A New Mechanism-Based Inhibitor of Photosynthetic Water Oxidation: Acetone Hydrazone. 1. Equilibrium Reactions†

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ABSTRACT: The process of photosynthetic water oxidation has been investigated by using a new type of water oxidation inhibitor, the alkyl hydrazones. Acetone hydrazone (AceH), $(CH_3)_2CNNH_2$, inhibits water oxidation by a mechanism that is analogous to that of NH_2OH. This involves binding to the water-oxidizing complex (WOC), followed by photoreversible reduction of manganese (loss of the $S_1 \rightarrow S_2$ reaction). At higher AceH concentrations the S_1 state is reduced in the dark and Mn is released, albeit to a lesser extent than with NH_2OH. Following extraction of Mn, AceH is able to donate electrons rapidly to the reaction center tyrosine radical Z^+ (^{161}Tyr–D_1 protein), more slowly to a reaction center radical C^+, and not at all to the dark-stable tyrosine radical D^+ (^{160}Tyr–D_2 protein) which must be sequestered in an inaccessible site. Manganese, Z^+, and C^+ thus appear to be located in a common protein domain, with Mn being the first accessible donor, followed by Z^+ and then C^+. Photooxidation of Cyt *b*-559 is suppressed by AceH, indicating either reduction or competition for donation to $P680^+$. Unexpectedly, Cl^- was found not to interfere or compete with AceH for binding to the WOC in the S_1 state, in contrast to the reported rate of binding of *N*,*N*-dimethylhydroxylamine, $(CH_3)_2NOH$ [Beck, W., & Brudvig, G. (1988) *J. Am. Chem. Soc. 110*, 1517–1523]. We interpret the latter behavior as due to ionic screening of the thylakoid membrane, rather than a specific Cl^- site involved in water oxidation. AceH appears not to bind to the acceptor side of PSII as evidenced by normal EPR signals both for Q_A^-Fe(II), the primary electron acceptor, and for the oxidized Fe(III) acceptor (Q_{400} species), in contrast to that observed with the NH_2OH. AceH can be oxidized in solution by a variety of oxidants including Mn(III) to form a reactive diazo intermediate, $(CH_3)_2CNN$, which reacts with carbonyl compounds. Oxidation to this diazo intermediate is postulated to be responsible for inhibition of the WOC.

This study introduces a new inhibitor of photosynthetic water oxidation, acetone hydrazone (AceH).[1] Alkyl hydrazones react with oxidizing agents to form carbenes and diazo intermediates and can therefore be potentially useful for the exploration of the active sites in redox enzymes.

Photosynthetic oxygen evolution occurs following four consecutive electron-transfer steps at a special cluster of four manganese ions which comprise the water-oxidizing complex (WOC) of photosystem II (PSII) in higher plants and cyanobacteria. The WOC also requires calcium and chloride which, together with manganese, bind at the lumenal surface of the PSII reaction center complex, and at the interface of two protein domains: the membrane-bound reaction center complex (Miyao & Murata, 1985; Ghanotakis & Yocum, 1985; Ikeuchi et al., 1985) and an extrinsic 33-kDa manganese-stabilizing protein (Kuwabara & Murata, 1982; Yamamoto et al., 1984; Abramowicz & Dismukes, 1984). Reaction center complexes capable of O_2 evolution are comprised of five protein subunits in addition to the manganese-stabilizing protein: the three subunits which comprise the D_1D_2Cyt *b*-559 reaction center core (Nanba & Satoh, 1987; Barber et al., 1987) and two chlorophyll *a* binding proteins. The protein sites where manganese, calcium, and chloride bind remain unknown, although proposals have been advanced (Padhye et al., 1986; Dismukes, 1988) and some initial data from protein labeling studies have begun to appear (Tamura et al., 1989; Seibert et al., 1989).

Our motive for investigating the alkyl hydrazones lies in their potential use as protein labeling reagents which are selective for redox-active sites capable of multielectron oxidations:

$$RR'CNNH_2 \xrightarrow{-2e^-/2H^+} RR'C{=}N^+{=}N^- \leftrightarrow RR'C^-{-}N_2^+ \quad (1)$$

Dehydrogenation of the hydrazone with a variety of two-electron oxidants, including MnO_2, produces the reactive diazo intermediate in solution (eq 1) (Regitz, 1978). The diazo species can undergo both electrophillic and nucleophilic addition reactions, protonation and N_2 elimination to form the carbocation, as well as addition reactions derived from formation of the carbene:

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[1] Abbrevations: PSII, photosystem II; WOC, water-oxidizing complex; Chl, chlorophyll; MES, 2-(*N*-morpholino)ethanesulfonic acid; OGP, *n*-octyl β-D-glucopyranoside; EPR, electron paramagnetic resonance; AceH, acetone hydrazone; DDQ, dichlorodicyanobenzoquinone; DPC, diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; DCBQ, 2,5-dichloro-*p*-benzoquinone; PPBQ, *p*-phenylbenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Q_A, primary plastoquinone acceptor; Q_B, secondary plastoquinone acceptor; Z^+, ^{161}Tyr on D_1 subunit; D^+, ^{160}Tyr on D_2 subunit; C^+, photooxidizable radical near reaction center; P680, special reaction center chlorophyll of PSII; TLC, thin-layer chromatography.

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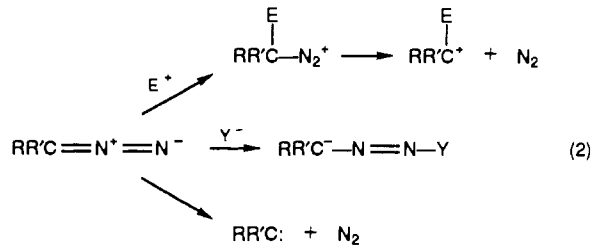


(2)

In protic solvents the carbonium ion pathways are favored (Hegarthy, 1978). This chemistry can be used as the basis for exploring the active sites of redox proteins, such as the binding site for manganese in the WOC.

At least three classes of electron transport inhibitors are known which target sites between water and P680, the reaction center photooxidizable chlorophyll of PSII. One class consists of small molecules such as NH_2OH, N_2H_2, H_2O_2, and H_2S. These act as redox-active substrate analogues which release manganese by chemical reduction in the dark (Bennoun & Joliot, 1969; Cheniae & Martin, 1971). At limiting concentrations of inhibitor, release of Mn does not occur and inhibition is reversible. Instead, a limited two-electron reduction of the Mn cluster occurs which is initiated upon formation of the S_2 state following illumination, producing an S_0 oxidation state (Bouges, 1971; Velthuys & Kok, 1978; Mano et al., 1987; Frasch & Mei, 1987; Guiles et al., 1990; Sivaraja & Dismukes, 1988a,b; Sivaraja et al., 1988). Evidence supporting reversible reduction in the dark of S_1 to S_{-1} has also been given (Beck & Brudvig, 1987).

