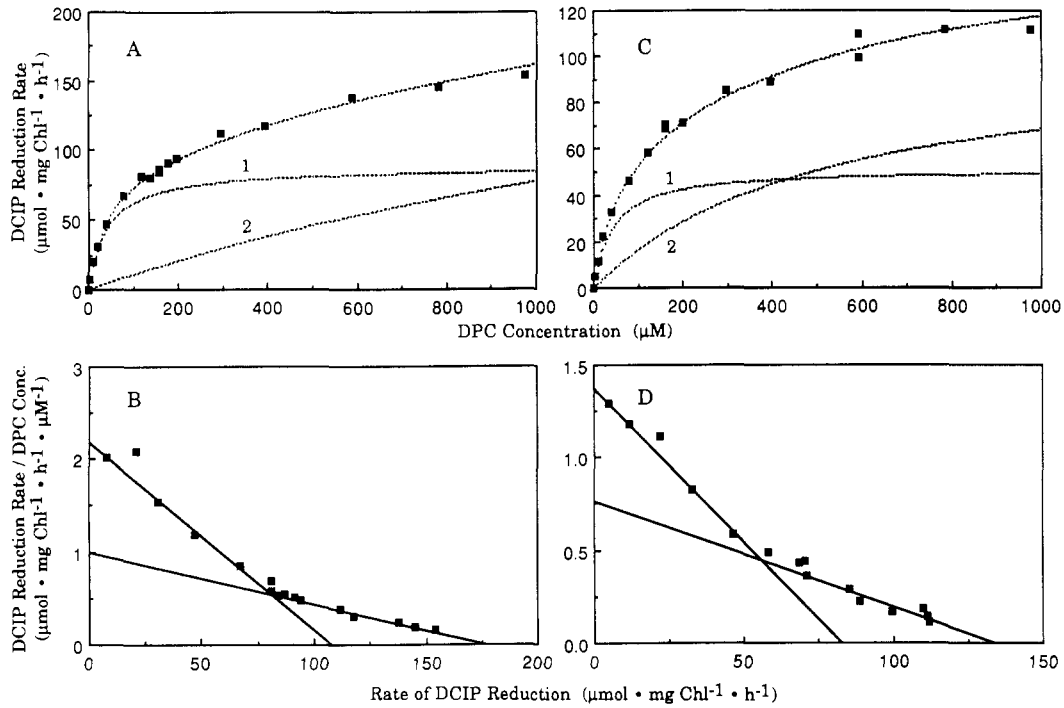

FIGURE 3: DPC concentration dependence of DPC photooxidation in wheat NH_2OH-PSII, after 0 min (A, B) and 30 min (C, D) of photoinhibition, expressed as velocity curves (A, C) and as Eadie–Scatchard plots (B, D). All details are as described in the legend to Figure 2, except that DPC was the electron donor, instead of Mn^{2+}.

Table I: Effects of Photoinhibition on Kinetic Constants of DPC, Mn^{2+}, and I^- Electron Donations to PSII[a]

electron donor	duration of photoinhibition (min)	site 1		site 2		total velocity	
		K_m (mM)	V_{max}[b]	K_m (mM)	V_{max}[b]	calculated[c]	observed[d]
DPC	0	0.042	88 (1.00)	2.0	236 (1.00)	163 (1.00)	155 (1.00)
	5	0.057	47 (0.53)	0.93	166 (0.70)	131 (0.80)	128 (0.83)
	30	0.042	52 (0.59)	0.53	105 (0.44)	119 (0.73)	112 (0.72)
Mn^{2+}	0	0.010	34 (1.00)	0.20	61 (1.00)	51 (1.00)	51 (1.00)
	30	0.0025	2 (0.06)	0.20	14 (0.22)	7 (0.14)	11 (0.22)
I^-	0	4.9	15 (1.00)	174	94 (1.00)	15 (1.00)	19 (1.00)
	30	5.8	10 (0.66)	117	58 (0.62)	11 (0.73)	14 (0.74)

[a] The kinetic constants were determined from Eadie–Scatchard plots as described in the text. Numbers in parentheses are relative values. [b] Micromoles of DCIP per milligram of chlorophyll per hour. [c] Calculated according to Michaelis–Menten kinetics at two independent sites, using the kinetic constants shown and the concentrations of DPC, Mn^{2+}, and I^- employed in the experiments of Figure 1. [d] From the curves of Figure 1.

rapidly photodamaged component ($t_{1/2}$ = 2–3 min) and site 2 to the slowly damaged component ($t_{1/2}$ = 1–4 h).

Similar analyses, but with the neutral, lipophilic DPC as electron donor, gave the results shown in Figure 3. As with Mn^{2+}, the Eadie–Scatchard plots gave evidence for two independent sites of electron donation by DPC. A comparison of the calculated velocity curves before (Figure 3A) and after (Figure 3C) 30 min of photoinhibition suggests that the velocity through site 1 is affected more than that through site 2, at least at concentrations of DPC ≤1 mM. Similar evidence was obtained for two independent sites of electron donation by I^- (data not shown), again with the more rapid photodamage assigned to site 1.

Table I summarizes the kinetic constants obtained in these and other analyses. In nonphotoinhibited membranes, the K_m values are 20–47 times smaller at site 1 than at site 2, while the V_{max} values are 1.8–6.3 times greater at site 2. Several anomalous behaviors are apparent, which indicate a certain complexity to the photoeffects on sites 1 and 2: (1) for both DPC and I^-, but not for Mn^{2+}, the V_{max} at site 1 is still substantial after 30 min of photoinhibition, despite a half-time of 2–3 min attributed to this site (Figure 1); (2) for all of the donors, the V_{max} at site 2 is diminished to a significantly greater extent after 30 min of photoinhibition than would be expected from the half-time of 1–4 h for the slow component in Figure 1; and (3) some, but not all, of the K_m values become significantly altered during photoinhibition. In particular, the K_m's for both DPC and I^-, but not for Mn^{2+}, are lowered at site 2. With respect to the donors, this situation is reversed at site 1.

