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In the Oxygen-Evolving Complex of Photosystem II the S_0 State Is Oxidized to the S_1 State by D^+ (Signal II_{slow})†

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ABSTRACT: A study of electron paramagnetic resonance (EPR) signals from components on the electron donor side of photosystem II has been performed. By measurement of EPR signal II_{slow} (D^+) it is shown that, after three flashes, D^+ decays slowly in the dark at room temperature in the fraction of the centers that was in the S_0 state ($t_{1/2}$ of 20 min in thylakoid membranes and 50 min in photosystem II enriched membranes). This reaction is accompanied by a conversion of S_0 to S_1. The concentration of S_1 was estimated from the amplitude of the S_2-state multiline EPR signal that could be generated by illumination at 200 K. These observations indicate that D^+ accepts an electron from S_0 in a dark reaction in which D and S_1 are formed. In addition, the reactions by which D donates an electron to S_2 or S_3 have been directly measured by monitoring both signal II_{slow} and the multiline signal. The redox interactions between the D/D^+ couple and the S states are explained in terms of a model in which D/D^+ has a midpoint potential between those of S_0/S_1 and S_1/S_2. In addition, this model provides explanations for a number of previously unrelated phenomena, and the proposal is put forward that the reaction between D^+ and Mn^{2+} is involved in the so-called photoactivation process.

Photosystem II (PSII)[1] catalyzes the light-driven oxidation of water and the reduction of plastoquinone in plants, green algae, and cyanobacteria. It is a multisubunit enzyme that spans the thylakoid membrane and contains a chain of interconnected redox components of diverse chemical nature [for a recent review, see Babcock (1987)]. Four consecutive charge separations in the reaction center of PSII create the oxidizing equivalents that are necessary for the oxidation of two molecules of water to one molecule of molecular oxygen. According to the current models, the redox-active center in the oxygen-evolving system is composed of a cluster of Mn atoms (Dismukes, 1986; Babcock, 1987). The oxygen-evolving complex is thought to exist in five different oxidation states, the so-called S states, S_0–S_4 (Kok et al., 1970), which probably reflect different oxidiation states of the Mn atoms. The S_2 state is paramagnetic and gives rise to two EPR signals, the so-called multiline signal, and a more recently discovered signal centered around $g = 4.1$. These signals have been studied extensively, and the multiline signal in particular has proved to be a useful mechanistic probe (Dismukes, 1986; Babcock, 1987).

The donor side of PSII also contains a component, Z, which is thought to be the immediate electron donor to the photooxidized special chlorophyll P_{680}^+. In the oxidized form (Z^+) this component gives rise to a radical-type EPR spectrum with fast reduction kinetics called signal $II_{very\ fast}$ (SII_{vf}) (Blankenship et al., 1975). In the native state Z^+ is rapidly reduced by the oxygen-evolving complex, while the kinetics of Z^+ are slower in preparations where the oxygen-evolving complex is damaged [the EPR signal is now called SII_{fast} (SII_f)] (Babcock & Sauer, 1975).

Also present on the oxidizing side of PSII is another component D, which, in its oxidized form, gives rise to an EPR spectrum that is very similar to SII_f. However, the signal is very stable in the dark (Babcock & Sauer, 1973) and is consequently called SII_{slow} (SII_s). D^+ has been shown to

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[1] Abbreviations: Chl, chlorophyll; DAD, diaminodurene; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II.

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interact magnetically with the Mn cluster (DeGroot et al., 1986; S. Styring and A. W. Rutherford, unpublished results), indicating their relative proximity. In early work it was observed that D, in its reduced form [present either in the dark (Babcock & Sauer, 1973) or formed by chemical reduction (Velthuys & Visser, 1975)], could reduce the S_2 and S_3 states with rate constants of approximately 1 s^{-1} (Babcock & Sauer, 1973). However, the normal redox state of D seems to be the oxidized form, D^+, and this has not been observed to interact chemically with any of the S states.

