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

I. Vass,‡ J. Tso,§ and G. C. Dismukes*,§

*Biological Research Center, Institute of Plant Physiology, Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary, and Department of Chemistry, Princeton University, Princeton, New Jersey 08544*

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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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‡ Hungarian Academy of Sciences.
§ Princeton University.

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AceH[1] is an inhibitor of water oxidation and, in some ways, functions analogously to reducing agents such as $NH_2OH$ and $N_2H_4$. However, unlike $NH_2OH$, AceH does not compete with chloride for binding to the site which inhibits manganese oxidation, suggesting a different binding site for $NH_2OH$ vs AceH. Another unexpected difference is that inhibition by AceH does not cause a shift in the EPR signal for the primary electron acceptor, $Q_A^-Fe$, in contrast to $NH_2OH$. These studies left unresolved the identity of the S state which is reactive in the initial reductive loss of the $S_2$ state. Either a dark reaction of the $S_1$ state or a light-induced reaction of the $S_2$ state could account for these results. Also, the very different interaction with the primary electron acceptor was not understandable considering the similar mode of action on the donor side.

In order to resolve these questions, we have used single-turnover flashes to examine the reaction of the individual S states with AceH. Our previous results on inhibition of steady-state $O_2$ evolution rates and EPR of the $S_2$ state have been extended to include additional probes of S-state cycling such as thermoluminescence, fluorescence induction, and $O_2$ yield measurements.

## Materials and Methods

*Sample Preparation.* Spinach thylakoid membranes and Triton X-100 extracted PSII-enriched membranes (so-called BBY type) were prepared according to established procedures (Berthold et al., 1981; Ford & Evans, 1983). These were stored at −70 °C in the sample buffer comprised of 0.4 M sucrose, 40 mM MES, 20 mM NaCl, pH 6.5, and 30% glycerol.

Treatment with AceH and standard assays were performed as previously described (Tso et al., 1990). Briefly, this involved incubation with AceH in the dark at 0.5 mg of Chl/mL in the buffer above without glycerol for 3 min, followed by centrifugation and resuspension in the same buffer. It was important to maintain the samples in the dark throughout this treatment in order to examine the reaction of the dark ($S_1$) state.

Flash $O_2$ yield measurements were performed by using a Joliot type platinum electrode, digitized with a multichannel analyzer (ICA70, KFKI) and analyzed with fitting routines on a computer (IBM-AT) (Vass et al., submitted for publication). Xenon flashes of 3-μs duration and 0.5-J intensity were used for excitation (Radio Stroboslave). No added electron acceptors were included, and initial sample concentrations were at 1 mg/mL Chl. For the measurement of $S_2$ and $S_3$ decay, samples were preilluminated with one or two flashes (4 Hz), respectively, followed by various intervals of dark relaxation and by a train of measuring flashes (4 Hz). The relative concentrations of $S_2$ and $S_3$ were calculated from the normalized oxygen yields on the first and second flash, respectively, as described earlier (Vermaas et al., 1984).

Thermoluminescence (TL) measurements were performed at 0.1 mg/mL Chl in the sample buffer with 30% glycerol to avoid distortion of the curves arising from thawing of the sample at 0 °C. Samples were excited with the xenon flash at 5 °C with 1-Hz frequency or with continuous illumination from a tungsten source (10 W/m²), followed by rapid freezing to 77 K. TL curves were measured between −40 and +70 °C with a heating rate of 20 °C/min as previously described (Vass et al., 1981).

[1] Abbreviations: Chl, chlorophyll, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethylbenzoquinone; AceH, acetone hydrazone; DPC, diphenylcarbazide.

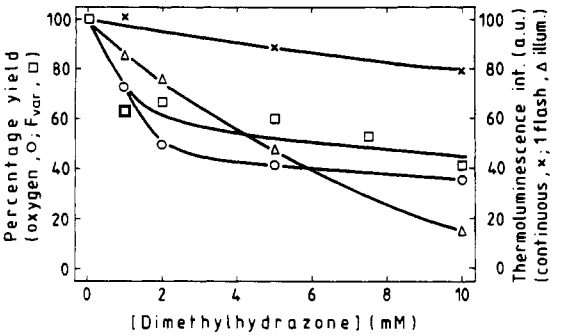



FIGURE 1: Concentration dependence for inhibition of PSII by AceH. Electron transport activity was probed by measuring $O_2$ evolution rate in continuous light (○), variable fluorescence (□), and thermoluminescence after a single flash (△) or after continuous illumination (×).