Because of these apparent anomalies, we asked first whether the kinetic constants in Table I are consistent with the time courses of Figure 1. Shown in Table I are the calculated expected velocities, determined from the kinetic constants and the concentrations of DPC, Mn^{2+}, and I^- used in the experiments of Figure 1, along with the actual velocities observed after equivalent durations of photoinhibition in the experiments of Figure 1. In all cases, the agreement is excellent, indicating that the observed changes in K_m and V_{max} values are indeed consistent with the observed time courses of photoinhibition.

The observed changes in K_m can be explained by a diminishing forward rate constant, k_p, for electron donation to each site (see the Appendix), perhaps a consequence of increased back-reactions between the oxidizing and reducing sides of PSII. To what extent such a change in k_p would affect K_m depends on the relative magnitudes of other rate constants in the expression for K_m. Thus, the change in K_m should be variable with different donors, as observed. Since V_{max} is also dependent on k_p, in addition to its dependence on the concentration of functional sites, this could also explain the

anomalously large decrease in V_{max} values at site 2.

We do not have a cogent explanation for the large V_{max} values for DPC/I^- at site 1 after 30-min photoinhibition. However, we offer two possibilities: (1) site 2 of some reaction centers is modified during photoinhibition, and the K_m's for DPC and I^- become similar to those of site 1, thereby obscuring the actual loss of V_{max} for these donors at site 1; or (2) DPC and I^-, but not Mn^{2+}, donate electrons through a third, non-photodamaged site with K_m's similar to those at site 1.

Effect of Photoinhibition on Flash-Induced Variable Chlorophyll a Fluorescence. Weak-light photoinhibition of NH_2OH-treated leaves and chloroplasts does not significantly affect the initial or maximal fluorescence yields measured in the presence of NH_2OH and DCMU (Callahan & Cheniae, 1985), the number of atrazine-specific receptor sites, or the atrazine/Q_B binding constant, but does diminish the quantum yield of DCMU-insensitive photoreduction of silicomolybdate with DPC as electron donor (Callahan et al., 1986). These observations tend to exclude the Q_A/Q_B locus as a site of weak-light photoinhibition and suggest photodamage to the secondary donors to P_{680}^+. Furthermore, weak-light photoinhibition of NH_2OH-PSII results in a loss of the EPR signals corresponding to the oxidized secondary donors Z^+ and D^+, at rates essentially equivalent to the rates of loss of site 1 and site 2, respectively (Blubaugh & Cheniae, 1990). Preliminary EPR/spectroscopic measurements with photoinhibited chloroplasts [reported by Tamura and Cheniae (1988)] also indicate a loss of Z^+, followed by loss of D^+, with no significant loss of the P_{680}^+/Q_A^- couple. In efforts to verify and extend these conclusions, we examined the effects of photoinhibition on the flash-induced variable Chl *a* fluorescence, measured in the presence or absence of an exogenous electron donor.

Normally, the relative amplitude of the variable fluorescence is an indictor of $[Q_A^-]$ formed and stabilized during the flash, and its decay reveals the kinetics of Q_A^- reoxidation [for a review, see Van Gorkom (1986)]. In the absence of photoinhibitory effects, illumination of NH_2OH-PSII yields reduction of Q_A and the oxidations of D and Z. Charge recombination between Z^+ and Q_A^- occurs in the dark ($t_{1/2}$ < 100 ms), but D^+ remains stable for ≥1 h in the absence of exogenous reductants (Babcock & Sauer, 1973). Typically, the reduction of P_{680}^+ by Z is much faster than P_{680}^+/Q_A^- charge recombination (e.g., Reinman et al., 1981), though the reduction of P_{680}^+ by D in a Z-less mutant of *Synochocystis* appears to be much slower (Metz et al., 1989). From these considerations, one may predict the following: (1) with D^+ oxidized, photodamage to Z would eliminate the flash-induced fluorescence amplitude (P_{680}^+/Q_A^- charge recombination is faster than our instrument-limited delay in fluorescence detection after the flash); (2) any restoration of the fluorescence amplitude by an exogenous electron donor to PSII would reflect electron donation either to P_{680}^+ directly or via the secondary donor D, in competition with the Q_A^-/P_{680}^+ charge recombination; and (3) barring formation of a quencher, damage to the Q_A/Q_B locus would result in a high fluorescence amplitude and diminished rate of decay.

Figure 4A shows the flash-induced fluorescence yield and its decay in nonphotoinhibited NH_2OH-PSII. The lower traces of Figure 4B–D show similar measurements made after the indicated durations of photoinhibition and a 10-min dark adaptation. D would have been oxidized during the photoinhibition treatments and would not have been reduced to any significant extent before the measurements. Therefore, the amplitudes reflect the stabilization of Q_A^- due to P_{680}^+ reduction by Z. During 1 min of photoinhibition, the amplitude

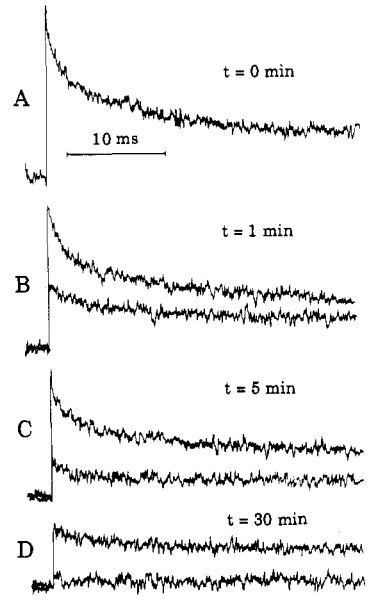

FIGURE 4: Decay of Chl *a* fluorescence yield after a saturating flash in NH_2OH-PSII from wheat. Shortly (~10 min) before the measurement, the membranes were photoinhibited, as described under Materials and Methods, for (A) 0, (B) 1, (C) 5, and (D) 30 min. In (A), there are no additions. In (B–D), the lower trace is with no additions; the upper trace is with 15 mM (B) or 30 mM (C, D) NH_2OH. The Chl concentration was 250 μg/mL.

