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The inhibition of photosynthetic oxygen evolution by ammonia probed by EPR

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EPR was used to study the binding of NH_3 to the photosynthetic O_2-evolving center. NH_3-treated, Ca^{2+}-depleted Photosystem II (PS II) membranes exposed to continuous light at 250 K showed a 10 mT-wide asymmetric EPR signal, centered around $g = 2$. When dark-adapted material was illuminated with a sequence of laser flashes the same signal appeared after the second flash, indicating that the $g = 2$ signal arises from a modified S_3 state. The signal is different from the 15–16.5 mT-wide EPR signal at $g = 2$ ascribed to the S_3' state in Ca^{2+}-depleted material in the absence of NH_3, indicating that NH_3 perturbs the structure of the O_2-evolving complex in the S_3' state. Illumination of native NH_3-treated PS II membranes with continuous light results in the appearance of an EPR signal at $g = 2$ with a width similar to that in Ca^{2+}-depleted, NH_3-treated membranes. The conditions for the formation of the signal and its properties suggest that it also arises from a perturbed S_3 state with NH_3 in close association with the manganese.

Introduction

Repeated withdrawal of single electrons from the oxygen-evolving complex in Photosystem II (PS II) results in the advancement from one of the five (S_0–S_4) intermediate states of the complex to the next in a cyclic sequence with oxygen evolved from water on the S_4–S_0 transition. The different S states represent successively higher oxidation states of the complex where the accumulated charges are stored on or in the vicinity of the four manganese ions in PS II. In fact, evidence has accumulated indicating that oxidation of manganese occurs on the S_0–S_1 and S_1–S_2 transitions, whereas the oxidation state of the metal ions remain unchanged going from S_2 to S_3 (reviewed in Ref. 1). The latter transition has instead been suggested to lead to the oxidation of an organic entity, possibly one directly ligated to the manganese [2] (see also Refs. 1 and 3 for reviews). The S_2 state is reasonably well characterized spectroscopically mainly owing to its association with two EPR signals, one, the multiline signal, centered around $g = 2$, and another broad signal known from the g value of its absorption peak as the $g = 4.1$ signal. It is now generally recognized that each of the signals arises from 2 to 4 magnetically coupled manganese ions (reviewed in Ref. 4) [5]. Recently, a 13–16.5 mT-wide signal from a modified S_3 state (S_3') was detected after inhibitory treatments of PS II, involving either removal of functional Ca^{2+} [6–8] or replacement of bound Cl^- with F^- [9]. The signal was suggested to arise from a radical, formed on oxidation of a histidine residue [6–10], in magnetic interaction with the manganese cluster of the oxygen-evolving center. Histidine as a redox-active ligand in water oxidation was, in fact, considered already several years ago [11].

Ammonia inhibits oxygen evolution by binding to two different sites on the PS II donor side. One of these is also a binding site for chloride [12]. The two EPR signals from the S_2 state are perturbed by ammonia binding [13,14] and for at least one of these, the multiline signal, this results from the ligation of NH_3 directly to the manganese [15].

Binding of ammonia to both sites has been reported to occur mainly in the S_2 state where it is strong and rapid [13,16,17] but weak binding to the S_1 state has also been suggested from the effects on the EPR signals [13,14,17,18].

Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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When ammonia is bound to the oxygen-evolving complex the advancement of the S-state cycle is inhibited. An increase in luminescence was observed after the third flash with dark-adapted, ammonia-treated chloroplasts, indicating that the S_4–S_0 transition was blocked, causing the S_4 state to decay to S_3 with the emission of light [19]. Two ammonia molecules bound in the S_3 state were required for inhibition [16]. In a recent study of the effects of ammonia on S-state turnover, S-state cycling was observed despite the presence of ammonia [20]. This was explained as being due to sluggish binding of the second (inhibiting) ammonia molecule to the S_3 state.

Here we have used EPR to study the effect of ammonia binding on the formation of intermediates occurring in the photosynthetic water-splitting reaction and their spectroscopic properties.