In the original S-state model for flash-induced oxygen evolution it was porposed that S_1 was present in the majority of the centers and that S_0 was present in 25–30% of the centers (Kok et al., 1970). The observation that D could donate an electron to S_2 or S_3 led Velthuys and Visser (1975) to ask whether the S_0 centers thought to be present in the dark were really those in which D was present. Subsequently, it was shown that indeed a 100% S_1 oscillation pattern of oxygen evolution was observed if flashes were given with a short flash interval (0.25 s) while the usual 25% S_0 and 75% S_1 pattern was obtained when more widely spaced flashes were used (2 s between flashes) (Vermaas et al., 1984). This effect was correlated with donation of an electron from D in 25% of the centers (Vermaas et al., 1984). It was also shown that when S_0 was formed in the majority of centers by illumination with three flashes, it was slowly converted to S_1 in the dark (Vermaas et al., 1984). The electron acceptor in this reaction was not identified, but it was later hypothesized that D^+ could oxidize S_0 to S_1 in the dark (Zimmermann & Rutherford, 1985).

In this paper we demonstrate that this reaction indeed takes place in thylakoid membranes and PSII-enriched membranes. We have also characterized the reactions between the reduced form of D and the higher S states in PSII-enriched membranes.

Materials and Methods

Thylakoid membranes were prepared from market spinach as described by Ford and Evans (1983). Before experimental use the membranes were resuspended to the desired chlorophyll concentration in 20 mM HEPES buffer at pH 7.5 containing 15 mM NaCl, 5 mM $MgCl_2$, and 30% ethylene glycol and kept on ice in the dark for 3 h. PSII-enriched membranes were prepared from spinach as described by Ford and Evans (1983) and were a kind gift of L.-G. Franzén (University of Göteborg, Göteborg, Sweden). They were stored in liquid nitrogen at 10 mg of Chl/mL. Before the experiments, the membranes were diluted 20 times in 20 mM MES buffer at pH 6.3 containing 0.4 M sucrose, 1 mM $CaCl_2$, 10 mM NaCl, and 30% (v/v) ethylene glycol. The membranes were pelleted by centrifugation and resuspended in the same buffer to the desired chlorophyll concentration. Immediately before use the membranes were kept in the dark on ice for 2 h. All chemicals used were of the highest quality commercially available. PPBQ and DAD were added from 20 and 100 mM solutions in dimethyl sulfoxide, respectively.

Samples in calibrated EPR tubes were incubated 1 min at 20 °C before they were given a saturating preflash. After the preflash they were allowed to equilibrate at 20 °C. When used, PPBQ was added 9 min after the preflash. Ten minutes after the preflash the samples were given the desired number of flashes. After the flashes the samples were kept at room temperature for a defined time, after which time they were frozen in an ethanol/solid CO_2 bath and immediately transferred to liquid nitrogen. In some experiments D^+ was partially reduced chemically with 5 mM sodium ascorbate and 1 mM DAD by incubation in the dark for 30 min. In these cases the reducing chemicals were removed after the reaction by dilution of the sample in 10 volumes of buffer without ascorbate and DAD. The membranes were thereafter collected by centrifugation and suspended to the desired concentration in buffer without reducing chemicals. All sample handling including addition of acceptor, chemical reductions, centrifugation, and room temperature incubations was performed in darkness.

The samples were illuminated with either continuous white light from an 800-W projector lamp for 4 min at 200 K or with saturating flashes from a Nd-YAG laser (15 ns, 100 mJ, 530 nm) at 20 °C with an interval between the flashes of 1 s. The light intensity of the laser flash was confirmed to be saturating by comparing the amplitude of the S_2-state multiline signal formed after one flash with the amplitude of the signal in the same sample after further illumination at 200 K. No increase in the multiline signal amplitude after the 200 K illumination was observed. The observed oscillation of the multiline signal with the flash number (Figure 1C) also indicates that the flash was saturating.