diminished by ~63%, which was much larger than the extent of loss of either site 1 or site 2 in the same time period, and may be related to the component with $t_{1/2}$ < 1 min, involved in photoactivation (Figure 1). Thereafter, the amplitude declined at a rate roughly equivalent to that for the loss of site 1, becoming negligible after 30 min. The upper traces of Figure 4B–D show the results obtained when 15–30 mM NH_2OH was added after the photoinhibition. Although NH_2OH did not affect the fluorescence behavior of nonphotoinhibited membranes (trace not shown), it did restore most of the amplitude lost after 1 min of photoinhibition. This probably indicates some charge separations between site 2 and Q_A, stabilized by electron donation to site 2 [see, however, Metz et al. (1989)]. From 1 to 30 min of photoinhibition, the extent of this restoration declined at a rate apparently intermediate between the rates of loss of sites 1 and 2, as would be expected if both sites were involved. Virtually identical results were obtained with 1 mM DPC in place of the NH_2OH.

Because the initial levels of fluorescence were equivalent in all of the samples [see also Callahan and Cheniae (1985)], we can rule out the possibility that a nonphotochemical quencher was generated during photoinhibition. These results strongly suggest an identification of site 1 with Z: (1) charge separation between Z and Q_A was inhibited; (2) the inhibition did not appear to be between P_{680} and Q_A, since an electron donor restored formation/stabilization of Q_A^-; and (3) from 1 to 30 min, this inhibition paralleled the loss of site 1, as well as loss of the EPR signal corresponding to Z^+ (Blubaugh & Cheniae, 1990). Similarly, identification of site 2 with D or P_{680} seems warranted. We are not able at this time to explain the change(s) occurring in the first minute of photoinhibition.

With the possible exception of the 30-min photoinhibited sample, the kinetics of Q_A^- reoxidation in the presence of NH_2OH do not appear different from the nonphotoinhibited sample. Therefore, weak-light photoinhibition does not appear to damage the electron acceptor side of PSII, at least at short times sufficient to cause significant loss of Z^+. We note, also, that the fluorescence decay of the 30-min photoinhibited sample, measured in the presence of NH_2OH, is virtually

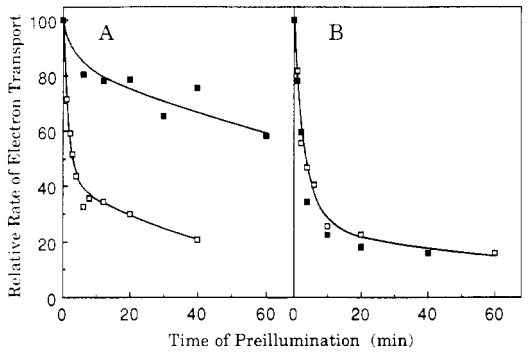

FIGURE 5: Effect of assay light intensity on the apparent time course of photoinhibition in wheat NH_2OH-PSII. Shown are relative rates of electron transport from DPC (A) or Mn^{2+} (B) to DCIP, assayed in saturating light (■) or in light that was approximately 10% saturating (□). The rates corresponding to 100 on the relative scale, in micromoles of DCIP reduced per milligram of chlorophyll per hour, were (A) (■) 115, (□) 30; (B) (■) 49.8; (□) 5.4.

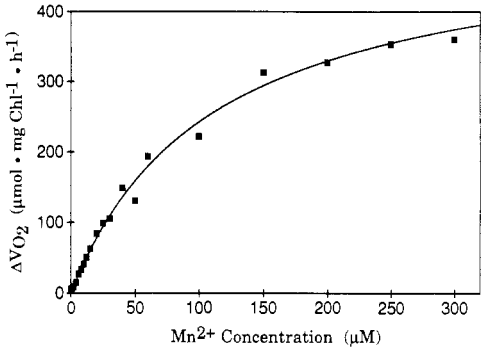

FIGURE 6: Rate of O_2 evolution after photoactivation of wheat NH_2OH-PSII, as a function of the Mn^{2+} concentration present during the photoactivation. 25 mM Ca^{2+}, competitive with Mn^{2+} (K_I = 1 mM; Boussac et al., 1986), was present throughout. The samples were not photoinhibited prior to photoactivation. The solid line is the velocity curve calculated for a single site with an apparent K_m of 112 μM (true K_m = 4 μM) and a V_{max} of 514 μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}.

identical with that of the 1-min sample, measured in the absence of NH_2OH. Therefore, although photodamage to Q_A/Q_B cannot be ruled out after ≥30 min of photoinhibition, our data do not favor this; rather, the diminished fluorescence amplitude is more consistent with photodamage to the secondary donor (i.e., D) than it is to photodamage to either P_{680} or Q_A/Q_B (Van Gorkom, 1986).

Effects of Photoinhibition on Quantum Yields of Mn^{2+} and DPC Photooxidations. The apparently large contribution of site 2 to DPC/I^- photooxidations (Figure 1) contrasts to the conclusion that Z^+, which we assign to site 1, is the principal site of oxidation of exogenous donors [e.g., see Babcock (1987)]. Moreover, the slower and less extensive loss of DPC/I^- photooxidations, compared to Mn^{2+}, during photoinhibition of NH_2OH-PSII (Figure 1) contrasts to the extensive (~80%) loss of DPC photooxidation observed during photoinhibition of NH_2OH-treated chloroplasts and attributed to photodamage to Z (Callahan et al., 1986). The above studies implicating DPC photooxidation by Z^+ were made with assay conditions that yield low rates of charge separation in the reaction center. In contrast, a high rate of charge separation (saturating light) was employed in the experiments described in previous sections.

In the experiments of Figure 5, rates of DPC and Mn^{2+} photooxidation by NH_2OH-PSII were determined at both a saturating and a subsaturating light intensity, following various durations of photoinhibition. When DPC photooxidation was assayed at subsaturating intensity (Figure 5A, open symbols), both the rate and extent of activity loss were much greater than when determined in saturating light (Figure 5A, closed symbols). Moreover, its rate and extent of loss were similar to the site 1 dominated loss of Mn^{2+} photooxidation assayed at either light regime (Figure 5B). Apparently, the rate of equilibration of DPC at Z (site 1) is much slower than at site 2; thus, with decreasing rates of reaction center turnover, the relative contribution of Z to DPC photooxidation increases.