Materials and Methods

PS II membranes were prepared from spinach as described earlier [21] and suspended in 20 mM Mes-NaOH (pH 6.3)/15 mM NaCl/5 mM $MgCl_2$/400 mM sucrose. The membranes were frozen as small beads in liquid nitrogen and stored at 77 K until used.

For ammonia treatment, PS II membranes were washed with 20 mM Hepes-NaOH (pH 7.5)/15 mM NaCl/5 mM $MgCl_2$/400 mM sucrose and transferred to EPR tubes. After 1 h in darkness at 0°C, 2 mM phenyl-*p*-benzoquinone was added to the samples which were further incubated in the dark with 100 mM NH_4Cl for 1 min and frozen for later illumination with continuous light. Samples to be illuminated with light flashes were incubated in darkness with NH_4Cl for 1 min immediately before being subjected to the illumination.

Depletion of Ca^{2+} bound to the oxygen-evolving complex was performed essentially as described in Ref. 22: the PS II membranes were incubated for 30 min in room light at 4°C at 0.5 mg chlorophyll/ml in 25mM Mes-NaOH (pH 6.5)/1.2 M NaCl/0.3 M sucrose/50 μM EGTA. After centrifugation, the pellet was washed twice with 25 mM Mes-NaOH (pH 6.5)/30 mM NaCl/50 μM EGTA followed by one wash and final resuspension in the same buffer but with 10 mM EGTA. For experiments at pH 7.5, the washing was done with a buffer containing 20 mM Hepes.

Illumination of the EPR samples (about 10 mg chlorophyll/ml) with continuous light (2000 W/m^2) was done in a nitrogen gas-flow system which allowed the temperature of the gas to be set at any temperature between 100 and 300 K. The temperature within a PS II sample during illumination was checked in separate experiments with a thermocouple in the sample.

Flash illumination of EPR samples was done as follows: after dark adaptation and addition of NH_4Cl the samples, at a chlorophyll concentration of 2.2 mg/ml, were illuminated at 250 (frozen samples), 273 or 293 K by flashes from a frequency-doubled, Q-switched Nd:YAG laser [23]. The illuminated samples were frozen immediately or after a delay in darkness in an ethanol-solid CO_2 slush and then cooled to 77 K in liquid nitrogen.

EPR measurements were performed with a Bruker ER 200D-SRC instrument equipped with an Oxford Instruments ESR-9 helium-flow cryostat.

Results

NH_3 modification of Ca^{2+}-depleted material

The 13–16.5 mT-wide signal at $g = 2$ observed after Ca^{2+} depletion has been suggested to arise from the S_3' state modified by the absence of the cofactor. Using this EPR signal as a probe, the binding properties of the S_3' state towards NH_3 were studied. In the absence of NH_3 the 14.4 mT-wide S_3' state EPR signal at pH 7.5 (Fig. 1B) is similar to that at pH 6.5. We have, however, noticed that the signal at both pH values appears to have a more complex shape than observed earlier (see for example Refs. 7 and 10) as indicated by the presence of a shoulder at fields slightly above the negative peak in the signal at about 0.345 T. Ca^{2+}-depleted PS II membranes treated with NH_4Cl at pH 7.5 and illuminated at 250–270 K with continuous light showed an asymmetric and more narrow EPR signal compared to the signal obtained in the absence of NH_3