EPR spectra were recorded at liquid helium temperatures with a Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments cryostat. The amplitude of the multiline signal was calculated from the added amplitudes of the well-defined peaks marked in Figure 2C. All samples were prepared in the presence of 30% (v/v) ethylene glycol, which prevents the formation of the signal around $g = 4.1$ when the illumination is performed at 200 K or room temperature, leaving only the multiline signal in the EPR spectrum from the S_2 state (Zimmermann & Rutherford, 1986). The amount of SII_s was estimated from the amplitude of the peak at 3344 G (marked in Figure 1A) in the nonsaturated spectrum. SII_s saturates differently in the different S states (DeGroot et al., 1986; S. Styring and A. W. Rutherford, unpublished results). To avoid errors in the quantification of SII_s due to this saturation behavior, the spectrum of SII_s was always run at two different low microwave powers and the function log [amplitude/(power)$^{1/2}$] was calculated. In the nonsaturated region of the saturation curve for an EPR signal this ratio is constant. All amplitudes for SII_s presented in this work have been tested with this criterion and found to be measured under nonsaturating conditions.

Results and Discussion

Reduction of D^+ in the Dark. Dependence on the S State. To test if D^+ can act as an electron acceptor in the S_0 state, an experiment to investigate the lifetime of D^+ in the different S states was performed. Photosynthetic membranes were given a different number of flashes, and the samples were frozen immediately. The amplitude of the S_2-state multiline signal was measured to verify that a good oscillation pattern was achieved in the experiment (Figure 1C,D). The oscillation of the multiline signal was slightly better in the PSII-enriched membranes where an exogenous acceptor was used [the oscillatory pattern could be satisfactorily fitted, assuming 13% misses (not shown)] than in the thylakoid membranes in which no acceptor was used. The amplitude of SII_s was also measured and found to be nearly equal in all samples (set as 100% in Figure 1B). Then the samples were thawed to room temperature and allowed to equilibrate in total darkness (60 min for thylakoid membranes and 90 min for PSII-enriched membranes) whereafter the samples were refrozen. The multiline signal had decayed completely (not shown) while SII_s remained nearly unchanged after zero, one, two, five, and six flashes (Figure 1A,B). However, SII_s had decayed significantly after three flashes, i.e., in the S_0 state. In the thylakoid

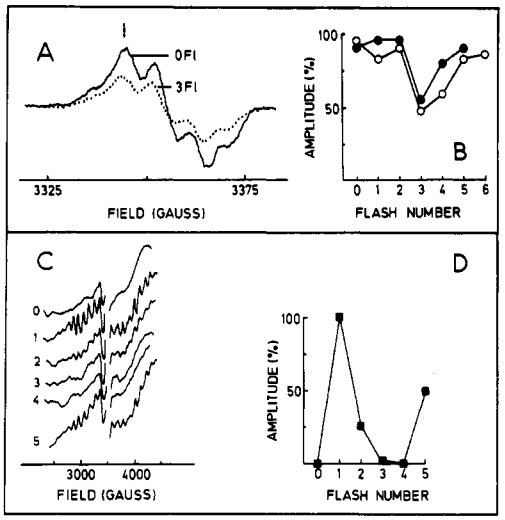


FIGURE 1: (A) EPR spectra of SII_s in PSII-enriched membranes after zero and three flashes followed by dark incubation for 90 min at 20 °C before freezing. (B) Amplitude of SII_s after a varying number of flashes followed by incubation in the dark for 90 min (PSII-enriched membranes, closed symbols) or 60 min (thylakoid membranes, open symbols) before freezing. The amplitude of SII_s immediately after the flash was set as 100%. (C) The S_2-state multiline signal formed immediately after a varying number of flashes given to PSII-enriched membranes. (D) Oscillation with the flash number of the multiline signal in (C). The amplitude of the signal after one flash was set as 100%. SII_s (A, B) and the multiline signal (C, D) were measured in the same samples. The chlorophyll concentration was 2 mg/mL. In the experiments with PSII-enriched membranes 0.25 mM PPBQ was used. Spectroscopic conditions: (A and B) temperature 15 K, microwave power 0.5 μW, and modulation amplitude 2.8 G; (C) temperature 8 K, microwave power 31.7 mW, modulation amplitude 22 G, and microwave frequency 9.44 GHz.