For fluorescence induction measurements samples were diluted to 10 μg of Chl/mL and illuminated with a tungsten source (10 W/m²) through a Corning CS 4-36 glass filter. The emitted light was filtered (Corning 2-64) and detected at right angles with a photomultiplier (EMI 9558B).

## Results and Discussion

*Oxygen Evolution Rate.* Figure 1 compares the concentration dependence for inhibition by AceH as probed by different techniques. The initial $O_2$ evolution rate (○), observed in the presence of the electron acceptor DMBQ, is compared to the intensity of variable fluorescence (□) and to thermoluminescence following a single flash (△) or under continuous illumination (×). The biphasic loss of $O_2$ evolution rate with 50% inactivation at 2 mM AceH occurs with both DMBQ and DCBQ as electron acceptors and also corresponds closely to the inhibition of variable fluorescence intensity. The former rate measures electron transport activity with water as the terminal electron donor, while the latter is a measure of the extent of photoinduced reduction of the primary electron acceptor, $Q_A$. Thus, both the terminal donor and acceptor of electrons report the same extent of loss of PSII electron transport activity. The biphasic concentration dependence could reflect either sample inhomogeneity (differential susceptibility to inhibition) or a reduced inhibition in the presence of multiple bound AceH molecules. The latter interpretation is favored by the observation that this same behavior is observed in all samples we have examined.

*Thermoluminescence.* Thermoluminescence (TL) intensity after a single flash is a measure of the extent of recombination from the single-state $S_2Q_B^-$ (Rutherford et al., 1982; Demeter & Vass, 1984). This exhibits essentially a monophasic loss of intensity, having both a higher threshold (50% loss at 4.5 mM AceH) and a greater total loss (83% at 10 mM AceH). The single-flash TL monitors the $S_1 \rightarrow S_2$ advancement, while $O_2$ rate and variable fluorescence monitor the ability to do continuous electron transfer. Therefore, the latter monitors changes affecting turnover anywhere from the S states through the reaction center up to reduction of the terminal electron acceptor. Observation of these changes induced by AceH requires incubation with the samples on the order of several minutes. A 3-min incubation time was used to ensure that the amplitude of the change had reached equilibrium.

The origin of the difference between steady-state $O_2$ evolution and single-flash TL is suggested by the much lower inhibition of TL intensity observed during continuous illumination, where multiple turnovers have occurred and TL arises from a mixed population of $S_2Q_B^-$ and $S_3Q_B^-$ centers (Rutherford et al., 1982; Demeter & Vass, 1984) (Figure 1,

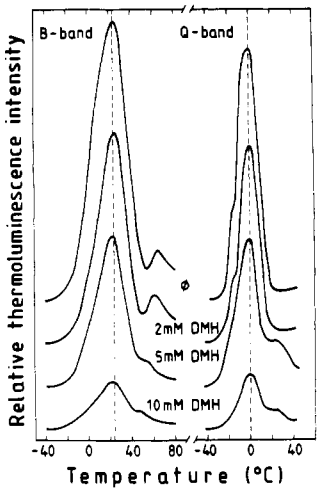



FIGURE 2: Inhibition of thermoluminescence intensity at various AceH concentrations. PSII membranes were treated with 0–10 mM AceH. Thermoluminescence was measured in the absence (B band) or the presence of 5 μM DCMU (Q band) after illumination by a single flash at +5 and −10 °C, respectively, followed by freezing.

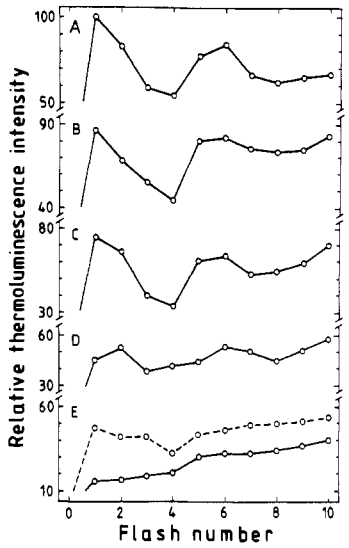



FIGURE 3: Flash-induced oscillation of thermoluminescence intensity in AceH-treated PSII membranes. Thermoluminescence peak intensity was measured after a series of single-turnover flashes fired at +5 °C with 1-Hz rate. (A) No addition; (B, C, and D) treatment with 1, 2, and 5 mM AceH, respectively; (E) 7.5 mM AceH treatment (solid line) and the same after 10 preflashes followed by 3-min dark incubation at room temperature (dashed line).