In a separate class are small amines such as NH_3 (Sandusky & Yocum, 1983, 1984, 1986). NH_3 inhibits O_2 evolution by displacement of chloride from the WOC. A second low-affinity site for NH_3 binding also exists (Beck et al., 1986; Andreasson et al., 1988) which appears to bind directly to the manganese cluster (Britt et al., 1989). This may also correspond to the binding site of the substrate, water.

The most powerful class of inhibitors is the so-called "ADRY" reagents which can reduce the PSII electron donors at substoichiometric levels. These inhibitors selectively deactivate the higher oxidation states of the WOC, namely, S_2 and S_3, and include lipophilic electron donors such as $B(Ph)_4^-$, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), various derivatives of 2-anilinothiophenes, and phenylhydrazone derivatives (Renger & Inoue, 1983; Velthuys, 1983; Hanssum et al., 1985; Packham & Ford, 1986). Mechanistically, some of these appear to inhibit water oxidation by direct reduction of the tyrosine radical Z^+ (Ghanotakis et al., 1982).

Some small molecules that reduce the rate of water oxidation may do so by interacting directly with the electron acceptor side of PSII. This can lead to complications in their use as probes of the donor side reactions. This appears to be the case for NH_2OH which binds to PSII and perturbs both the manganese cluster and the primary electron acceptor Q_A^-Fe (Sivaraja & Dismukes, 1988a,b).

In order to understand the basis for inhibition of water oxidation by the alkyl hydrazones, we have examined the reactions of AceH with the PSII electron donors and acceptors using equilibrium titrations and electron paramagnetic resonance (EPR) spectroscopy. This has been compared to its reaction with a synthetic dimanganese(III) complex, which is a partial model of the WOC.

Materials and Methods

Acetone hydrazone (AceH) was synthesized according to the procedure of Day and Whiting (1970) with the following modification. A one-step synthesis was performed by using a molar ratio of 1.0 mol of acetone to 1.1 mol of anhydrous hydrazine and refluxed for 12 h at 100 °C. The hydrazone was purified by distillation over a molecular sieve at a boiling range of 124–126 °C and its purity was confirmed by proton NMR. The chemical shifts were $\delta = 1.7$ (CH_3) and 1.8 (CH_3) and $\delta = 4.7$ (NH_2), in agreement with those reported in the literature (Karabatsos & Osborne, 1968). A 5% yield of the azine dimer $(CH_3)_2NN(CH_3)_2$ was also detectable. Because AceH slowly hydrolyzes to acetone and hydrazine after prolonged standing at room temperature in air, the compound was stabilized by storage at −80 °C until further use. It is stable in aqueous solutions for a period of at least 30 min, as monitored by NMR.

Spinach BBY–PSII membranes were prepared according to the procedure outlined by Berthold and co-workers (Berthold et al., 1981), except with the modification of Ford and Evans (1983), and stored at −80 °C in buffer A containing 0.4 M sucrose, 50 mM MES, 25 mM NaCl, pH = 6.5, and 30% glycerol. These preparations typically evolve oxygen at rates of 300–400 μmol of O_2 (mg of Chl^{-1}) h^{-1}. Oxygen-evolving core particles were prepared from PSII membranes by using the nonionic detergent *n*-octyl β-D-glucopyranoside (OGP), according to the procedure outlined by Ghanotakis et al. (1987), except that a 40 mM OGP solution was used for extraction (Sivaraja & Dismukes, 1988c). Buffer B, which was used to measure oxygen rate activity in the PSII core complexes, contained 0.4 M sucrose, 50 mM MES, 10 mM NaCl, and 15 mM $CaCl_2$, pH = 6.0. These particles typically exhibited an oxygen rate activity in the range of 700–900 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Oxygen evolution was monitored by using a Clark-type oxygen electrode in the presence of 1–2 mM 2,5-dichloro-*p*-benzoquinone (DCBQ) as the exogenous electron acceptor.

The release of manganese from the PSII membranes upon treatment with AceH was detected by graphite furnace atomic absorption spectroscopy.

PSII membranes were treated with DMH by first resuspending in buffer A. Appropriate concentrations of acetone hydrazone were added from a 0.1 M stock solution in DMSO (dimethyl sulfoxide) and incubated at a final Chl concentration of 0.5 mg/mL. The samples were subsequently dark-adapted for 3–5 min with gentle shaking and then centrifuged at 10000*g* to remove the excess AceH. In the case of the PSII core particles, incubation with the inhibitor occurred at a [Chl] = 0.1 mg/mL in buffer B.

For measurement of the PSII acceptor Fe(III) EPR signals, samples were resuspended to a final [Chl] = 3–4 mg/mL in buffer C containing 0.4 M sucrose, 50 mM HEPES, and 25 mM NaCl, pH, = 7.2–7.5, and dark-adapted at 4 °C for 1–2 h. AceH was added to final concentrations of 1 and 2 mM. *p*-Phenylbenzoquinone (PPBQ) was then added as the terminal electron acceptor and brought to a final concentration of 1 mM. Subsequent procedures for oxidation of Fe(II) were performed according to Petrouleas and Diner (1987). The procedure for salt washing of the PSII membranes with 2 M NaCl or 1 M $CaCl_2$ at pH = 6.5 to remove the extrinsic 17- and 23-kDa proteins or all three extrinsic polypeptides (17, 23, and 33 kDa), respectively, was adapted from Miyao and Murata (1983) and Kuwabara and Murata (1982), respectively. Removal of the extrinsic proteins and manganese by 0.8 M Tris washing was also performed by incubation of PSII membranes in Tris base at pH = 9.0 for 30 min and at a [Chl] = 0.5 mg/mL (Yamashita & Butler, 1968). These depleted membranes were subsequently centrifuged at 10000*g* and resuspended in buffer B (except for $CaCl_2$-washed samples

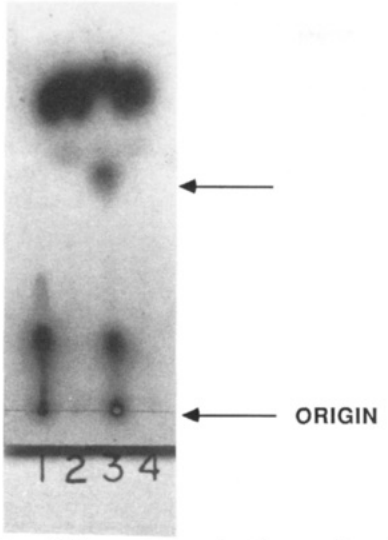


FIGURE 1: Silica gel chromatogram showing reaction of AceH with dichlorodicyanobenzoquinone (DDQ) in the presence of benzaldehyde. Lane 1: AceH. Lanes 2 and 4: AceH + benzaldehyde. Lane 3: Benzaldehyde + DDQ + AceH.

which were resuspended in buffer B containing 200 mM NaCl). Incubation of protein-depleated samples with AceH proceeded in the dark at 4 °C for 10–15 min prior to electron transport and EPR measurements.