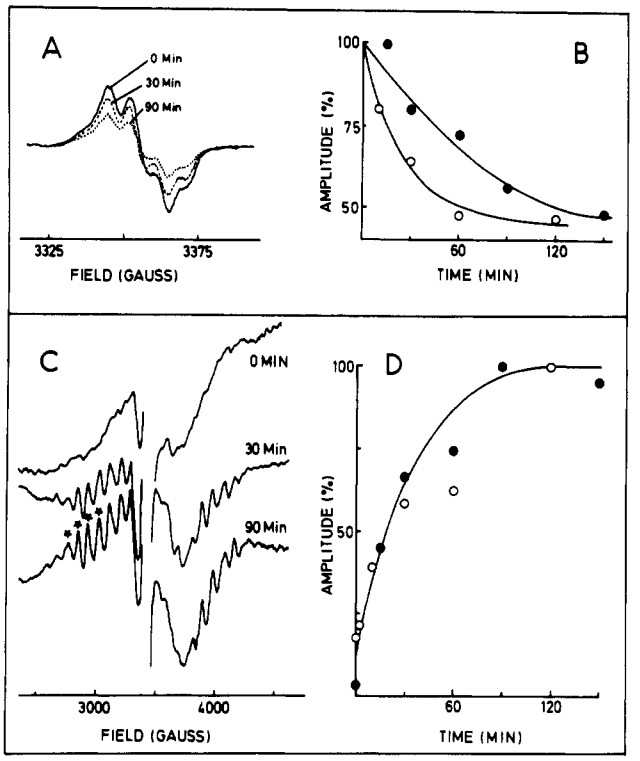


FIGURE 2: (A) EPR spectra of SII_s in PSII-enriched membranes after three flashes followed by 0, 30, and 90 min of dark incubation at 20 °C before freezing. (B) Time course for the decay of SII_s in PSII-enriched membranes (closed symbols) and thylakoid membranes (open symbols) following three flashes. The amplitude immediately after the flashes is set as 100%. (C) The S_2-state multiline signal formed by illumination at 200 K in the same samples as in (A). (D) Formation of the S_2-state multiline signal generated by illumination at 200 K of the same samples as in (B). PSII-enriched membranes, closed symbols; thylakoid membranes, open symbols. The amplitude of the multiline signal in a sample that was given one flash is set as 100%. Experimental conditions and EPR settings for SII_s and the multiline signal were as in Figure 1.

membranes 48% of SII_s remained after 60 min while 56% of the original amplitude remained in the PSII-enriched membranes after 90 min. The observed reduction of D^+ also after four flashes (40% in thylakoid membranes and 20% in PSII-enriched membranes) was due to the damping in the oscillatory pattern, which resulted in a fraction of the centers being in the S_0 state.

Decay of SII_s in the S_0 State. Formation of the S_1 State. SII_s disappears only in the S_0 state while it is essentially stable in the other S states (Figure 1B). Since the S_0 state has been found to disappear in approximately 20 min in pea thylakoids (Vermaas et al., 1984), it was important to determine the kinetics for the reduction of D^+ in the S_0 state in order to correlate these observations.

Figure 2A,B shows the decrease in the amplitude of SII_s that takes place in the dark after three flashes. In PSII-enriched membranes, SII_s was diminished to 47% of its original amplitude with an approximate half-time of 50 min. The reduction is faster in thylakoid membranes (Figure 2B) where the decay half-time is approximately 17 min. From the oscillatory pattern for the multiline signal in the PSII-enriched membranes (Figure 1D) it can be estimated (assuming 13% misses) that 66% of the centers were in the S_0 state immediately after the three flashes. Thus, SII_S disappeared in 70% of the centers that were in the S_0 state. A similar number can be calculated also for the thylakoid membranes (data not shown).