×). This recovery of TL intensity following multiple turnovers is attributable to the consumption of AceH that is achieved by cycling through the S states. This was also evident in the progressive recovery of TL intensity observed in inhibited samples (10 mM AceH) that were preflashed with 1, 2, ..., and 10 saturating flashes and dark adapted for 3 min prior to being given a single flash before freezing and measuring (data not shown). This same photoreversibility was also evident in samples that were not centrifuged to remove excess AceH prior to measurements.

By examining the concentration dependence of inhibition of TL, it is possible to determine if the relative free energies of the recombining partners have been affected by the inhibitor. TL originating from recombination within the state $S_2Q_B^-$ yields the so-called B band peaking at about 25 °C (Rutherford et al., 1982; Demeter & Vass, 1984). As shown in Figure 2, the B band disappears monotonically with increasing AceH concentration with 50% lost by 3–4 mM AceH. It does not change in peak position or shape, indicating that AceH has no effect on charge recombination in those centers that are still active after AceH treatment. In other words, the relative redox potentials of $S_2/S_1$ and $Q_B^-/Q_B$ are unaffected (Vass et al., 1981).

If samples are illuminated in the presence of DCMU, the photoelectron stops at $Q_A$ and recombination within $S_2Q_A^-$ leads to the so-called Q (or D band) peaking at around 0 °C (Rutherford et al., 1982; Demeter & Vass, 1984). Figure 2 shows that AceH decreases the Q band intensity monotonically with 50% loss at 3–4 mM. Again, there is no evidence for a shift in the peak position, indicating by analogy with the B band that the redox potential of the $Q_A^-/Q_A$ couple relative to $S_2/S_1$ is also not affected by AceH treatment. It seems, however, that AceH decreases the affinity of DCMU binding as indicated by the induction of a small B band at 25 °C above 5 mM AceH (Figure 2).

For both the B and Q bands both continuous illumination and a series of preflashes followed by 3-min dark period were effective in reversing the AceH inhibition, with continuous illumination being more efficient.

TL intensity after each flash in a series is proportional to the total population in the $S_2Q_B^-$ and $S_3Q_B^-$ states. If the concentration of $Q_B^-$ was constant in a series of flashes, then TL oscillation would reflect only the $S_2 + S_3$ population, showing maxima after 2, 6, 10, ..., etc., flashes. This is not expected in dark-adapted samples where $[Q_B] \gg [Q_B^-]$ and the first maximum occurs after the first flash in the state $S_2Q_B^-$ (Rutherford et al., 1982; Demeter & Vass, 1984). As shown in Figure 3, this pattern with periodicity four becomes progressively decreased in amplitude as AceH is added, but with little change in the depth of the oscillation until above about 2 mM (curve C), where the damping increases greatly. This concentration dependence is reminiscent of the biphasic inhibition of steady-state $O_2$ evolution (Figure 1). From the reasoning given above, the loss of intensity without change in relative oscillation depth or phase should reflect inactive centers which can no longer photoreduce $Q_B$, or which can no longer form $S_2$ or $S_3$. Above 2 mM AceH the oscillations are heavily damped, reflecting increased misses for S-state turnover in that population of centers which can reach $S_2$ and $S_3$. A partial recovery of oscillation amplitude and depth is produced by preillumination with 10 preflashes, as shown in Figure 3E. This light-induced recovery of S-state function again reflects the photoreversible character of AceH inhibition and localizes the site of inhibition within the water-oxidizing complex. TL measurements thus show two modes of inhibition: one involving complete obstruction of formation of $S_2 + S_3$, and another at higher AceH concentration >2 mM, involving a reduced quantum yield for formation of $S_2$ and $S_3$.

*Fluorescence Induction.* The loss of variable fluorescence intensity from chlorophyll produced by AceH inhibition of PSII (Figure 1) is associated with a slowing of the kinetics for reduction of $Q_A^-$, as shown in Figure 4A. This phenomenon is typically associated with reduction in the electron donation rate on the donor side of PSII. The same phenomenon is seen in the AceH-induced decrease of fluorescence intensity in the presence of 2 μM DCMU (data not shown).

Following AceH inhibition electron transport through the reaction center can be restored partly by the addition of the electron donors such as DPC (curve e), $NH_2OH$ (curve f), and $MnCl_2$ and even excess AceH (curve g), as seen in Figure 4B by the increase in variable fluorescence. $MnCl_2$ has a similar restoring effect on variable fluorescence (not shown).