Photoinduced electron transport rates through PSII were measured as either the Hill reaction, $H_2O \rightarrow$ 2,6-dichlorophenolindophenol (DCIP), or the reaction 2,5-diphenylcarbazide (DPC) → DCIP, following the extraction of manganese. The kinetics were measured spectrophotometrically by following the change in the intensity of the 600-nm absorption band of DCIP. A cuvette containing a dark-adapted suspension of PSII membranes at [Chl] = 20 μg/mL in buffer B (except for $CaCl_2$-treated samples, which were resuspended in buffer B containing 200 mM NaCl) was mixed with a DCIP stock solution in buffer to obtain a final concentration of 0.20 mM.

For TLC the running solvent was a 50:50 mixture of toluene and ethyl acetate and the plates were visualized by iodine staining.

Results and Discussion

Oxidation of Acetone Hydrazone by Manganese. The reaction of the manganese complex $HBPz_3Mn^{III}O(CH_3CH_2CO_2)_2Mn^{III}Pz_3BH$ [Pz_3BH = hydrotris(*N*-pyrazolyl)borate; Sheats et al., 1987] with acetone hydrazone gave as the major manganese products $Mn^{II}(HBPz_3)_2$ and an unidentified Mn(II) species along with a gaseous product presumed to be N_2. $Mn^{II}(HBPz_3)_2$ was identified by thin-layer chromatography (TLC) and its characteristic EPR spectrum (Unni Nair et al., 1989), while the other Mn(II) species gave rise to a symmetric EPR signal at $g = 2$ characteristic of high-spin Mn(II) in ligand fields of axial symmetry or higher. These results are compatible with the expected pathway given in eq 1.

Formation of the short-lived reactive diazo species could not be followed directly. Instead, we used a chemical trapping scheme to verify its presence and reactivity. Generation of the diazo species in the presence of aldehydes and ketones is known to lead to the formation of epoxides and carbene insertion adducts (Hegarty, 1978).

Formation of a covalent adduct between the diazo intermediate and benzaldehyde is indicated by TLC, as shown in Figure 1. This experiment uses dichlorodicyanobenzoquinone (DDQ) as the oxidant. The sample running in lane 1 is AceH. Lanes 2 and 4 are mixtures containing AceH + benzaldehyde (lane 4 contains a higher concentration), and lane 3 is the reaction mixture of benzaldehyde + DDQ + AceH. The most striking feature on the TLC plate is the appearance of a new compound which forms in the presence of all three reagents, as shown by the upper arrow in lane 3. In particular, the formation of this new compound occurs only if AceH is added to the mixture after the addition of benzaldehyde and DDQ. No detectable reaction occurred between DDQ and benzaldehyde (data not shown). An unresolved higher mobility band is observed in all lanes. Identification of the new species of lower mobility in lane 3 was not pursued. It is sufficient for our purpose to note that a covalent adduct forms in the oxidative coupling of the hydrazone with benzaldehyde.

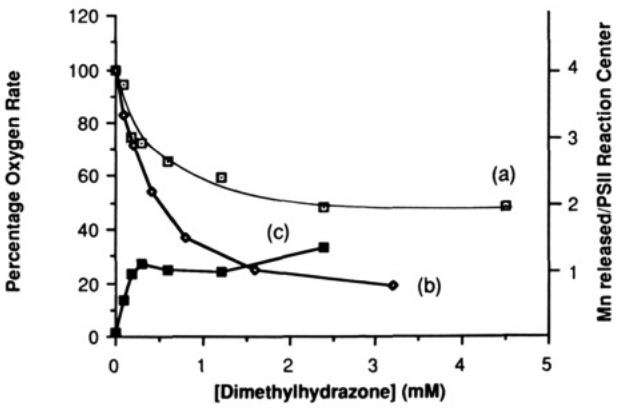


FIGURE 2: Comparison of the inhibition of steady-state oxygen evolution in PSII membranes and PSII core complexes as a function of AceH concentration. Curves: (a) PSII membranes; (b) PSII core complexes; (c) Mn release from PSII membranes. Assay media contain 0.4 M sucrose, 50 mM MES, 25 mM NaCl, 15 mM $CaCl_2$, pH = 6.5, and 1–2 mM DCBQ as the electron acceptor [Chl] = 30–40 μg/mL.

An important point to note is that the order of addition of the three reagents controls the formation of the covalent adduct. The new species in lane 3 appeared only when AceH was added to a mixture of DDQ + benzaldehyde. In the case where AceH was added to benzaldehyde prior to addition of DDQ, no resolvable product was detected on TLC. This shows that the new species in lane 3 is derived by oxidation with DDQ in the presence of the hydrazone and benzaldehyde, as expected it its formation requires the diazo intermediate. These experiments show that AceH undergoes two-electron oxidation to yield a reactive intermediate, presumed to be the diazo species, which can be covalently trapped with carbonyl compounds. This predicts its potential for labeling proteins which harbor oxidizing reactive sites.

Inhibition of O_2 Evolution by Acetone Hydrazone. AceH inhibited the steady-state rate of O_2 evolution with a concentration dependence as shown in curves a and b of Figure 2 for PSII membranes and PSII core complexes, respectively. In both cases the dependence was biphasic with a 50% inhibition concentration, I_{50}, of approximately 0.25 mM, which is 4-fold greater for PSII membranes (2 mM) than for PSII core complexes (0.5 mM). However, the fraction of centers that were inhibited in both PSII membranes and core complexes were equal with an I_{50} of ~0.25 mM. This biphasic behavior was also seen in the yield of variable fluorescence (Vass et al., 1990), and in samples in which the excess hydrazone was not separated prior to assay. Therefore, it is a characteristic of the inhibition process and not due to depletion of the electron acceptor upon addition of AceH.