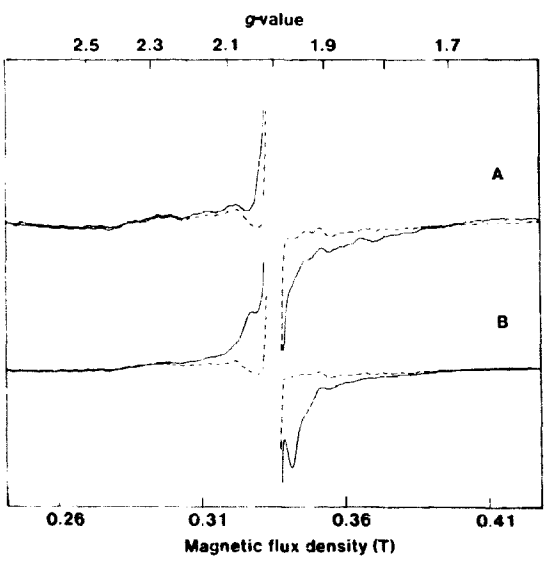

Fig. 1. Effect of NH_4Cl on the light-induced EPR spectrum of Ca^{2+}-depleted PS II membranes. (A) Treated with 100 mM NH_4Cl at pH 7.5. Dashed line, dark-adapted; solid line, 4 min illumination at 270 K. (B) Spectra obtained as in A but without NH_4Cl. Conditions for EPR spectra: microwave frequency, 9.36 GHz; microwave power, 20 mW; modulation amplitude 2 mT; temperature 11 K.

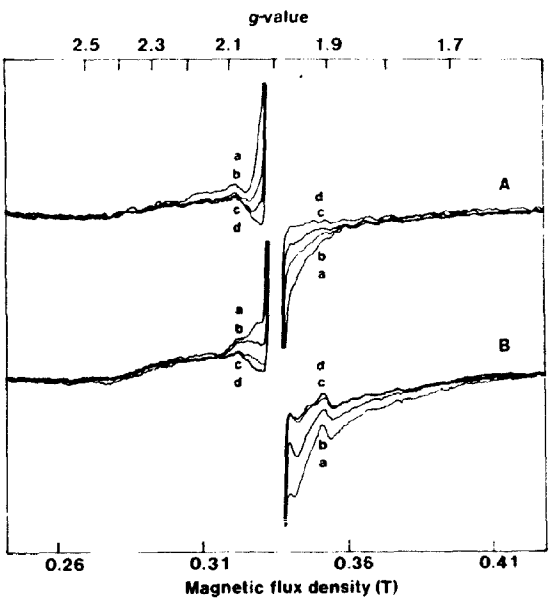

Fig. 2. Relaxation of the light-induced EPR signal in Ca^{2+}-depleted PS II membranes. (A) a, membranes treated with 100 mM NH_4Cl and illuminated at 250 K; b–d, after relaxation at 273 K for 1, 3 and 15 min, respectively. (B) Membranes illuminated as in A but without NH_4Cl; b–d, after dark-relaxation for 0.5, 1 and 3 min, respectively. Conditions for EPR spectra as in Fig. 1.

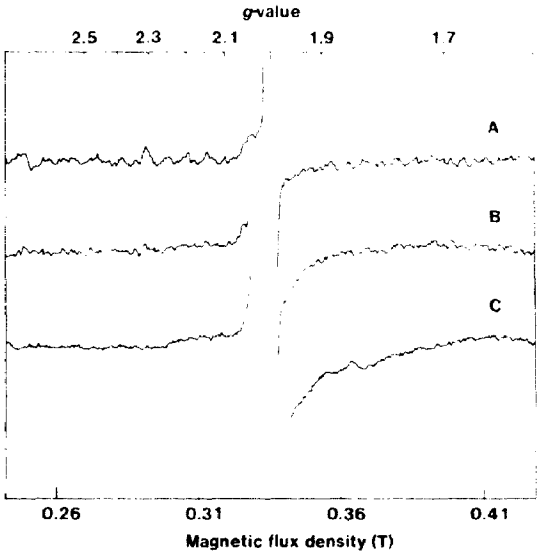

Fig. 3. Flash-induced EPR spectra from Ca^{2+}-depleted, NH_4Cl-treated PS II membranes. Spectra obtained after one (A) and two (B) light flashes given to dark-adapted PS II membranes at 293 K. For comparison the EPR spectrum obtained after continuous illumination at 270 K is shown (C). The spectra are presented as difference illuminated minus dark-adapted. Conditions for EPR as in Fig. 1.