The demonstration that D^+ decays in the dark after three flahses (Figures 1 and 2) supports the idea that it accepts an electron from S_0. The product in this reaction is predicted to be S_1, which is not directly detectable by EPR. However, the concentration of the S_1 state present can be estimated from the amplitude of the S_2-state multiline signal that is photoinduced at 200 K (Brudvig et al., 1983). Therefore, the samples from the experiments in Figure 2B were illuminated at 200 K. Figure 2C shows the multiline signal formed after 0, 30, and 90 min of incubation in the dark after the three flashes. It is clear that the longer the sample was allowed to react the more the multiline signal was formed upon illumination at 200 K. This shows that the S_1 state was formed during the incubation in the dark following the three flashes. The time course for the formation of the multiline signal in PSII-enriched membranes and thylakoid membranes is shown in Figure 2D. The rise half-time for the formation of the multiline signal was approximately 20 min in both materials. In thylakoid membranes this seems to correlate with the half-time for the decay of D^+. However, in PSII-enriched membranes the decay of D^+ was significantly slower than the formation of the S_1 state. The rate of S_1 formation is expected to differ from the rate of D^+ decay due to deactivation of S_2 and S_3 (which is faster than the decay of D^+; see below) in a fraction of the centers. This fraction was calculated from Figure 1D to be approximately 34% in the PSII-enriched membranes (assuming 13% misses). Despite this complication the formation of the S_1 state upon prolonged incubation in the dark after three flashes supports the hypothesis that the substrate for the reduction of D^+ is S_0 and that this is oxidized to S_1 in the reaction.

Reoxidation of D in the S_2 and S_3 States. Velthuys and Visser (1975) reduced D^+ in chloroplasts with ascorbate and DCIP and demonstrated that D^+, after a single flash, was

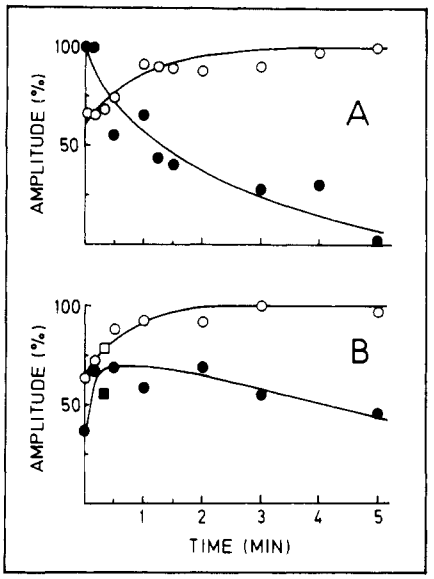


FIGURE 3: (A) Formation of SII_s (open symbols) and decay of the multiline signal (closed symbols) after one flash in PSII-enriched membranes when D^+ was reduced chemically in 50% of the centers from the start. (B) Formation of SII_s (open symbols) and the multiline signal (closed symbols) after two flashes in PSII-enriched membranes when D^+ was reduced chemically in 50% of the centers from the start. After the flashes the samples were incubated in darkness at 20 °C for the times indicated before they were frozen. The squares in (B) indicate the amounts of the multiline signal and SII_s that were present in the sample from zero time after it had been thawed in the dark to room temperature for 10–20 s before it was refrozen. The amplitude of SII_s before chemical reduction and the amplitude of the multiline signal in a sample given one flash after chemical reduction and frozen immediately were set as 100%, respectively. The experimental conditions and the EPR settings were the same as in Figure 1.

restored to 80% with a half-time of about 2 s. This was similar to the half-time for D^+ formation after one flash in untreated chloroplasts prepared from dark-stored spinach leaves (Babcock & Sauer, 1973). The same reaction, i.e., the oxidation of D to D^+ by S_2 reduction to the S_1 state, was observed in the experiments presented in Figure 2 when, following illumination at 200 K, the samples were thawed to room temperature in the dark (data not shown). In this experiment SII_s was nearly quantitatively restored within 10 s in thylakoid membranes concomitant with the loss of the multiline signal. The reaction in PSII-enriched membranes was much slower and at least 2 min of reaction in the dark at room temperature was needed to allow complete reoxidation of D.

The reoxidation of D in the S_2 state in PSII-enriched membranes was characterized in an experiment in which D^+ was reduced to half its original amplitude with sodium ascorbate and DAD. The chemicals were removed by dilution and centrifugation in darkness (see Materials and Methods). The samples were then given one saturating flash and frozen at different times after the flash. SII_S and the multiline signal were measured. The results are presented in Figure 3A and show that SII_S was restored to its amplitude before reduction with a half-time for its formation of 30–40 s. A rapid increase of SII_S occurred within the freezing time after a flash (from 50% to 65% of its original amplitude). In a separate experiment this was found to be due to rapid reoxidation of D after a flash in 28% of the centers in which D was originally reduced (data not shown).