*Flash $O_2$ Evolution in PSII.* The effect of AceH on the yield of $O_2$ following each flash in a train given at 1-Hz rate

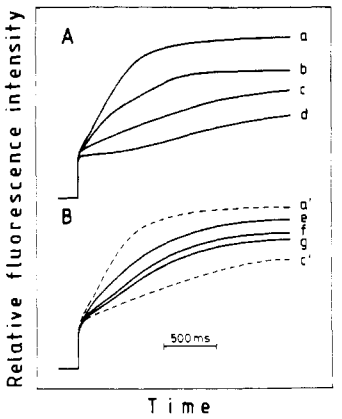



FIGURE 4: Inhibition of variable fluorescence by AceH and its restoration by exogenous electron donors. (A) Fluorescence induction kinetics were measured on PSII membranes without treatment (a) and after treatment with 1 (b), 5 (c), and 10 mM AceH (d). (B) PSII membranes treated with 5 mM AceH (c') were supplemented with 2 mM DPC (e), 2 mM $NH_2OH$ (f), or 20 mM AceH (g). The induction curve of untreated samples is also indicated (a').

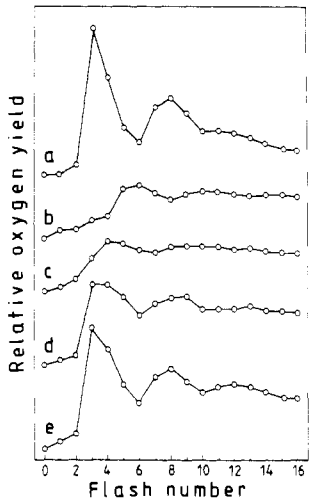



FIGURE 5: Flash-induced oscillation of $O_2$ evolution in AceH-treated PSII membranes: (a) untreated control, no preflash; treated with 2.5 mM AceH and given the following: (b) no preflash, (c) one preflash, (d) two preflashes, or (e) five preflashes. The preflashed samples were dark adapted after preflashing for 3 min before the measuring flash series.

was measured in order to separately measure the $O_2$ evolution step. Figure 5a,b shows that the maximum in $O_2$ yield in the presence of 2.5 mM AceH is delayed from the third to the fifth or sixth flash and reduced overall. The steady state of flash $O_2$ yield is declining with increasing flash numbers as a result of the limited plastoquinone pool in BBY–PSII preparations (Siebert & Lavorel, 1983). The yield and oscillation pattern for $O_2$ release are diminished by AceH to an even greater extent than for TL. This inhibition was largely reversed by preflashes given after AceH treatment followed with a 3-min dark readaptation period prior to the measuring flash train, as shown in Figure 5c–e. Restoration of the characteristic 3, 7, 11, ... oscillation pattern seen in the uninhibited sample is first seen following two preflashes and is fully developed after five preflashes. Both the two-flash delay in $O_2$ yield and the restoration of the original oscillation pattern after two preflashes indicate that AceH reduces the water-oxidizing complex by two electrons in a majority of centers and that further reduction between flashes is minimal at 1-Hz flash rate. At greater AceH concentrations (10 mM) this photoreversibility is almost completely lost (data not shown).

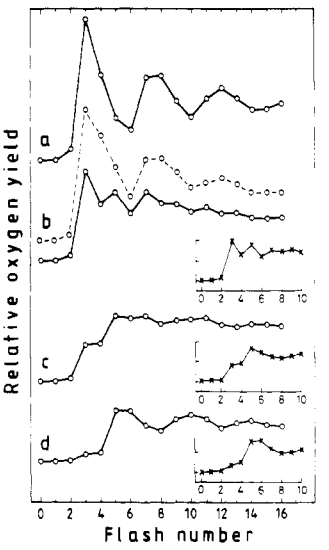



FIGURE 6: Flash-induced oscillation of $O_2$ evolution in AceH-treated thylakoid membranes: (a) untreated membranes; (b) +1 mM AceH (solid line) and the same after a single preflash followed by 3-min dark adaptation (dashed line); (c) +2 mM AceH; and (d) +5 mM AceH. The insets for curves b, c, and d show the results of computer simulations using a Kok model and assuming a two-flash retardation of $O_2$ evolution in 40, 60, and 80% of the centers, respectively. The value of double hits used in the fits was 3% for all the calculations. The miss parameter for the $S_1 \rightarrow S_2$ transition was increased from 12% (b) to 42% (c) and 62% (d), while the miss parameter for all the other S-state transitions was fixed at 12%.