(Fig. 1). The width of the new signal in NH_3-treated membranes was estimated to be 10 mT or a little less from the illuminated minus dark difference spectra although the absence of resolved peaks in spectrum and the overlap of the strong, dark-stable, 2 mT-wide signal from Tyr-D^+ (Signal II_s), also centered at this g value, makes this value somewhat uncertain. The difference in shape between the signals obtained in the absence and presence of NH_3 is also evident in Fig. 2. The NH_3-modified signal decayed with a half-time of 1 min at 273 K and was replaced by the spectrum of the dark-adapted starting material (Fig. 2A). Also the S_3' state EPR signal, induced at this pH (7.5) in the absence of ammonia, was converted to the dark spectrum but with a somewhat shorter half-time, about 30 s, on warming at 273 K (Fig. 2B). This represents a significant difference to what was observed at pH 6.5 where the decay of the S_3' state in the absence of ammonia resulted in the conversion to the dark-stable S_2 state, characterized by a multiline EPR signal (not shown), in accordance with observations made earlier [6,7].

Power saturation studies of Ca^{2+}-depleted PS II membranes treated with ammonia revealed a $P_{1/2}$ of 2 mW at 11 K, close to the value obtained earlier for the modified S_3 state in the absence of ammonia [6,9].

Illumination at 200 K of dark-adapted Ca^{2+}-depleted, NH_3-treated PS II membranes at pH 7.5 did not result in the formation of significant amounts of a multiline-type EPR signal usually associated with the S_2 state. A multiline signal could be observed in the absence of NH_3, although it was weaker than at pH 6.5 (not shown).

To identify the S-state responsible for the $g = 2$ EPR signal in Ca^{2+}-depleted, NH_3-treated PS II membranes, dark-adapted material was exposed to a sequence of saturating laser flashes. Hardly any change compared to the dark spectrum was seen on the first flash (Fig. 3A), apart from an increase in the amplitude of Signal II at $g = 2$, but after a second flash a broad $g = 2$ signal appeared (Fig. 3B) which was indistinguishable from that seen after continuous illumination (Fig. 3C). Similar results were obtained, irrespective of whether the samples were frozen (250 K) or liquid (293 K) when illuminated.

NH_3-treated Ca^{2+}-sufficient PS II membranes

Illumination at 250 K of PS II membranes treated with 100 mM NH_4Cl resulted in the formation of a modified multiline signal with the same properties as after illumination at temperatures around 200 K (Fig. 4), i.e., with a width of about 200 mT and multiple lines spaced about 6–7 mT apart [13]. In addition, an EPR signal, centered around $g = 2$ not previously reported appeared, which is seen as a broadening at the base of

Signal II_s, similar to the effect observed with NH_3-treated PS II membranes depleted of bound Ca^{2+} (Fig. 1A). Illumination of oxygen-evolving PS II membranes in the absence of NH_4Cl under otherwise similar conditions resulted in the formation of a normal unmodified multiline EPR signal and an increase in the amplitude of Signal II_s with no indications of the broad signal around $g = 2$ (not shown). The width of the new $g = 2$ signal is similar to that in Ca^{2+}-depleted PS II membranes or about 10 mT but the shape is more symmetric lacking the pronounced tail on the high-field side seen after removal of Ca^{2+} (see Figs. 1A and 4B). The spectrum does not show any resolved peaks (but see below) and a lowered modulation amplitude of 0.5 mT did not result in a significant improvement in resolution of the spectral region around $g = 2$.

At an illumination temperature of 240 K the 10 mT-wide $g = 2$ signal decreased in amplitude to less than half of that at 250 K, whereas the NH_3-modified multiline signal ascribed to the S_2 state doubled in intensity (not shown). When the illumination temperature was raised to 260 K the reverse was observed: a slight increase of the broad $g = 2$ signal accompanied by a decrease-in the NH_3-modified multiline signal.