The decay of the multiline signal seems to be biphasic. Approximately half of the initial amplitude of the signal disappears with a half-time of around 30 s. This is attributed to donation from D to S_2 in those centers in which D was present in its reduced form in the dark. The rest of the multiline signal is more stable and disappears in a slow reaction, which takes several minutes to complete. This corresponds to the decay of the S_2-state multiline signal ($t_{1/2}$ is approximately 4 min at 20 °C) in untreated PSII-enriched membranes in the presence of 0.5 mM PPBQ as an electron acceptor (unpublished results).

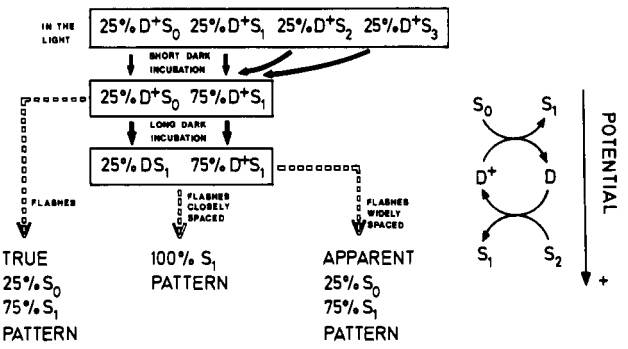
This experiment shows that the reduction of D in the S_2 state is slower in PSII-enriched membranes (in the majority of the centers) than in chloroplasts or thylakoids. The reaction is sufficiently slow to allow studies also of the oxidation of D in the S_3 state using our experimental technique. Again D^+ was reduced chemically to 50% of its original amplitude, and the chemicals were removed by dilution and centrifugation. The samples were then given two flashes to produce the S_3 state. After incubation in darkness for different times the samples were frozen and the amplitudes of SII_s and the multiline signal were measured. Figure 3B shows that D^+ was restored to its full amplitude after about 1 min with an approximate half-time for its formation of 25 s (also in this experiment D was oxidized within the freezing time after the flash in about 15% of the total centers). The electron donation by D to the S_3 state has earlier been studied by Babcock and Sauer (1973), who measured a rate constant for the reaction of 1 s^{-1} in untreated chloroplasts prepared from dark-adapted spinach leaves. It is clear that also this reaction was slower in PSII-enriched membranes.

Figure 3B also shows the amplitude of the multiline signal during the time course in this experiment. The kinetic behavior of the multiline signal was more complex with formation and decay kinetics from centers in which D was present in either its oxidized or its reduced form. Also, centers that only reached the S_2 state with the two flashes (approximately 30% originating from misses and from centers in which D was rapidly oxidized after the first flash) complicate the analysis. However, it is clear that the multiline signal was increased by approximately 28% during the first 30 s of the reaction. It should also be noted that when the sample that had been frozen immediately after the flashes (time = 0) was thawed to room temperature for 10–20 s, the amplitudes of SII_S and the multiline signal were increased (Figure 3B, squares). This increase in the multiline signal can mainly be attributed to the electron donation from D to S_3 to form the S_2 state in the 26% of the centers that were predicted to be in the state DS_3 immediately after the flashes. In addition, the decay of the S_3 state in the centers in which D^+ was present contributes to the transient formation of the multiline signal [the decay half-time of the S_3 state in PSII-enriched membranes in the presence of 0.5 mM PPBQ was 4–5 min as measured by EPR (unpublished results)]. The S_2 state formed from the S_3 state thereafter decays in a slow reaction (see above). This experiment represents the first direct observation of S_2 formation from S_3. In earlier work Zimmermann and Rutherford (1984), working in the absence of an exogenous acceptor, did not observe any increase in the amplitude of the multiline signal during the decay of the S_3 state. Their interpretation was that S_2 decays faster than S_3 or that S_3 does not deactivate via the S_2 state. In our experiment (Figure 3B), however, D was reduced in approximately half of the centers and provided a rapid deactivation pathway for S_3 that allowed a transient formation of the multiline signal to be observed. In addition, this experiment was performed in the presence of an exogenous electron acceptor, which prevents recombination between the S_2 state and Q_B^- from taking place.