Oxygen evolution requires chloride, and inhibition of activity

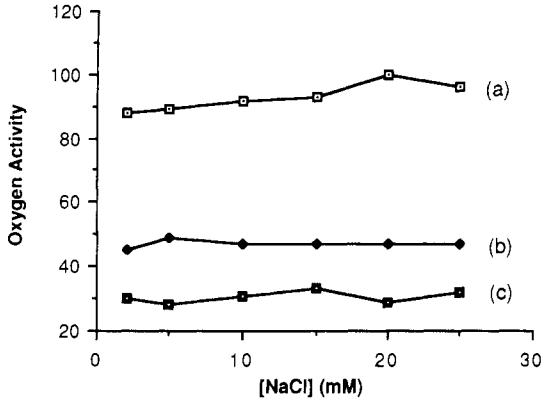


FIGURE 3: Oxygen evolution in AceH-treated PSII membranes as a function of Cl^- concentration. Curves: (a) 0 mM AceH; (b) 1 mM AceH; (c) 2 mM AceH. Assay media contained 0.4 M sucrose, 50 mM MES, pH = 6.5, and 1–2 mM DCBQ as the electron acceptor. [Chl] = 30–40 μg/mL.

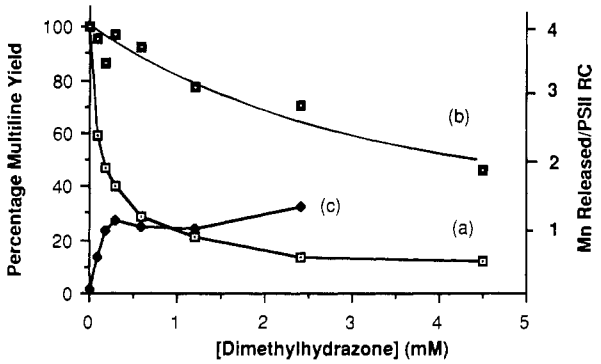


FIGURE 4: Effect of AceH on the formation of the S_2 multiline EPR signal in PSII membranes at 200 K and the recovery from inhibition by multiple turnovers at 273 K. Curves: (a) illumination at 200 K; (b) sample from curve a warmed to 273 K, illuminated under dim light for 30 s, dark-adapted for 30 min, and reilluminated at 200 K; (c) amount of Mn release as a consequence of AceH treatment. [Chl] = 4–5 mg/mL; [DCBQ] = 1 mM. EPR conditions: T = 8 K; MA = 32 G; TC = 1 min.

may arise from displacement of Cl^- by the inhibitor, as in the case of NH_3 (Sandusky & Yocum, 1983, 1984, 1986). To test for this, we examined the dependence of the O_2 evolution rate as a function of Cl^- concentration at 1 and 2 mM AceH. Samples were washed free of excess Cl^- by dilution and centrifugation. The amount of residual Cl^- was estimated to be <8 μM. The data given in Figure 3 show that in the range of 1–25 mM NaCl there is no evidence for competition between AceH and Cl^-. This result was not expected since the similar inhibitor *N,N*-dimethylhydroxylamine, $(CH_3)_2NOH$, has been found to exhibit competitive kinetics with Cl^- in the range of 20–50 mM for the rate of binding to its site of inhibition (Beck & Brudvig, 1988).

AceH Inhibits the $S_1 \rightarrow S_2$ Reaction. Figure 4a shows that the yield of the S_2 multiline EPR signal for the Mn cluster, produced by a single-turnover illumination at 200 K, also decreases biphasically with increasing concentrations of AceH and reaches 50% at 0.2 mM AceH. This is a factor of 10 lower than observed for inhibition of the initial rate of O_2 evolution for the same concentration of AceH. This inhibition was partly photoreversible as shown in Figure 4b. In this experiment, the illuminated sample was warmed to 273 K and illuminated under dim light in order to produce multiple turnovers. This was followed by dark adaptation for 30 min and reillumination at 200 K. The S_2 multiline signal was partly restored by this treatment, to an extent that is equal to or less than the fraction

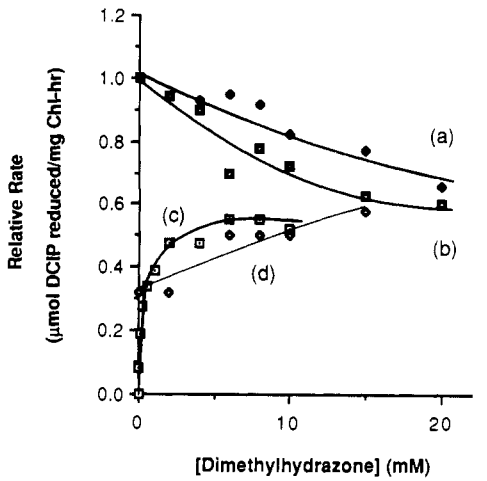


FIGURE 5: Effects of AceH on the reduction kinetics of DCIP in PSII membranes obtained from various salt treatments. Curves: (a) control PSII membranes; (b) 2 M NaCl washed PSII; (c) 0.8 M Tris-washed PSII; (d) 1 M $CaCl_2$ washed PSII. [Chl] = 20 μg/mL; [DCIP] = 200 μM. A relative rate of 1 = 200 μM DCIP reduced/(mg of Chl·h). The samples were incubated with the indicated [AceH] for 2–3 min prior to measurement.

of centers which retain functional Mn. Figure 4c shows that about 1 atom of (nonspecific) Mn is released from PSII membranes at an AceH concentration that yields about a 60% oxygen evolution rate. Release of the three extrinsic proteins (17, 23, and 33 kDa), as determined by SDS–PAGE, was observed upon incubation with AceH and increased qualitatively with the extent of loss of O_2 evolution. Protein release by AceH was always less than complete.