Evidence for a Single Site of Mn^{2+} Photooxidation in Photoactivation of O_2 Evolution. The oxidation of Mn^{2+} to $Mn^{\geq 3+}$ is believed to be required in the formation of the water-oxidizing tetra-Mn complex by photoactivation (Radmer & Cheniae, 1977; Tamura & Cheniae, 1986, 1987a). Cytochrome *b*-559 (Cramer et al., 1986), tyrosine Z (Callahan et al., 1986), and tyrosine D (Nugent et al., 1987; Styring & Rutherford, 1987) have all been proposed as the sites of Mn^{2+} photooxidation in photoactivation. In the experiments of Figure 6, we asked whether the extent of photoactivation as a function of Mn^{2+} concentration gave evidence for a single site or a greater number of sites of Mn^{2+} photooxidation in photoactivation. These data were obtained with addition of 25 mM Ca^{2+} to the incubation mixture. Though Ca^{2+} is not required for ligation of the $Mn^{\geq 3+}$ formed during photoactivation, it is required for expression of water-oxidizing activity of any tetra-Mn complex formed (Tamura & Cheniae, 1987a, 1988; Tamura et al., 1989; Miller & Brudvig, 1989). However, Ca^{2+} also is a competitive inhibitor of Mn^{2+} photooxidation in the photoactivation process (Tamura & Cheniae, 1987a; Miller & Brudvig, 1989).

Under such conditions, and over a wide range of Mn^{2+} concentrations, the yield of photoactivation versus Mn^{2+} concentration exhibited simple Michaelis–Menten kinetics (Figure 6). The Eadie–Scatchard plot (not shown) showed only one linear region, in sharp contrast to the analyses of DCIP-supported photooxidation of Mn^{2+} (Figure 2B). Thus, we conclude that only one site of Mn^{2+} photooxidation is involved in photoactivation. We exclude participation of site 2 in photoactivation on the basis of these arguments: (1) the measured apparent K_m for Mn^{2+} in photoactivation (112 μM; Figure 6 legend), which is increased above the true K_m by the competitive Ca^{2+}, is ~2-fold less than the K_m for Mn^{2+} photooxidation by site 2 (205 μM, Table I), determined in the absence of Ca^{2+}; (2) during photoinhibition, the complete loss of photoactivation is much more rapid than the slow kinetic component attributed to loss of site 2 (Figure 1).

A K_I value of 1 mM has been reported for the competitive inhibition by Ca^{2+} of Mn^{2+} photooxidation in spinach NH_2-OH-PSII (Boussac et al., 1986). From this value and the apparent K_m of 112 μM for Mn^{2+} in photoactivation, obtained in the presence of 25 mM Ca^{2+}, a K_m of 4 μM was calculated, which is reasonably close to the K_m for Mn^{2+} photooxidation at site 1 (Table I). These considerations suggest that site 1, attributed to tyrosine Z, is the only site of Mn^{2+} photooxidation involved in photoactivation. However, from the kinetic analyses of the photoinhibition of photoactivation (Figure 1), a component was detected whose decay ($t_{1/2} \lesssim 1$ min) is more rapid than the loss of site 1 ($t_{1/2}$ = 2–3 min). This suggests that another PSII component, susceptible to photodamage, contributes nonessentially to the photoactivation process.

Discussion

Ordinarily, P_{680}^+ extracts an electron from tyrosine Z with a certain probability of extracting an electron from tyrosine D. Reduction of Z^+ by WOC resupplies electrons for subsequent turnovers of the reaction center. Selective inactivation of WOC is known to alter the kinetics of P_{680}^+ reduction

(Conjeaud et al., 1979; Boska et al., 1983; Weiss & Renger, 1986), to decrease the Z/Z^+ redox potential (Yerkes et al., 1983), and to permit facile observation of Z^+, D^+ (Babcock, 1987) and a component, possibly cytochrome *b*-559, with a midpoint potential of ~240 mV (Golbeck & Warden, 1985). At 77 K, no photooxidation of Z is observed, and a single turnover of P_{680} oxidizes cytochrome *b*-559 (Vermeglio & Mathis, 1974), or a chlorophyll/carotenoid molecule (DePaula et al., 1985), or possibly D (Nugent et al., 1987). All serve as secondary donors of P_{680}^+, albeit with differing rate constants. In the absence of either functional WOC or artificial PSII electron donors, the secondary donors become susceptibile to weak-light photoinhibition by a mechanism still to be established (Callahan et al., 1986; Theg et al., 1986; Becker et al., 1987).

Our analyses of the effects of photoinhibition on electron donation by Mn^{2+}, DPC, and I^- indicate two sites of photooxidation, with different susceptibilities to photodamage. On the basis of the rates of loss of charge separations between the secondary donors and Q_A, as reflected in the amplitude of the flash-induced variable Chl *a* fluorescence, we assign the rapidly damaged site 1 ($t_{1/2}$ = 2–3 min) to tyrosine Z and favor assignment of the slowly damaged site 2 ($t_{1/2}$ = 1–4 h) to tyrosine D. These assignments are supported by EPR studies showing loss of the signals corresponding to Z^+ and D^+ with equivalent half-times as the loss of sites 1 and 2, respectively (Blubaugh & Cheniae, 1990). However, in the absence of definitive proof of D^+ reduction by exogenous electron donors, we cannot exclude other possible oxidants, such as P_{680}^+ or Chl^+, as candidates for site 2.