To investigate the stability of the new EPR signal a sample prepared by 250 K illumination was kept in the dark at 273 K for 25 min. This resulted in the complete disappearance of the broad $g = 2$ signal while the amplitude of the NH_3-modified multiline signal decreased only slightly (Fig. 5). Even if the new species decayed via the S_2 state this behavior may be expected because of the comparably low amount of the new species (judged from the signal amplitude) and the limited stability of the S_2 state. (The half-life of the NH_3-modified S_2 state under similar conditions was measured to about 25 min in a separate experiment.) The change in amplitude of the narrow Tyr-D$^+$ EPR signal at $g = 2$ is insignificant during the decay of the wide $g = 2$ component. Therefore, the difference spectrum obtained from the decay reaction (Fig. 5B) is better resolved than that recorded after illumination (Fig. 4B) where the signal is partially masked by the large increase in Signal II_s. The peak-to-peak distance of the signal in Fig. 5B is slightly less than 10 mT.

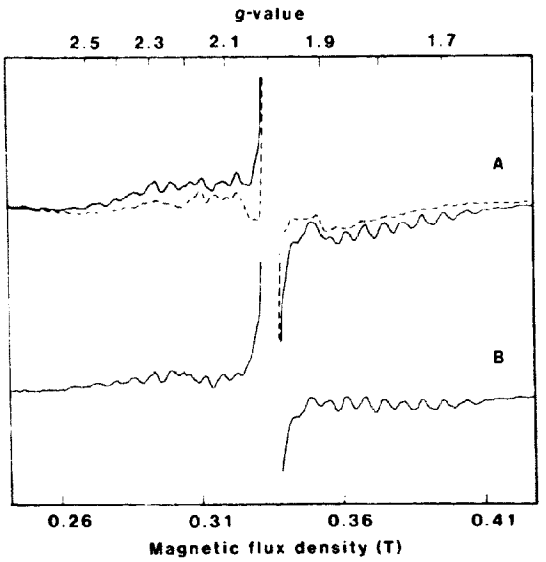

Fig. 4. Light-induced changes in the EPR spectrum of Ca^{2+}-sufficient PS II membranes treated with NH_4Cl at pH 7.5. (A) dashed line, dark-adapted membranes; solid line, after illumination at 250 K for 4 min. (B) Difference spectrum, illuminated minus dark-adapted. Conditions for EPR as in Fig. 1.

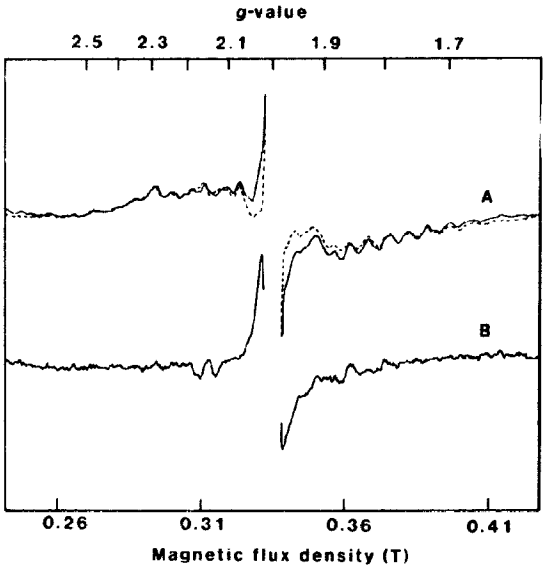

Fig. 5. Effect of warming on the light-induced EPR spectrum in NH_4Cl-treated PS II membranes. (A) Solid line, illuminated as in Fig. 4; dashed line, after relaxation in the dark at 273 K for 25 min. (B) Difference spectrum, illuminated minus relaxed (gain twice that in A). Conditions for EPR as in Fig. 1.

The power saturation of the new signal was measured at a field position on the high-field side of $g = 2$ where interference from the Tyr-D$^+$ signal was negligible and gave a value for $P_{1/2}$ at 11 K of 1 mW, similar to the value earlier reported for the modified S_3 state (see above).

If the new 10 mT-wide signal in NH_3-treated, native PS II membranes corresponds to a modified S_3 state one would expect it to appear after illumination with two light flashes. However, no such signal was observed after the second flash at 293 K or on the following flashes (7 flashes in total), only a NH_3-modified multiline signal in agreement with earlier observations [20]. As the warming experiment described above indicated that the state responsible for the signal was unstable,

the flash illumination was repeated at 273 and 250 K in attempts to trap the signal. These experiments also gave negative results.