Acetone Hydrazone Does Not Inhibit Electron Transport through the PSII Reaction Center. The effect of AceH on the reaction center can be monitored by the reduction kinetics of an exogenous electron acceptor, DCIP. This experiment is shown in Figure 5. PSII membranes that have been treated with 2 M NaCl to remove the 17- and 23-kDa proteins have only slightly greater sensitivity to AceH than the control PSII membranes (Figure 5, curves b and a, respectively). This indicates that these extrinsic proteins do not impose a kinetic barrier to the photooxidation of AceH, in contrast to the 10–20-fold greater sensitivity to inhibition of O_2 evolution seen for NH_2OH in salt-washed PSII membranes (Ghanotakis et al., 1984). The lower rate of DCIP reduction in both samples most probably reflects the replacement of the endogenous Mn electron donor with AceH. An important point to note is that the PSII-mediated electron transport rates in control membranes (curve a) and in membranes lacking the 17- and 23-kDa polypeptides (curve b) remain much higher than oxygen evolution rates at comparable concentrations of AceH (Figure 2). This implies that the reaction center remains functional between the electron donor, Tyr–Z, and the primary acceptor, Q_A, although O_2 evolution is partially inhibited. This places the site of inhibition prior to Z oxidation, namely, in the Mn cluster. Removal of all three of the extrinsic proteins (17, 23, and 33 kDa) by washing in 1 M $CaCl_2$ resulted in an initial DCIP reduction rate that was 30% of the control rate. This rate increased with addition of AceH, approaching the same rate as that observed in the NaCl-treated PSII sample above 10 mM AceH (curve d). Electron transport is known to be specifically disrupted within the WOC by removal of the 33-kDa protein, the so-called "manganese-stabilizing protein". These data show that AceH can act as an alternate donor either to the exposed Mn site or, following the release of Mn, to Tyr–Z^+. Another possibility is the solubilization of func-

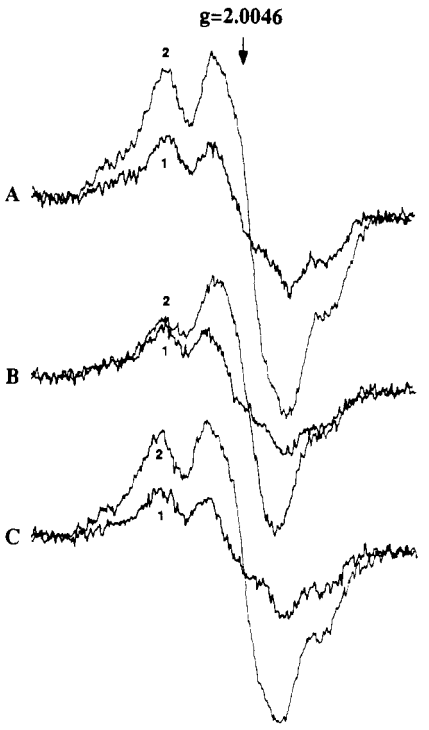


FIGURE 6: Room temperature EPR spectra of signal II (slow and fast) in Tris-treated PSII membranes: (1) dark; (2) continuous illumination. (A) Tris–PSII; (B) Tris–PSII + 1 mM AceH; (C) same as (B) except centrifuged and resuspended in AceH-free buffer in the dark. [Chl] = 2–3 mg/mL. EPR conditions: gain = 8×10^3; power = 10.2 mW; MA = 2 G.

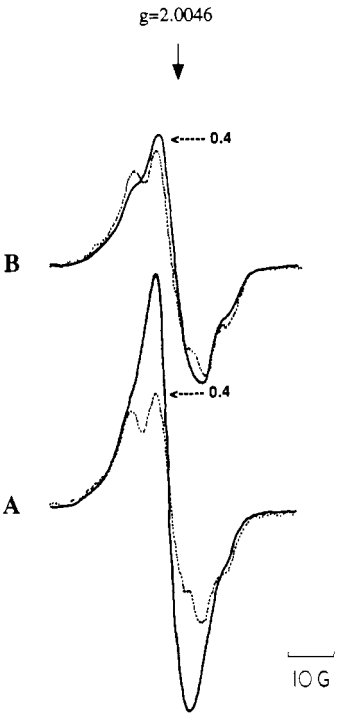


FIGURE 7: Low-temperature EPR spectra of Tris-washed PSII membranes illuminated at 200 K. Solid lines (gain × 0.4) show the spectra after 200 K illumination, and dotted lines show the dark spectra after a 273 K dark period for 30 s. (A) Tris–PSII; (B) Tris–PSII + 2 mM AceH; [Chl] = 2–3 mg/mL, and 1 mM PPBQ. EPR conditions: gain = 1.25×10^5 (dotted) and 5×10^4 (solid); power = 36 dB; MA = 4 G; T = 12 K; 9.49 GHz.

tional Mn and its replacement by AceH.

AceH was found to partially restore PSII-mediated electron transport in membranes that had been initially depleted of all three extrinsic polypeptides and 3 or 4 Mn atoms by washing with 0.8 M alkaline Tris (Figure 5c). These data show that AceH can act as an alternate electron donor to the reaction center following removal of Mn. The likely site of donation is at Tyr–Z^+. This assumption was tested by EPR (vide infra).

The saturated rate for AceH → DCIP electron transport was slower than that for DPC → DCIP, indicating a slower oxidation rate for AceH by Z^+ than for DPC (data not shown). PSII-mediated electron transport was completely inhibited by addition of 0.5 mM DCMU, a Q_B analogue herbicide (data not shown). These results demonstrate that the AceH → DCIP reaction involves electron flow through the PSII reaction center rather than a spurious photosensitized reaction.

Acetone Hydrazone Reduces Tyrosine Donor Z^+ but Not the Dark-Stable Tyrosine D^+. As previously reported by Babcock et al. (1986), a doubling of the intensity of the dark-stable EPR signal II, arising from tyrosine donor D^+, can be observed under continuous illumination conditions in Tris-treated membranes without an added artificial electron acceptor. This is shown in Figure 6A. In contrast, when AceH was present, the light-induced contribution to signal II was absent, presumably due to electron donation by AceH to Z^+ (Figure 6B). The dark signal for tyrosine D^+ was unaffected. The suppression of Z photooxidation by AceH could be reversed in the dark by dilution and centrifugation to remove the unreacted AceH (Figure 6C).

At room temperature both the control and the AceH-treated samples exhibited a light-induced symmetric signal at the g = 2.0030 region with a line width, ΔH_{pp}, of 7.5–8.0 G. This is due to a free-radical species of unknown origin. It has previously been suggested that this species may be associated with photooxidation in the presence of $K_3Fe(CN)_6$ to form a chlorophyll radical at g = 2.0026 having ΔH_{pp} of 8 G (Malkin & Bearden, 1973) or, in the presence of K_2IrCl_6, a species having ΔH_{pp} of 10 G (dePaula et al., 1985), or even to form a carotenoid species close to the PSII reaction center (Nugent et al., 1981). The yield of this photooxidized radical, here called C^+, was not affected by AceH when illuminated at room temperature. The C^+ radical decays promptly in the dark.