Here and throughout the vast literature dealing with the photooxidation of exogenous electron donors by PSII, the maximum and dissimilar rates of Mn^{2+} and DPC photooxidations are less than the rates of water oxidation via the tetra-Mn cluster of the water-oxidizing complex. Yet at sufficiently low rates of quantum absorption, the absolute quantum efficiencies for the oxidation of both water and most exogenous electron donors are high and equivalent. These simple considerations suggest that during photoinhibition, in which Z and site 2 are lost at different rates, the relative steady-state rates of photooxidation of various donors by PSII might be governed by a combination of (1) the rates of equilibration of the donors with Z vs site 2, and (2) the abundance of Z vs site 2, and (3) the average probability of formation of Z^+ vs oxidized site 2 in a given light regime.

According to Babcock (1987), Z^+ is the principle site of electron donation by exogenous donors in nonphotoinhibited thylakoids and PSII membranes, based on analyses of the effects of various donors on the decay of Z^+/D^+ EPR signals formed during a saturating flash. If we accept this conclusion, then we must reconcile and explain the following observations on Mn^{2+}/DPC donation to PSII under steady-state illumination of nonphotoinhibited and variously photoinhibited NH_2OH-PSII:

(1) With nonphotoinhibited NH_2OH-PSII, the relative contributions of Z and site 2 to DPC photooxidation measured in saturating light (1 mM DPC) were 52% and 48%, respectively (from curves 1 and 2 of Figure 3A or from calculation of the independent velocities using the K_m and V_{max} values in Table I); however, in weak light, the relative contributions of Z and site 2 were 85% and 15%, respectively (from velocity data collected in weak light, not shown).

(2) Although Z^+ and site 2 both contribute to Mn^{2+} photooxidation by nonphotoinhibited NH_2OH-PSII in saturating light (60% and 40%, respectively, for 100 μM Mn^{2+}), only one site for Mn^{2+} was involved in photoactivation, for which Mn^{2+} photooxidation is necessary (Radmer & Cheniae, 1977; Tamura & Cheniae, 1987a); after correction for Mn^{2+}/Ca^{2+} competition at this site, the K_m (4 μM) was similar to the 10 μM value for Mn^{2+} at Z (site 1) measured here and by Hoganson et al. (1989), who used single saturating flashes to generate Z^+.

(3) During photoinhibition of the secondary donors to P_{680}, both Mn^{2+} and DPC photooxidation decrease biphasically; however, the decrease of DPC photooxidation was dominated by the loss of Z (site 1) only in analyses of DPC photooxidation at subsaturating light (Figure 5A), whereas a Z-dominated loss of Mn^{2+} photooxidation was observed in analyses done at either saturating or subsaturating light (Figure 5B).

Observations 1 and 2 are interpreted to indicate that the relative rates of Z and site 2 turnover are governed by both the probability of formation of Z^+ vs oxidized site 2 and the rate of Mn^{2+} and DPC equilibration with these oxidized species. According to this interpretation, observation 1 showing a 50% vs 15% contribution of site 2 to DPC photooxidation by nonphotoinhibited NH_2OH-PSII in saturating vs subsaturating light can be attributed to a slow rate of DPC $\rightarrow Z^+$ reaction and a high intrinsic probability of Z^+ vs oxidized site 2 formation. Observation 2 showing a 40% vs 0% contribution of site 2 to the photooxidation of Mn^{2+} at saturating light vs photoactivation in low light is similarly explained. Thus, extrapolation of our steady-state data to an infinitely low light intensity leads to conclusions similar to those reached from single flash-induced turnovers of the secondary donors to P_{680}^+ (Yerkes & Babcock, 1980; Ghanotakis et al., 1982; Hoganson et al., 1989); namely, Z^+ is the principle site of photooxidation of exogenous PSII donors at conditions of low rates of quantum absorption by nonphotoinhibited PSII.

To explain observation 3, we invoke a kinetic model (Appendix) which, along with the experimentally determined kinetic constants for Mn^{2+}/DPC/I^- photooxidations after various durations of photoinhibition (Table I), permits conclusions regarding the changing contributions of Z and site 2 to electron donor photooxidations due to differential photodamage to these species. In this model, two opposing factors affect the contribution of site 2: (1) as centers lose Z, the average probability of formation of oxidized site 2 increases, which tends to increase the rate through site 2 initially during photoinhibition; (2) the forward rate constant, k_p, for electron donation to site 2 diminishes during photodamage to Z and tends to decrease the rate through site 2; the magnitude of this second effect depends on the relative value of the reverse rate constant, k_{-1}, for donor binding at site 2 and is probably maximal when Mn^{2+} is the donor (Appendix).

In saturating light, factor 1 predominates for DPC photooxidation, while factor 2 predominates for Mn^{2+} photooxidation. Thus, although the initial relative contributions of Z vs site 2 are not much different for the two donors, the effect of photoinhibition of Z on the velocity through site 2 differs dramatically for the two. At low light intensity, the diminishing quantum yield of the photooxidations with increasing extent of photoinhibition (Callahan et al., 1986), like factor 2, contributes to a decrease in the rate through site 2.

If we postulate that site 2 is tyrosine D, then our data lend no support to hypotheses (Nugent et al., 1987; Styring & Rutherford, 1987) suggesting a function of D in the photoactivation of the WOC: (1) the loss of photoactivation capacity by photoinhibition is ~20–200 times faster than the loss of site 2 (Figure 1); (2) the calculated K_m values for Mn^{2+} in photoactivation (Figure 6) and Mn^{2+} photooxidation by Z