To investigate the possibility that a low rate of formation, e.g., due to slow binding of NH_3, was responsible for the failure to form the 10 mT-wide signal after the second flash, two laser flashes were given at 273 K to NH_3-treated PS II membranes to generate the S_3 state, followed by a dark interval between 2 and 40 s before freezing to allow for the formation of the signal. The 10 mT wide signal was also not observed with this method.

Discussion

The approximately 15 mT-wide EPR signal at $g = 2$ seen after depletion of Ca^{2+} has been suggested to arise from a modified S_3 state (S_3') accumulated as a consequence of the inhibition of oxygen evolution [6,7]. It is interesting to find that in the presence of ammonia, which interferes specifically with the oxygen-evolving process, Ca^{2+}-depleted PS II membranes show a similar but not identical signal, the main difference being a considerably decreased width. Although ammonia has been suggested to bind to several of the possible intermediates, i.e., the S states, in the oxygen-evolving process, the S_2 state has been the only intermediate in which ammonia binding has been observed directly by spectroscopic techniques so far [5,13,14–17]. Therefore, the modification by ammonia of the EPR signal induced after depletion of Ca^{2+} may represent the first direct spectroscopic evidence of ammonia binding to an S state other than S_2. The similarity of this signal with that observed after Ca^{2+} depletion suggests that it also arises from an S_3' state, although ammonia-modified. These observations are in line with earlier studies where binding of ammonia to S_3 was inferred indirectly from luminescence and EPR measurements [16,20]. Another piece of evidence which also suggests that the broad $g = 2$ signal originates from an S_3' state is the formation of the signal on the second flash given to dark-adapted, Ca^{2+}-depleted and ammonia-treated PS II membranes, assuming that dark-adapted material resides preferentially in the S_1 state. The modification of the S_3' signal by NH_3 is also a strong indication that the inhibitor binds near or directly to the manganese cluster in the S_3' state although one cannot exclude that a change in conformation from a remotely bound NH_3 molecule could weaken the interaction between the radical and manganese with a decrease in splitting and signal width as a result.

Our results may indicate that high pH renders the S_2 state in Ca^{2+}-depleted PS II membranes temperature labile. Ca^{2+} depletion by itself has been shown to make the S_2 state unusually dark stable so that relaxation of the S_3' state leads to an accumulation of the S_2 state with a multiline EPR signal modified by the absence of Ca^{2+} [6,7,24]. At pH 7.5 no multiline signal appeared on the decay of the broad $g = 2$ signal regardless of whether ammonia was present or not; only the kind of relatively featureless spectrum which usually characterizes a dark-adapted PS II sample in the S_1 state resulted (Fig. 2). If the S_2 state is more labile than the S_3' state in these samples no accumulation of the former state would be expected to occur.

It is also possible that the S_2 state in these samples has been modified in a way to make its EPR signal difficult to detect. This may be one reason for the absence of a strong EPR signal after the first flash (Fig. 3), also when the illumination was given at 250 K. Again, if the S_2 state is labile at this temperature and above, rapid decay of the S_2 state may prevent the trapping of significant amounts. Sometimes a weak multiline EPR signal was detected after the first flash in the ammonia-treated samples (Fig. 3A). However, because of its low amplitude it was not possible to decide whether this came from an S_2 state modified by ammonia or if it represented a small population of centers in the unmodified, Ca^{2+}-depleted S_2 state.

The absence of an S_2 EPR signal after illumination at 200 K of the Ca^{2+}-depleted, NH_3-treated PS II membranes may be interpreted as showing that this state is extremely labile under these conditions, even at this low temperature. A more likely explanation is that the S_2 state does not even form under these conditions since it has been observed that Ca^{2+} depletion may result in an inhibition of the S_1 to S_2 transition at low temperatures [22,24].