In Tris-washed PSII membranes depleted of Mn and illuminated at 200 K, low-temperature EPR revealed the same light-induced radical species (Figure 7A). However, in the presence of AceH this radical was greatly reduced (Figure 7B), showing that AceH can replace Mn as a more effective donor than the oxidizable reaction center donor, C. In line with the previously observed normal functioning of the PSII reaction center, these data show that both tyrosine Z^+ and the C^+ radical can be reduced by bound AceH in the absence of Mn. However, electron donation from AceH under continuous turnover conditions at room temperature does not compete well with photooxidation of donor C (Figure 6B), suggesting a slow steady-state reduction rate of C^+ from AceH in solution. No direct evidence for a stable $AceH^+$ radical that should form upon reduction of Z^+ or C^+ was found. This is presumably due to the instability of the radical analogous to that observed for oxidation of NH_2OH by PSII (Beck & Brudvig, 1987; Sivaraja & Dismukes, 1988a–c). It has also been found that certain ADRY reagents such as carbonyl cyanide (*m*-chlorophenyl)hydrazone (CCCP) reduce Z^+ in Tris-washed chloroplasts. These oxidized compounds in turn are susceptible to reduction by a reductant pool which consists of carotenoids, chlorophyll, and Cyt *b*-559 (Ghanotakis, et al., 1982).

Acetone Hydrazone Does Not Bind to the Iron–Quinone Acceptor in PSII. Because NH_2OH influences the primary electron acceptor as seen by a shift of the g = 1.9 EPR signal for Q_A^-Fe, we searched for evidence of a similar reaction with

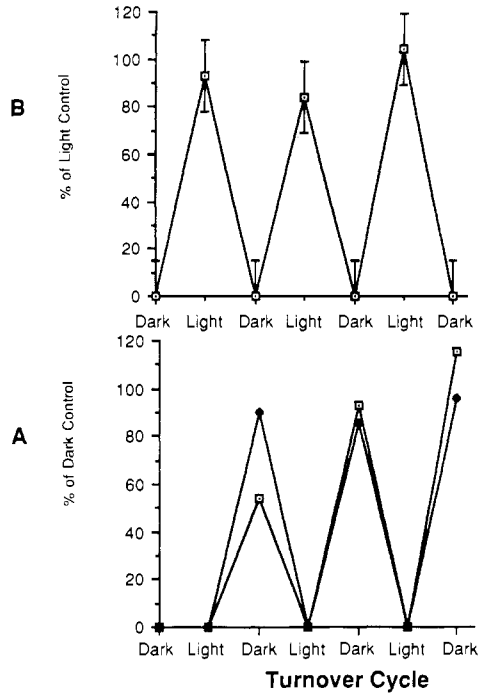


FIGURE 8: Comparison of the yields of the $g = 8.1$ Fe(III) and $g = 1.9$ Q_A^-Fe(II) EPR signals in AceH-treated PSII membranes relative to the yields in control PSII as a function of sequential electron turnover events by a 200 K illumination followed with a dark adaptation at 273 K. (A) $g = 8.1$ EPR signal yield for (◆) 1 mM AceH and (⊡) 2 mM AceH; (B) $g = 1.9$ EPR signal yields at 1 mM AceH. EPR conditions: gain = 3.2×10^5; MA = 32 G; power = 8 dB; $T = 4.6$ K; 9.46 GHz.

AceH. We used the EPR signals for both Q_A^-Fe(II) and the oxidation state in which the iron is oxidized to Fe(III) (Petrouleas & Diner, 1986; Zimmermann & Rutherford, 1986). As previously reported, continuous illumination at 200 K of PSII membranes in buffer at pH = 7.2 produces a single turnover, which is demonstrated by the characteristic Q_A^-Fe EPR signal appearing at $g = 1.9$. Upon warming to 273 K for 30 s in the dark in the presence of the exogenous quinone PPBQ, oxidation of the Fe(II) occurs and new EPR signals appear at $g = 8.1$ and $g = 5.6$ for Fe(III). No significant change in the yield of the S_2 multiline signal was observed during the dark warming cycle to 273 K in the control, in agreement with the known stability of the S_2 state under these conditions. A second turnover by illumination at 200 K converts the Fe(III) form back to Fe(II). In a series of three turnovers, the intensity of the Fe(III) signals maximizes on the first cycle, decreases on the second cycle, and peaks again on the third cycle. Figure 8A plots the yield of the $g = 8.1$ Fe(III) EPR signal relative to the yield in the control as a function of successive turnovers for PSII membranes treated with 1 and 2 mM AceH prior to the addition of PPBQ. The oxidized Fe(III) EPR signals (both $g = 8.1$ and $g = 5.5$ signals) were generated in ~90% (1 mM AceH) and 54% (2 mM AceH) yields on the first turnover, but with no significant changes in yield relative to the control on the second and third turnovers. The line shapes of the EPR signals for both Fe(III) and Q_A^-Fe did not change relative to the untreated material, and there were no new signals as a consequence of the AceH addition. These results suggest that AceH does not bind in the vicinity of the acceptor side iron.

In the presence of 2 mM AceH, the yield of Fe(III) in the dark cycle following the first turnover was 40–50% of the control value (Figure 8A). The reduced yield of Fe(III) can be understood if ca. 50–60% of the centers undergo either no turnover, as a result of the binding of more than one molecule of AceH, or a double turnover on the first illumination cycle at 200 K. Normal yields on the second and third turnovers could then be accounted for by slow or incomplete displacement of oxidized AceH by new AceH molecules on the donor side. This explanation is supported by the observation noted above that AceH was capable of reducing the reaction center donor C^+ under single-turnover conditions, but not as efficiently under steady-state turnover conditions. Moreover, AceH does not dampen the oscillations in the yields of the Fe signals like the classical Q_B herbicides; hence it does not act like an inhibitor of the acceptor side. An alternative but less likely explanation is that AceH binds directly to and reduces Fe(III) and suppresses semiquinone formation, but without influencing either of their EPR spectral line shapes.