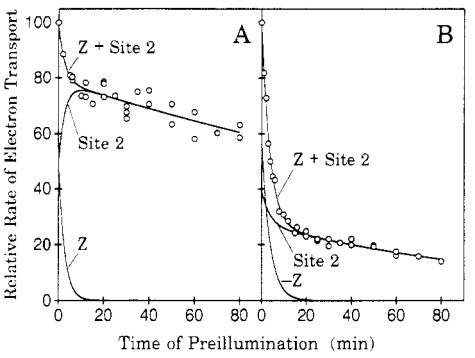

FIGURE 7: Predicted time courses for the photoinhibition of DPC or Mn^{2+} photooxidations in saturating light [(A) 1 mM DPC; (B) 100 μM Mn^{2+}]. The curves marked "Z" and "Site 2" are the predicted contributions to the overall rate from tyrosine Z and site 2, respectively, according to eq A-6 and A-7 (Appendix). The curve marked "Z + Site 2" is the sum of the other two, as described by eq A-4. The parameter values V_X', k_Z, and K_D were determined directly from the time courses of Figure 1 (for parameter definitions and the methods of their determination, see the Appendix). Values for qV_Z and $(1 - q)V_X$ were determined from the velocities through sites 1 and 2, respectively, at zero time of photoinhibition, calculated from the kinetic constants of Table I. The following values were used: (A) qV_Z = 52, $(1 - q)V_X$ = 48, V_X' = 79.2, k_Z = 0.368 min^{-1}, k_X = 3.29 × 10^{-3} min^{-1}. (B) qV_Z = 60, $(1 - q)V_X$ = 40, V_X' = 27.1, k_Z = 0.262 min^{-1}, k_X = 7.63 × 10^{-3} min^{-1}.

(site 1, Table I) are nearly equal and ~20-fold less than the K_m for its oxidation by site 2 (Table I); (3) no Mn becomes ligated during Mn^{2+} photooxidation by NH_2OH-PSII previously subjected to ≥30-min photoinhibition to extensively photodamage Z but not site 2 (unpublished results). These arguments and those derived from site-directed mutagenesis of tyrosine D and tyrosine Z of the D_2 and D_1 polypeptides, respectively (Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989), strongly suggests a direct important function of Z, but not D, in photoactivation.

Our K_m value of 10 μM for Mn^{2+} photooxidation at Z (site 1, Table I) compares favorably with the value of 12 μM determined by Izawa (1970), who used chloroplasts subjected to heat treatment to inactivate the WOC, and to a 10 μM value reported by Hoganson et al. (1989) for Tris-washed PSII membranes. These values, as well as the K_m value for Mn^{2+} photooxidation at site 2 (Table I), are appreciably greater than those determined either by Mn^{2+}/DPC competition assays (0.15 μM; Hsu et al., 1987), by assays of Mn^{2+}-supported photodecomposition of H_2O_2 [1.3 μM (Boussac et al., 1986); two sites, 0.033 and 0.36 μM (Inoué & Wada, 1987; Inoué et al., 1987)], or by various assays at pH 8.0 in the presence of Tris buffer (<0.2 μM; Klimov et al., 1982). We propose that the 10 μM K_m value for Mn^{2+} is typical for PSII membranes capable of photoactivation of the WOC and is, in fact, the K_m for Mn^{2+} photooxidation by Z^+ in the second proposed photoact of the photoactivation mechanism (Tamura & Cheniae, 1987a). According to this interpretation, the $Mn^{\geq 3+}$ ligated near Z on the first photoact is a (non)competitive inhibitor of Mn^{2+} photooxidation in the second photoact. Chemical reduction (e.g., by H_2O_2) of the first formed $Mn^{\geq 3+}$, or its destabilization (e.g., by high Tris concentration at alkaline pH), eliminates the competition, thereby permitting the observation of K_m values of ≤1 μM for Mn^{2+} photooxidation.

Our data (Figure 3) indicating two sites of DPC photooxidation with K_m values of 42 μM and 2.0 mM might seem to contrast with those of Hsu et al. (1987) showing only a single site of DPC photooxidation with a K_m of 150 μM. However, since these workers used only relatively large concentrations of DPC, the low-K_m site (site 1) would have been overlooked and the K_m of site 2 would have been grossly underestimated (Spears et al., 1971); their actual reported data are not in significant disagreement with ours.

The studies reported here and those by Callahan et al. (1986) suggest that photoinhibition of PSII occurs when the rate of P_{680}^+ turnover exceeds the rate of reduction of the native donors to P_{680}. According to the hypothesis of Thompson and Brudvig (1988), the primary event in photoinhibition is the formation of a presumed Chl^+ radical, and the secondary events arise from oxidations of amino acid residues by Chl^+ in its near vicinity. Alternatively, we suggest that P_{680}^+ itself may be the causative agent for photodamage resulting in formation of Chl^+, then loss of Z, followed by loss of site 2.

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APPENDIX: DERIVATION OF THE RATE EQUATION TO DESCRIBE THE TIME COURSE OF PHOTOINHIBITION

Under steady-state conditions, a certain fraction (q) of the reaction centers will have made Z^+ on any given turnover, while the remainder, $1 - q$, will have made X^+, where q is the probability of a single center making Z^+ and X is site 2. The value of q will depend, in part, on the rate of reduction of Z^+ by an exogenous donor. In the absence of any photoinhibition effects, the total rate of electron flow is $qV_Z + (1 - q)V_X$, where V_Z and V_X are the rates that would be observed if all of the centers made only Z^+ or only X^+, respectively. As Z becomes photodamaged, an increasing proportion of centers are able to make only X^+. At the same time, V_X approaches a new value, V_X', due to a change in the forward rate constant, k_p, for electron donation to X, reflected in a lower K_m for some donors at site 2 (Table I).

The relative significance of k_p to V_X depends on the relative values of the forward and reverse rate constants, k_1 and k_{-1}, respectively, for binding of the donor, as is apparent from the Briggs–Haldane expression:

$$V = \frac{k_p[E]_t[S]}{(k_{-1} + k_p)/k_1 + [S]} \qquad \text{(A-1)}$$

where $(k_{-1} + k_p)/k_1$ is K_m. In this example, $[E]_t$ is the concentration of functional site 2, [S] is the exogenous donor concentration, and V is V_X. Thus, the ratio of V_X'/V_X ought to vary for different electron donors. The change in k_p will have maximum effect on V_X when $[S] \gg K_m$ or $k_p \ll k_{-1}$. In the latter case, the change in K_m would be negligible, as was observed for Mn^{2+} at site 2 (Table I). Therefore, V_X'/V_X may be at a minimum in the case of Mn^{2+}. There is a small but finite probability, in any given center, that photodamage will occur to site 2 before Z; thus, after a certain duration of photoinhibition, some centers would be able to make only Z^+ (with rate V_Z').