It is also noteworthy that after AceH treatment a small oxygen signal (5% maximum) is seen even at the first flash which is not reversed by preflashes (Figure 5b–e). No $O_2$ release is observed in the control following one flash. This effect may indicate that AceH can reduce $S_1$ in the dark to an $S_{-1}$ state which may be capable of binding $O_2$:

$$S_1 + (CH_3)_2CNNH_2 \rightarrow$$

$$S_{-1} + (CH_3)_2CN_2 + 2H^+ \overset{O_2}{\Leftrightarrow} O_2{\cdot}S_{-1} \xrightarrow{h\nu} S_0 + O_2 \quad (1)$$

An alternative explanation in which $H_2O_2$ forms after one flash and then undergoes disporpotionation, as appears to occur in salt-washed PSII membranes, could also account for $O_2$ formation after one flash (Schroeder & Akerlund, 1986; Seibert & Lavorel, 1983).

*Flash $O_2$ Evolution in Thylakoid Membranes.* A different behavior is seen when thylakoid membranes are used instead of Triton-extracted PSII membranes. Progressive addition of AceH leads to a change in the oscillation pattern from the initial 3, 7, 11, ... pattern to a binary pattern having reduced amplitudes at 3, 5, 7, 11, 13, ... at 1 mM AceH, as shown in Figure 6a,b. At higher AceH concentration (Figure 6c,d) this converts to the same behavior as seen in PSII membranes (Figure 5b), but with an increasing proportion of inactive centers. Contrary to PSII membranes, AceH-treated thylakoids did not show oxygen evolution after the first flash. The binary oscillation seen at low AceH concentration in Figure 6b can be understood to arise from a mixed population of centers, some exhibiting the normal 3, 7, 11, ... pattern and another population exhibiting a pattern delayed by two flashes: 5, 9, 13, ..., characteristic of the inhibited centers, as shown by computer simulations given in the two insets. The dashed curve in Figure 6b shows that a single preflash followed by a 3-min dark period is sufficient to completely abolish the binary oscillation pattern by restoring a normal period four oscillation pattern, 3, 7, 11, ..., in almost 100% of centers. This has two implications. First, a low amount of bound AceH

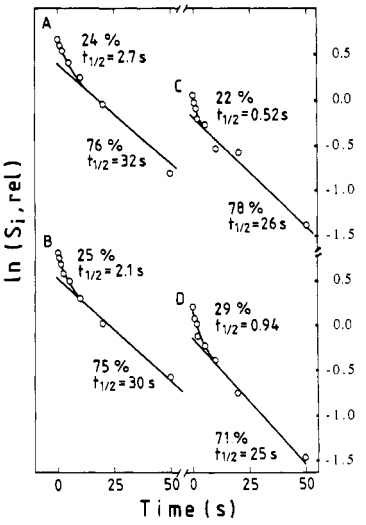



FIGURE 7: Effect of AceH on the decay kinetics of the $S_2$ (A, C) and $S_3$ (B, D) states in thylakoid membranes as monitored by $O_2$ yield. The kinetics of decay of $S_2$ and $S_3$ were measured after one and two preflashes, respectively, by varying the dark interval between the preflashes and a series of ten measuring flashes from which the amounts of $S_2$ and $S_3$ were calculated. (A and B) Untreated thylakoid membranes; (C and D) +1 mM AceH.

creates a stable state in the dark as deduced from the stability of the binary oscillations upon dark storage. Second, the oxidation state reached after a single preflash (normally $S_2$) is unstable in the presence of AceH, such that within 3-min dark adaptation it restores the normal $S_1$ state observed in dark-adapted control samples—the inhibition is reversed. The $S_2$ state behaves as though it oxidizes AceH by one electron followed by dissociation of oxidized $AceH^+$ in the dark. We were unable to detect formation of this putative radical intermediate using EPR and continuous illumination. Triton-extracted PSII membranes did not exhibit recovery from inhibition by a single preflash.

In thylakoid membranes treated at higher AceH concentrations (>2 mM) two preflashes are required to restore the normal oscillation pattern to those centers which can be reactivated (data not shown). This behavior is similar to that seen for PSII membranes (Figure 5). Three preflashes do not significantly improve the recovery of the original oscillation pattern over that observed with two preflashes. These results suggest that one or more additional equivalents of AceH can bind at these higher concentrations to suppress the recovery from inhibition seen with a single preflash. It is not clear from these studies if this additional AceH is directly involved in further reduction of the water-oxidizing complex, or if it simply enhances the two-electron reduction of the initially bound AceH by preventing the dissociation of the one-electron-oxidized $AceH^+$ from the bound state, $S_2$·AceH, produced after the first flash.

*AceH Binds Slowly in the Dark in the $S_1$ State.* The time scale of AceH binding in the dark to the $S_1$ state was estimated to occur over 1–3 min and was dependent on the concentration, as determined by the time scale for induction of the delay in $O_2$ yield.