Correlation of Loss of Electron Donor and Acceptor Signals. At the 2 mM concentration of AceH used for this experiment there was 50% loss of the O_2 evolution rate (Figure 2) and 80% initial loss of the S_2 multiline signal (Figure 4). This concentration also gave a reduced yield of both Fe(III) and Q_A^-Fe(II) (data not shown) on the first turnover, indicating either disruption of electron transfer or reduction of the S_1 state on the donor side. Evidence supporting the latter origin is seen by the loss of the EPR multiline signal (Figure 4). At 1 mM AceH, illumination at 200 K produced acceptor side EPR signals that were comparable in yields to the control (Figure 8) and only nonspecific Mn release occurred (Figure 4), although the formation of the S_2 state was inhibited by 75–80%. These results are compatible with the observation that the level of inhibition by AceH is photoreversible to a greater extent at lower concentrations of AceH, as seen by the recovery of the S_2 multiline signal (Figure 4). The release of approximately 1 Mn atom even at low concentrations of AceH (<1 mM) does not seem to correlate with the loss of multiline signal, since the yield of this signal was photoreversible in these samples (Figure 4). This appears to be due to the release of nonspecific Mn not associated with the WOC. This behavior is analogous to that which is observed with NH_2OH at low concentrations (Sivaraja & Dismukes, 1988a). Above 1 mM AceH a shallow slope in the release occurs which appears to be associated with the WOC. This concentration also coincides with a sharp loss in the inhibition of O_2 evolution (Figure 2). This biphasic inhibition curve for O_2 evolution is a distinguishing feature of inhibition by AceH, in contrast to other reductive inhibitors like NH_2OH which has a nearly monophasic inhibition curve that follows in parallel with Mn release. This has also been observed in studies of thermoluminescence and fluorescence emission (Vass et al., 1990). The origin of this effect is unexplained but may be releated to the lowered extent of Mn release, even though Mn reduction remains monophasic.

Acetone Hydrazone Reduces Cyt b-559$^+$. Disruption of the normal sequence in electron donation by AceH can also be observed by a lower yield of the EPR signal at $g = 3.0$ for the photooxidized Cyt *b*-559$^+$. As shown in Figure 9 the yield of this signal increases in control membranes on the second and third turnover as donors closer to the reaction center chlorophyll donor, P680$^+$, are exhausted, while AceH-treated samples have lower yields on all turnovers. This behavior can be understood if AceH can donate electrons either directly to Cyt *b*-559$^+$ or to an endogenous donor which competes with it for oxidation by P680$^+$.

CONCLUSIONS

The evidence given here shows that AceH inhibits the WOC by a two-stage mechanism similar to that observed with

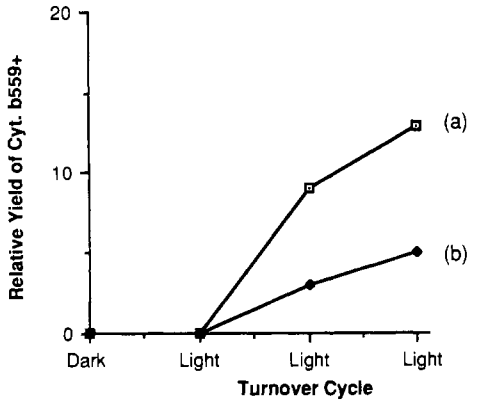
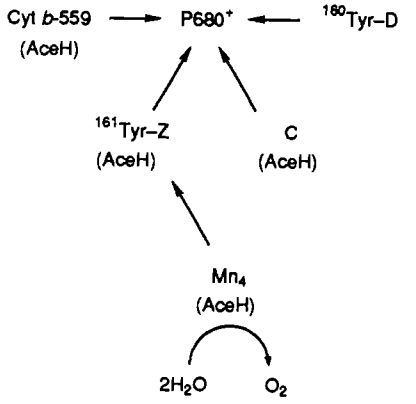


FIGURE 9: Comparison of the yield of Cyt b-559$^+$ (g = 3.0) as a function of sequential electron turnover events between (A) control PSII and (B) 2 mM AceH-treated PSII. EPR conditions are the same as for Figure 8 except T = 12 K.

Scheme I



NH_2OH (Bouges, 1971; Sivaraja & Dismukes, 1988a) and H_2S (Sivaraja et al., 1988). At low concentrations it inhibits O_2 evolution by binding in the dark S_1 state to the WOC, close to the manganese cluster, analogous to the behavior of NH_2OH (Andreasson & Hansson, 1987; Beck & Brudvig, 1987; Sivaraja & Dismukes, 1988a). Under these conditions illumination at 200 K does not form a normal S_2 state, as seen by loss of the S_2 multiline signal. This inhibition can be reversed by continous illumination upon warming to 273 K, which allows multiple turnovers and consumes the AceH. A reaction with either the dark S_1 state or the light-induced S_2 state could be responsible for the loss of the S_2 EPR signal. In the case of NH_2OH both kinetic evidence and Mn electronic absorption evidence show that, although binding occurs in the S_1 state, the S_2 state is the reacting partner (Guiles et al., 1990; Andreasson & Hansson, 1987; Sivaraja & Dismukes, 1988a). At higher concentrations both NH_2OH and AceH react in the dark with Mn, apparently by reduction of Mn(III) to Mn(II). No EPR evidence for binding of AceH to the acceptor side Fe(III) or Q_A^-Fe could be observed, in contrast to NH_2OH which causes a structural change at the acceptor side (Sivaraja & Dismukes, 1988b).

Scheme I is helpful in summarizing our results on the pathways for electron donation in PSII which are influenced by AceH. We see that, following removal of Mn and the extrinsic proteins of the WOC, AceH can donate electrons efficiently to Z^+. This result is compatible with the observed accessibility of Z^+ from the aqueous phase only after release of manganese (Ghanotakis & Babcock, 1983). The location and identity of the Z and D donors have been linked to the ^{161}Tyr residue on the D_1 subunit and the ^{160}Tyr on the D_2 subunit of the PSII reaction center, respectively (Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989). Although the Z^+ and D^+ radicals both appear to be derived from chemically identical tyrosine side chains, only Z^+ is reduced by AceH. This indicates a location for Z^+ that is much closer to the manganese cluster, while the dark-stable D^+ must exist in a more sequestered environment that is not accessible to AceH from the aqueous phase.

Following the release of manganese, photooxidation of the unidentified donor C occurs (Malkin & Bearden, 1973; Nugent et al., 1981; de Paula et al., 1985). The oxidized donor C^+ is accessible to AceH reduction from the aqueous phase which is exposed upon release of manganese. This reduction of C^+ is slow compared to its steady-state photooxidation rate, suggesting that it is kinetically less accessible than Tyr–Z^+. This suggests the existence of a common protein domain serving as the binding pocket for Mn, Z, and C which in its native form is accessible only to small neutral molecules. Upon release of Mn this domain is locally denatured and accessibility to the aqueous phase is increased. High-potential Cyt b-559 is also found to be reduced by AceH, but without the need for Mn removal. This indicates that the heme center must also be accessible to the aqueous phase, but not necessarily from the same protein domain that controls access to Mn, Z, and C.