Photodamage to both Z and site 2 exhibits first-order kinetics (Figure 1). Therefore, at any given time, the fraction of centers with functional Z is $\exp(-k_Z t)$, where k_Z is the first-order rate constant for damage to Z and t is the time of photoinhibition. The fraction with damaged Z is $1 - \exp(-k_Z t)$. Likewise, the fractions of centers with functional and damaged site 2 are $\exp(-k_X t)$ and $1 - \exp(-k_X t)$, respectively. By appropriate combination of these fractions, the fraction of centers in any particular state of photodamage can be calculated:

fraction of centers functional in	rate if all centers were in this state	
neither Z nor X = $(1 - e^{-k_Z t})(1 - e^{-k_X t})$	0	
X but not Z = $(1 - e^{-k_Z t})e^{-k_X t}$ $= e^{-k_X t} - e^{-(k_Z + k_X)t}$	V_X'	(A-2)
Z but not X = $(1 - e^{-k_X t})e^{-k_Z t}$ $= e^{-k_Z t} - e^{-(k_Z + k_X)t}$	V_Z'	
both Z and X = $e^{-k_Z t}e^{-k_X t} = e^{-(k_Z + k_X)t}$	$qV_Z + (1 - q)V_X$	

We conclude that the overall rate is

$$V = [qV_Z + (1 - q)V_X]e^{-(k_Z+k_X)t} + V_Z'[e^{-k_Z t} - e^{-(k_Z+k_X)t}] + V_X'[e^{-k_X t} - e^{-(k_Z+k_X)t}] \quad \text{(A-3)}$$

Our experimental data indicate that loss of Z occurs much faster than loss of site 2 (i.e., $k_Z \gg k_X$). The second term of eq A-3, describing the rate from centers that have lost site 2 but not Z, never contributes more than ~0.8% to the overall rate. Thus, eq A-3 simplifies to

$$V \approx [qV_Z + (1 - q)V_X - V_X']e^{-(k_Z+k_X)t} + V_X'e^{-k_X t} \quad \text{(A-4)}$$

This equation is identical in form with the empirical equation

$$V = A_f e^{-k_f t} + A_s e^{-k_s t} \quad \text{(A-5)}$$

describing a biphasic exponential decay. Thus, values for V_X', k_X, and k_Z can be determined directly from time courses such as those in Figures 1 and 5. By definition, qV_Z and $(1 - q)V_X$ are equal to the velocities through site 1 (Z) and site 2, respectively, in nonphotoinhibited membranes (Figures 2A and 3A; or calculated from the kinetic constants of Table I).

Equation A-4 can be broken into two separate equations representing the individual contributions of Z and site 2 to the overall velocity:

$$\text{rate through Z} = qV_Z e^{-(k_Z+k_X)t} \quad \text{(A-6)}$$

$$\text{rate through site 2} = [(1 - q)V_X - V_X']e^{-(k_Z+k_X)t} + V_X'e^{-k_X t} \quad \text{(A-7)}$$

In this derivation, the change in k_p at site 2 is attributed to changes induced by the loss of Z (i.e., V_X' does not continue to change after Z is lost). The possibility that k_p continues to change throughout the entire time course of photoinhibition cannot be excluded. Also, not included in the model is a decrease in quantum yield during photoinhibition, as noted by Callahan et al. (1986).

Figure 7 shows the calculated contributions of Z, site 2, and the sum of the two to the photooxidations of Mn^{2+}/DPC during photoinhibition. Panels A and B, for DPC and Mn^{2+}, respectively, show the calculated curves and experimental data points from saturating light measurements of DPC/Mn^{2+} photooxidations (data from Figure 1). These curves show that the relative contributions of site 1 (Z) and site 2 calculated from the experimentally determined kinetic constants of Table I can be reconciled with the observed biphasic time courses of photoinhibition.

Registry No. Chl P680, 53808-91-6; Mn, 7439-96-5; tyrosine, 60-18-4.

References

Armstrong, J. M. (1964) *Biochim. Biophys. Acta 86*, 194–197.

Babcock, G. T. (1987) in *Photosynthesis* (Amesz, J., Ed.) pp 125–158, Elsevier Science Publishers, Amsterdam.

Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 483–503.

Babcock, G. T., & Sauer, K. (1975a) *Biochim. Biophys. Acta 376*, 315–328.

Babcock, G. T., & Sauer, K. (1975b) *Biochim. Biophys. Acta 376*, 329–344.

Babcock, G. T., & Sauer, K. (1975c) *Biochim. Biophys. Acta 396*, 48–62.

Babcock, G. T., Blankenship, R. E., & Sauer, K. (1976) *FEBS Lett. 61*, 286–289.

Becker, D. W., Callahan, F. E., & Cheniae, G. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. IV, pp 31–34, Nijhoff Publishers, Dordrecht, The Netherlands.

Blubaugh, D. J., & Cheniae, G. M. (1988a) *Plant Physiol. 86*, S-131.

Blubaugh, D. J., & Cheniae, G. M. (1988b) in *Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models* (Stevens, S. E., & Bryant, D. A., Eds.) pp 368–372, The American Society of Plant Physiologists, Rockville, MD.

Blubaugh, D. J., & Cheniae, G. M. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 503–506, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Boska, M., Sauer, K., Buttner, W., & Babcock, G. T. (1983) *Biochim. Biophys. Acta 722*, 327–330.

Boussac, A., & Etienne, A.-L. (1982) *FEBS Lett. 148*, 113–116.

Boussac, A., Picaud, M., & Etienne, A.-L. (1986) *Photobiochem. Photobiophys. 10*, 201–211.

Callahan, F. E., & Cheniae, G. M. (1985) *Plant Physiol. 79*, 777–786.

Callahan, F. E., Becker, D. W., & Cheniae, G. M. (1986) *Plant Physiol. 82*, 261–269.

Cheniae, G. M., & Martin, I. F. (1971) *Biochim. Biophys. Acta 253*, 167–181.