*Kinetics and Yield of $S_2$ and $S_3$ Decay.* In order to obtain additional information about the interaction of AceH with the $S_2$ and $S_3$ states, their kinetics of decay were measured from the yield of $O_2$ with varying flash intervals. As Figure 7A,B shows, the decay of the $S_2$ and $S_3$ states in dark-adapted thylakoids is biphasic. A fast phase ($t_{1/2}$ = 2.5 s) in about 25% of centers reflects electron donation from the reduced tyrosine donor D to the Mn cluster, while the slow phase ($t_{1/2}$ = 30 s) seen in about 75% of centers reflects deactivation of $S_2$ and $S_3$ by the reduced electron acceptors (Vermaas et al., 1984; Siebert & Lavorel, 1983; Vass et al., 1990). In thylakoids which were treated with 1 mM AceH and exhibiting the binary oscillations as in Figure 6b, the decay of the fast phase of $S_2$ and $S_3$ was accelerated compared to the control (Figure 7A,B). This result indicates that the $S_2$ state formed in centers where AceH binding occurs decays faster than our oxygen measurements could resolve, i.e., oxidation of AceH by $S_2$ and $S_3$ is faster than about 0.5 s. In parallel, the $O_2$ yield under continuous illumination was only 55% of the control, which corresponds well to the amount of normally functioning centers estimated from computer simulations of the flash patterns (inset in Figure 6b).

## Conclusions

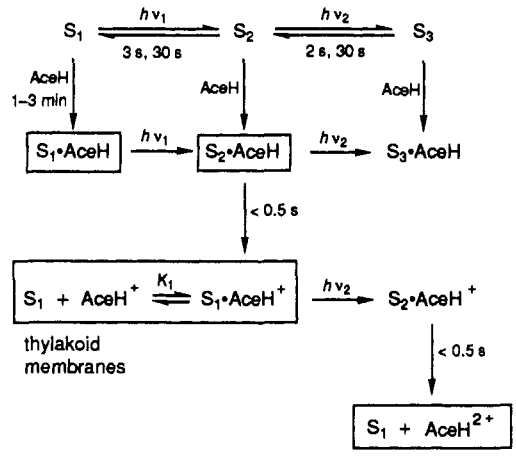
These results support and extend our earlier work showing that AceH inhibits water oxidation by a two-step process involving initial reversible reduction of manganese, followed by, at higher AceH concentrations, irreversible inhibition accompanied by release of manganese (Tso et al., 1990). The reversible loss of TL intensity and of variable fluorescence, at low AceH concentrations, both indicate loss of effective charge separation in PSII from the donor side. The recovery of TL intensity and $O_2$ evolution by illumination following AceH treatment parallels the previously observed recovery of the $S_2$ EPR multiline signal and the retention of manganese. The recovery of variable fluorescence, after pretreatment with AceH, by the addition of DPC, $MnCl_2$, $NH_2OH$, or AceH supports the view that bound manganese, the endogenous electron donor, can be replaced by these exogenous electron donors which function by reduction of the tyrosine radical $Z^+$ (Yerkes & Babcock, 1980; Tso et al., 1990). Thus, the PSII reaction center remains functional after AceH inhibition; inhibition must occur outside the reaction center.

*$S_2$ Reacts with AceH by Photoreversible Reduction.* The identity of the S state which is the target for the reduction by AceH is an important issue which was not resolved in our earlier work. The loss of the $S_2$ state multiline EPR signal which we reported in the preceding paper and the present results showing the loss of single-flash-induced TL directly show that the $S_1 \rightarrow S_2$ reaction is specifically inhibited. This inhibition cannot be an all or nothing inactivation of centers, since this would create a decrease in TL intensity and $O_2$ yield without a change in the pattern of oscillations produced by multiple flashes. Attempts to simulate the pattern of damped $O_2$ yields (Figure 6) shows that including an increased "miss" parameter in the Kok formulation for S-state turnovers (Kok et al., 1970) can account for the observed damping.

We interpret the two-flash shift in the maximum yield of $O_2$ observed in both PSII membranes (Figure 5) and thylakoids (Figure 6) as strong evidence for the two-electron reduction of the water-oxidizing complex by AceH. This is strictly analogous to the two-electron reversible inhibition observed with hydroxylamine (Bouges, 1971; Sivaraja & Dismukes, 1988a,b) for which the S state involved in manganese reduction is the $S_2$ state, as seen by the X-ray absorption edge results for manganese (Guiles et al., 1987). Thylakoids differ somewhat from PSII membranes in that below 2 mM AceH a single preflash followed by a 3-min dark period is sufficient to fully reverse the inhibition, while PSII membranes require two flashes. At higher AceH concentrations more preflashes are needed, and even this fails to restore all centers to their preinhibition activity.