A new possibility for the identity of the donor C is suggested from the data of Figures 6 and 7. Low-temperature photooxidation of PSII membranes oxidizes this donor, while room temperature photooxidation gives the normal Z^+ EPR line shape. The Z and C donors may be the same reaction center species, but having interconvertable EPR line shapes when they are formed by photooxidation at different temperatures. EPR signal II_s (D^+) has been shown previously to convert to a signal with symmetric line shape, ΔH_{pp} = 9–10 G, in the presence of strong chemical oxidants like K_2IrCl_6 (Boussac & Etienne, 1984) and $Ce(NH_4)_2(NO_3)_6$ (Tso et al., 1987).

Unlike the small amines such as NH_3 (Sandusky & Yocum, 1983, 1984, 1986) and NH_2OH (Beck & Brudvig, 1988), AceH does not compete with Cl^- for binding to the WOC (Figure 3). This unexpected result could mean that molecules as similar as $(CH_3)_2NOH$ (*N*,*N*-dimethylhydroxylamine) and $(CH_3)_2CNNH_2$ (AceH) really do have different binding sites to the WOC. Although this seems unlikely, it is supported by the observation that AceH is more effective than NH_2OH in inhibiting turnover of the reaction center (reduction of Q_A).

Another explanation may be responsible for the difference in the Cl^- competition of these inhibitors. The reported competition between Cl^- and $(CH_3)_2NOH$ for binding to PSII membranes was monitored by examining the initial rate of loss of the S_2 multiline EPR signal produced upon addition of *N*,*N*-dimethylhydroxylamine in the presence of increasing amounts of NaCl (Beck & Brudvig, 1988). This experiment is thus measuring the initial rate of binding of the inhibitor to the dark S_1 state as the ionic strength increases. Since the binding of NH_2OH and presumably its derivatives is irreversible in the dark, this experiment not only is monitoring the inhibitor binding site, but also is sensitive to any kinetic intermediate states which are precursors to inhibitor binding. It is therefore not possible to exclude the possibility that the apparent competition with chloride is due to general electrostatic screening of the membrane or protein which affects the kinetics of binding to the S_1 state. In this view, binding to a common site by both types of inhibitors could occur and this site would not directly involve the coordination of Cl^-.

Work is currently in progress to determine the fate of the anticipated diazo intermediate formed upon AceH oxidation with the proteins in PSII using radiolabeled acetone hydrazone.

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Registry No. AceH, 5281-20-9; L-Tyr, 60-18-4; Mn, 7439-96-5; O_2, 7782-44-7; $HBPz_3Mn(III)O(CH_3CH_2CO_2)_2Mn(III)Pz_3BH$, 106418-36-4; $Mn(II)(HBPz_3)_2$, 18131-15-2; acetone, 67-64-1; hydrazine, 302-01-2; cytochrome *b*-559, 9044-61-5.

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A New Mechanism-Based Inhibitor of Photosynthetic Water Oxidation: Acetone Hydrazone. 2. Kinetic Probes†

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ABSTRACT: The mechanism of photosynthetic water oxidation in spinach was investigated with a newly developed inhibitor of the water-oxidizing complex, acetone hydrazone (AceH), $(CH_3)_2CNNH_2$ [Tso, J., Petrouleas, V., & Dismukes, G. C. (1990) *Biochemistry* (preceding paper in this issue)], by using fluorescence induction and single-turnover flashes to monitor O_2 yield and thermoluminescence intensity. AceH binds slowly (1–3 min) in the dark to the S_1 (resting) oxidation state of the water-oxidizing complex in thylakoids and PSII membranes. Once bound, it causes a two-flash delay in the pattern of O_2 release seen in a train of flashes. This is initiated by reduction of manganese in the S_2 oxidation state of the complex in a fast reaction (<0.5 s). In thylakoid membranes which have been partially inhibited at low AceH concentrations (<2 mM) the inhibition can be reversed by a single flash and a subsequent dark period. This behavior can be explained by two sequential one-electron oxidation steps:

$$S_1{\cdot}AceH \xrightarrow{h\nu} S_2{\cdot}AceH \rightleftarrows S_1{\cdot}AceH^+ \xrightarrow{h\nu} S_2{\cdot}AceH^+ \rightarrow S_1 + AceH^{2+}$$

Dissociation of the unobserved radical intermediate, $AceH^+$, from S_1 is proposed to account for the recovery from inhibition after one flash. In contrast, recovery from inhibition after a single flash is not observed in detergent-isolated PSII membranes or in intact thylakoid membranes at higher AceH concentrations (>2 mM), where the two-flash delay in O_2 release is seen. This suggests either a concerted two-electron process, $S_2 \rightarrow S_0$, or tight binding of $AceH^+$ to S_1. Fluorescence induction shows that AceH inhibition does not affect the electron-transfer reactions within the photoreaction center protein. Thermoluminescence shows no evidence for abnormal activation barriers to recombination from both $(S_2 + S_3)Q_A^-$ and $(S_2 + S_3)Q_B^-$, indicative of a lack of observable structural alteration of these states by AceH. The binding of AceH lowers the binding affinity for DCMU, a herbicide that binds to the Q_B acceptor site. A small yield of O_2 (<5%) is observed on the first flash in AceH-inhibited PSII membranes, in contrast to untreated membranes where no O_2 forms. This suggests that O_2 may bind to S_{-1} centers which form in <5% of the S_1 centers that undergo two-electron reduction in the dark. These centers could then release O_2 after forming the S_0 state with a single flash.

Molecular inhibitors of photosynthetic water oxidation offer insight into the mechanism of water oxidation leading to O_2 evolution. Three classes of inhibitors are known which are directed at the water-oxidizing complex found in association with the photosystem II (PSII) reaction center protein complex in all green plants and algae (Babcock, 1987; Dismukes, 1986). These classes differ according to the mechanism of inhibition. They react selectively with specific oxidation states of the water-oxidizing complex, so-called S states, of which there are five: S_0, S_1, ..., S_4. They also bind to different sites within the water-oxidizing complex and are capable of direct reduction of either the tetramanganese complex involved in water oxidation, as in the case of hydroxylamine (Cheniae & Martin, 1971; Beck & Brudvig, 1987; Sivaraja & Dismukes, 1988a,b), or, in the case of ADRY reagents, reduction of the tyrosine donor Z^+ located between manganese and the reaction center (Ghanotakis et al., 1982).

In the preceding article in this issue we introduced a new inhibitor of the water-oxidizing complex, acetone hydrazone, $(CH_3)_2CNNH_2$ (Tso et al., 1990). These studies revealed that

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