Since NH_3 is an inhibitor of oxygen evolution one or more reactions in the catalytic process should occur with a lower rate than normally or not at all, leading to the accumulation of an intermediate before the inhibited step. The observation of a new EPR signal after illumination of Ca^{2+}-sufficient PS II membranes in the presence of NH_3 may indicate the presence of such an intermediate. In addition, it is possible that the binding of NH_3 has perturbed the structure of the oxygen-evolving complex so that the intermediate is more easily observable by EPR in analogy with the modified S_3 state formed after inhibition of oxygen evolution by depletion of Ca^{2+} [6–8] or replacement of Cl^- with F^- [9].

It is not possible at this stage to give a definite assignment of the intermediate responsible for the 10 mT EPR signal at $g = 2$ in NH_3-treated PS II membranes in the presence of Ca^{2+}. The observations that the signal forms preferentially above 240 K, where acceptor side limitations to electron transfer due to the temperature are relaxed [25], and at the expense of the S_2 multiline signal, argue for an S state later in the catalytic cycle. One possible candidate is a modified S_3 state in analogy with the earlier described S_3' states.

with EPR signals ascribed to a histidine radical [6–10] in magnetic interaction with the manganese center. Several lines of evidence would support such an assignment. In all these cases, the treatments which result in inhibition of oxygen evolution are believed to interfere with the oxidation of the S_3 state. Furthermore, in addition to similarities with the other S_3' states with regard to the illumination and temperature requirements for the formation of the 10 mT-wide EPR signal, the NH_3-dependent signal shows a limited stability and decays at elevated temperatures. Although more narrow than other S_3' signals observed so far, the signal has a width considerably larger than that of a typical radical. The difference in width compared to the earlier observed S_3' signals may be easily explained by a change in the magnetic coupling between the radical and the manganese cluster induced by NH_3 binding in analogy with the effect on the signal width of the extrinsic 16 and 23 kDa proteins [22]. Power saturation studies also show a more efficient relaxation than what is likely to be the case for an isolated radical and more typical of a magnetically interacting paramagnetic center. The similarity with the signal observed in NH_3-treated, Ca^{2+}-depleted PS II membranes might be taken to show that it actually arises from a population of centers inadvertently depleted of Ca^{2+}. However, the absence of the signal in the flash experiment (see below) argues against this possibility and instead suggests that the signals arise from distinct but closely related intermediates. The small dissimilarities between the two most likely reflect the difference in the availability of Ca^{2+}.

The absence of the 10 mT-wide signal in NH_3-treated, Ca^{2+}-sufficient PS II membranes after two flashes seems to argue for an origin other than a modified S_3 state. However, the signal was not detected anywhere else in the flash sequence but only after continuous illumination. This behavior is expected for an inactive intermediate which is formed slowly and in competition with a rapidly formed normal intermediate. In PS II membranes the formation of the inhibited state (S_3-NH_3) in the presence of NH_3 was reported to occur with a half-time of 30 s at room temperature [20], probably because of slow binding of an NH_3 molecule. At a flash frequency of 1 Hz there is little time for NH_3 to bind and S-state cycling can proceed almost unimpeded for a few cycles [20]. When the interval between the second flash and the freezing event is prolonged to give more time for NH_3 to bind, formation of significant amounts of the of the modified S_3 state is counteracted by its limited stability. This is a likely explanation why the 10 mT-wide EPR signal was not observed in the flash experiments. In continuous light, on the other hand, even if the average transit time for the S states is short, the repeated passage through the susceptible S_3 state should eventually lead to photoaccumulation of the inactive form of S_3 through NH_3 binding followed by the formation of the EPR signal. The actual amount observed should depend on factors such as the balance between the light intensity [20] and the stability of the NH_3-modified S_3 state at each particular temperature.

In conclusion, these experiments provide spectroscopic evidence that binding of NH_3 to the oxygen-evolving complex leads to the formation of a structurally perturbed and inactive S_3 state. The spectral changes induced by NH_3 suggests that the inhibitor binds near or directly to the manganese cluster in this state of the oxygen- volving center.

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