Cheniae, G. M., & Martin, I. F. (1972) *Plant Physiol. 50*, 87–94.

Cheniae, G. M., & Martin, I. F. (1973) *Photochem. Photobiol. 18*, 409–416.

Cleland, R. E., Melis, A., & Neale, P. J. (1986) *Photosynth. Res. 9*, 79–88.

Conjeaud, H., Mathis, P., & Paillotin, G. (1979) *Biochim. Biophys. Acta 546*, 280–291.

Cramer, W. A., Theg, S. M., & Widger, W. R. (1986) *Photosynth. Res. 10*, 393–403.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988b) *Biochemistry 27*, 9071–9074.

Dekker, J. P., Plijter, J. J., Ouwehand, L., & Van Gorkom, H. J. (1984) *Biochim. Biophys. Acta 767*, 176–179.

DePaula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114–8120.

Ghanotakis, D. F., Yerkes, C. T., & Babcock, G. T. (1982) *Biochim. Biophys. Acta 682*, 21–31.

Golbeck, J. H., & Warden, J. T. (1985) *Biochim. Biophys. Acta 806*, 116–123.

Hayden, D. B., Baker, N. R., Percival, M. P., & Beckwith, P. B. (1986) *Biochim. Biophys. Acta 851*, 86–92.

Hoganson, C. W., Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1989) *Photosynth. Res. 22*, 285–294.

Hsu, B.-D., Lee, J.-Y., & Pan, R.-L. (1987) *Biochim. Biophys. Acta 890*, 89–96.

Ikeuchi, M., & Inoue, Y. (1988) *Plant Cell Physiol. 29*, 695–705.

Inouē, H., & Wada, T. (1987) *Plant Cell Physiol. 28*, 767–773.

Inouē, H., Akahori, H., & Noguchi, M. (1987) *Plant Cell Physiol. 28*, 1339–1343.

Inoue, Y., Kobayashi, Y., Sakamoto, E., & Shibata, K. (1975) *Plant Cell Physiol. 16*, 327–336.
Izawa, S. (1970) *Biochim. Biophys. Acta 197*, 328–331.
Klimov, V. V., Allakhverdiev, S. I., Shuvalov, V. A., & Krasnovsky, A. A. (1982) *FEBS Lett. 148*, 307–312.
Kyle, D. J. (1987) in *Photoinhibition* (Kyle, D. J., Osmond, C. B., & Arntzen, C. J., Eds.) pp 197–226, Elsevier, New York.
Kyle, D. J., Ohad, I., & Arntzen, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4070–4071.
Lozier, R. H., & Butler, W. L. (1973) *Photochem. Photobiol. 17*, 133–137.
MacKinney, G. (1941) *J. Biol. Chem. 140*, 315–322.
Metz, J. G., Nixon, P. J., Rogner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.
Miller, A.-F., & Brudvig, G. W. (1989) *Biochemistry 28*, 8181–8190.
Nugent, J. H. A., Demetriou, C., & Lockett, C. J. (1987) *Biochim. Biophys. Acta 894*, 534–542.
Ono, T., & Inoue, Y. (1987) *Plant Cell Physiol. 28*, 1293–1299.
Press, W. H., Flannery, B. P., Teukolsky, S. A., & Vetterling, W. T. (1988) *Numerical Recipes in C: The Art of Scientific Computing*, Cambridge University Press, New York.
Radmer, R., & Cheniae, G. M. (1971) *Biochim. Biophys. Acta 253*, 182–186.
Radmer, R., & Cheniae, G. M. (1977) in *Primary Processes of Photosynthesis* (Barber, J., Ed.) Vol. 2, pp 303–348, Elsevier, Amsterdam.
Reinman, S., Mathis, P., Conjeaud, H., & Stewart, A. (1981) *Biochim. Biophys. Acta 635*, 429–433.
Schreiber, U. (1986) *Photosynth. Res. 9*, 261–272.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Spears, G., Sneyd, J. G. T., & Loton, E. G. (1971) *Biochem. J. 125*, 1149–1151.
Styring, S., & Rutherford, A. W. (1987) *Biochemistry 26*, 2401–2405.
Tamura, N., & Cheniae, G. M. (1985) *Biochim. Biophys. Acta 809*, 245–259.
Tamura, N., & Cheniae, G. M. (1986) *FEBS Lett. 200*, 231–236.
Tamura, N., & Cheniae, G. M. (1987a) *Biochim. Biophys. Acta 890*, 179–194.
Tamura, N., & Cheniae, G. M. (1987b) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 1, pp 621–624, Martinus Nijhoff, Dordrecht, The Netherlands.
Tamura, N., & Cheniae, G. M. (1988) in *Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models* (Stevens, S. E., & Bryant, D. A., Eds.) pp 227–242, The American Society of Plant Physiologists, Rockville, MD.
Tamura, N., Inoue, Y., & Cheniae, G. M. (1989) *Biochim. Biophys. Acta 976*, 173–181.
Theg, S. M., Filar, L. J., & Dilley, R. A. (1986) *Biochim. Biophys. Acta 849*, 104–111.
Thompson, L. K., & Brudvig, G. W. (1988) *Biochemistry 27*, 6653–6658.
Van Gorkom, H. J. (1985) *Photosynth. Res. 6*, 97–112.
Van Gorkom, H. J. (1986) in *Light Emission by Plants and Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 267–289, Academic Press, New York.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Vermeglio, A., & Mathis, P. (1974) in *Proceedings of the 3rd International Congress on Photosynthesis* (Avron, M., Ed.) pp 323–334, Elsevier, Amsterdam, The Netherlands.
Weiss, W., & Renger, G. (1986) *Biochim. Biophys. Acta 850*, 173–183.
Yamashita, T., Inoue, Y., Kobayashi, Y., & Shibata, K. (1978) *Plant Cell Physiol. 19*, 895–900.
Yerkes, C. T., & Babcock, G. T. (1980) *Biochim. Biophys. Acta 590*, 360–372.
Yerkes, C. T., Babcock, G. T., & Crofts, A. R. (1983) *FEBS Lett. 158*, 359–363.