These results can be summarized with the model presented in Scheme I. Slow binding of AceH to the $S_1$ state occurs

Scheme I




in the dark over 1–3 min. Following this slow binding phase the two-flash delay in $O_2$ release and the loss of $S_2$ multiline EPR intensity can be attributed to the reduction of the $S_2$ state by bound AceH to give initially the one-electron radical intermediate $AceH^+$ and the $S_1$ state. We have no direct evidence for the existence of this radical species which is likely to be unstable if released into solution and hence difficult to detect. One-electron oxidation by PSII of *N*-alkyl-substituted hydroxylamines to form long-lived nitroxide radicals has been observed (Beck & Brudvig, 1987). If a second flash is given within a few seconds of the first flash, a two-flash retardation in the maximum $O_2$ yield is seen. This occurs whenever photooxidation to produce $S_2$•$AceH^+$ on the second flash occurs more rapidly than dissociation of $AceH^+$ from $S_1$. For longer dark periods after the first flash, greater than 1–3 min in the case of thylakoids, release of $AceH^+$ is proposed to account for the observed recovery from inhibition. This produces $S_1$, and the system is restored to the uninhibited state by a single preflash (Figure 6). This behavior is strongly reminiscent of the mode of reduction of chloroplasts by hydroxylamine in which both one- and two-electron reductions of the $S_2$ and $S_3$ oxidation states could be observed through its effects on the $O_2$ yield (Hanssum & Renger, 1985). An alternative model in which the state $S_2$•AceH releases AceH and decays in less than 3 min to form a normal $S_1$ would also result in the recovery from inhibition observed in thylakoids following a single flash. However, such a mechanism is unable to account for the rapid (<0.5-s) loss of the $S_2$ and possibly $S_3$ states with AceH (Figure 7), nor can it account for the loss of the multiline EPR signal normally observed in the $S_2$ state (Tso et al., 1990).

No evidence for recovery from inhibition by a single preflash was found in thylakoids at higher AceH concentrations (>3 mM), or in Triton-extracted PSII membranes at any AceH concentration. Both samples exhibit the two-flash delay in $O_2$ yield and an increased miss parameter. This could be due to a tighter binding of AceH in detergent-extracted PSII membranes or, in the case of thylakoids, to the binding of additional AceH molecules. Evidence for multiple sites for hydroxylamine and hydrazine binding to the water-oxidizing complex are well-known (Hanssum & Renger, 1985; Forster & Junge, 1986; Sivaraja & Dismukes, 1988a). Inhibition in PSII membranes is less reversible, exhibiting a greater extent of inhibition and a smaller extent of recovery by preflashing as seen in both $O_2$ yield and TL intensity.

Because evidence for recovery from inhibition by a single flash was absent inn PSII membranes, we are unable to distinguish between inhibition mechanisms for $S_2$ involving a direct two-electron reduction to $S_0$, or two one-electron sequential steps involving the intermediates $S_1$•$AceH^+$ and $S_2$•$AceH^+$. The reaction of $S_3$ with AceH could not be followed independent of the fast reaction with $S_2$ by using the present methods.

Scheme II

$$S_1 + n\text{AceH} \longrightarrow S_{-1}(\text{AceH}^+)_n \xrightarrow{\text{AceH}} \text{free Mn(II)}$$

AceH appears to directly reduce the manganese site as evidenced by the loss of $S_2$ multiline EPR signal and, at higher concentrations, the release of manganese into solution. This differs from the mode of inhibition by the class of lipophillic anions called ADRY reagents, of which one member includes the aromatic hydrazone, carbonyl cyanide (*m*-chlorophenyl)hydrazone (Renger & Inoue, 1983). These reagents differ from AceH in several aspects. They partition into the membrane unlike AceH which remains in the aqueous phase. They bring about efficient inhibition of electron transport at substoichiometric concentrations, without releasing manganese. Mechanistically, they appear to short circuit manganese oxidation by direct reduction of $Z^+$ (Ghanotakis et al., 1982).

*Thermoluminescence Flash Yield.* The two-flash delay in the oxygen yield of AceH-treated thylakoids and PSII membranes would implicate a similar two-flash delay in TL oscillation pattern as well. TL oscillation, however, showed only a strong damping at high AceH concentrations (Figure 3). This apparent contradiction can be reconciled if one recognizes that TL arises only from centers which reach $S_2$ and $S_3$ and hence must survive reduction by AceH. Because the technique involves warming of the sample to temperatures where diffusion occurs freely, multiple reduction processes will occur over the 2-min warming period.

*$S_1$ Reacts with AceH by Irreversible Release of Manganese.* Scheme II summarizes the reactions of AceH with the dark $S_1$ state. As the concentration of AceH increases (10 mM), the $S_1$ state gets reduced in the dark, producing an $S_{-1}$ oxidation state. According to current interpretations of the $S_1$ and $S_2$ oxidation states, the $S_{-1}$ state should contain 2 Mn(II) and 2 M(III) ions (Dismukes, 1986, 1988). The $S_{-1}$ state is considered to be formed prior to release of manganese (Beck & Brudvig, 1987; Sivaraja & Dismukes, 1988a; Tso et al., 1990). Further reduction of $S_{-1}$ is considered to cause the release of Mn(II) into the solution.

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**Registry No.** $O_2$, 7782-44-7; Mn, 7439-96-5; $(CH_3)_2CNNH_2$, 5281-20-9.

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# $^{15}N$ NMR Spectroscopy of *Pseudomonas* Cytochrome *c*-551†

Russell Timkovich
*Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487-0336*
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ABSTRACT: $^{15}N$-$^{1}H$ correlation spectroscopy with detection at the $^{1}H$ frequency has been used at natural abundance to detect nitrogen nuclei bonded to protons in the ferrocytochrome *c*-551 from *Pseudomonas aeruginosa* (ATCC 19429). Side-chain aromatic nitrogens, main-chain amides, and side-chain amides have been assigned to specific residues by comparison to previous proton assignments. Assignment ambiguities arising from overlap in the proton dimension have been resolved by examining spectra as a function of temperature and pH. Nitrogen chemical shifts are reported at pH 4.6 and 9.4 and three temperatures, 32, 50, and 60 °C. Significant differences arise from the observed protein shifts and expected shifts in the random coil polypeptide.

Studies of $^{15}N$ nuclei in proteins have not been as common as NMR investigations of $^{1}H$ or even $^{13}C$, because of the severe sensitivity problem. Pioneering studies have been made [for example, see Smith et al. (1987) and their references to earlier literature], but they have required special circumstances, including obtaining material enriched over natural abundance. Techniques have now been developed for the detection of insensitive nuclei by polarization transfer from the insensitive nucleus to a sensitive one, usually $^{1}H$. These now make the $^{15}N$ nucleus more accessible. Recently, backbone amide $^{15}N$ chemical shifts have been reported for bovine pancreatic trypsin inhibitor, apamin (Glushka et al., 1989, 1990), interleukin (Marion et al., 1989a), the DNA binding protein Ner (Gronenborn et al., 1989), the inflammatory protein C5a (Zuiderweg & Fesik, 1989), and *Staphylococcus* nuclease (Torchia et al., 1989; Wang et al., 1990). Also, powerful spectroscopic methods have been developed to further elaborate overlapping amide protons in large macromolecules into a third dimension, the $^{15}N$ frequency region (Weber & Mueller, 1989; Marion et al., 1989a,b; Zuiderweig & Fesik, 1989), or by combinations of several two-dimensional spectra (Gronenborn et al., 1989; Glore et al., 1988; Westler et al., 1988; Stockman et al., 1989). These also provide chemical shift information for $^{15}N$ with bound protons. Currently, many of these techniques require enriched proteins. In principle, genes encoding for proteins from higher organisms can be cloned into microorganisms, but this remains a nontrivial task. For any protein produced in liquid cultures, there is high expense for culture media containing enriched $^{15}N$ compounds. Even for microorganisms that can be grown on chemically defined minimal media, there can remain problems of cell growth yields. For example, the strain of *Pseudomonas aeruginosa* discussed in this report grows on a complex medium (Parr et al., 1976) to a cell density of about 5 g wet wt/L, while on a minimal media utilizing ammonium chloride as the sole source of assimilated nitrogen (Bryan et al., 1983), the generation time doubles and cell yields drop to about 1 g/L. Therefore powerful motivation still exists for working at natural abundance.

While progress in $^{15}N$ NMR spectroscopy of proteins is accelerating, the data base of observed shifts is still small. The ferrocytochrome *c*-551 from *Pseudomonas aeruginosa* has been studied by inverse detection of its $^{15}N$ nuclei with attached protons, and extensive assignments have been made. Cytochromes *c*-551 from *Pseudomonas* function as electron transport proteins in an analogous manner to cytochrome *c* in mitochondria (Timkovich, 1979). The size (9300 kDa) of *P. aeruginosa* cyt$^{1}$ *c*-551 makes it amendable to investigation

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