CONCLUSIONS

It has previously been shown that D, the reduced form of

Scheme I



the component responsible for SII_s, can donate an electron to the S_2 or S_3 state of the oxygen-evolving enzyme (Babcock & Sauer, 1973; Velthuys & Visser, 1975). Here these observations have been confirmed by low-temperature EPR measurements, which have enabled us to monitor directly both SII_s and the S_2 state (the multiline signal). For the reaction after one flash, $S_2 + D \rightarrow S_1 + D^+$, the formation of SII_s and the accompanying decay of the multiline signal were observed (Figure 3A), while for the reaction after two flashes, $S_3 + D \rightarrow S_2 + D^+$, the simultaneous formation of SII_s and the multiline signal could be observed before the S_2 state itself decayed (Figure 3B). The kinetics for these two reactions are slower in PSII-enriched membranes (in the majority of the centers) than in chloroplasts (Babcock & Sauer, 1973) or thylakoid membranes (Vermaas et al., 1984). The reason for this is not known, but it is possible that the detergent treatment in the preparation of the PSII-enriched membranes modifies the donor side of PSII.

In this paper we have investigated the reaction in which S_1 is formed from S_0 in the dark, and it has been demonstrated that this occurs by reduction of D^+, i.e., loss of SII_s. The chemical interactions of D and D^+ with the S states are summarized in Scheme I. This scheme provides a cohesive explanation for a number of previously unrelated phenomena. In the light, D^+ is present in all centers and S_0, S_1, S_2, and S_3 are equally represented. After a short dark adaptation a distribution of S states close to 25% S_0 and 75% S_1 is present in accordance with the model of Kok et al. (1970). This is reflected in the pattern of flash-induced oxygen release and is designated true 25% S_0/75% S_1 pattern in Scheme I. However, after longer dark adaptation the S_0 state is converted to the S_1 state in the reaction $S_0 + D^+ \rightarrow S_1 + D$. This explains the decrease in S_0 that takes place upon dark adaptation (Vermaas et al., 1984). At the same time it rationalizes the existence of a stable SII_s in the dark in most centers and the decay (in 2 h) of SII_s in approximately 25% of the centers (Babcock & Sauer, 1973). A sequence of closely spaced flashes (frequency 4 Hz) given to centers that have been incubated in the dark for longer times results in an oscillation pattern of the oxygen release, which reflects the 100% of the S_1 state that was present in the dark (Vermaas et al., 1984). A sequence of more widely spaced flashes (frequency 0.5 Hz) allows D (which is present in 25% of the centers, i.e., those that were previously in the S_0 state) to donate to S_2 or S_3. This results in an oscillation pattern in which 25% of the centers require four flashes before oxygen is evolved (designated apparent 25% S_0/75% S_1 pattern in Scheme I) (Vermaas et al., 1984).

Also summarized in Scheme I are the redox interactions between D/D^+ and the lower S states. The components are placed on a potential scale since the reactions described here indicate that D/D^+ has a higher potential than the S_0/S_1 couple while it has a lower potential than both the S_1/S_2 and the S_2/S_3 couples.

The question now arises: why should a mechanism exist by which S_0 is oxidized to S_1 in the dark? It is possible that the manganese cluster in the S_0 state is more labile to loss of manganese than the S_1 state. This explanation would be supported by the fact that Mn^{2+} has been proposed to be present in the S_0 state [see, for example, Srinivasan and Sharp (1986)], and it has been pointed out earlier that the coordination of Mn^{2+} can be expected to be weaker than that of the higher oxidation states of manganese (Ghanotakis et al., 1984). In this case the function of D^+ would be to oxidize Mn^{2+} to Mn^{3+}, thereby stabilizing the Mn cluster (this was suggested to us by Dr. G. W. Brudvig, Yale University). This reaction might be considered to be a step in the so-called photoactivation process (Radmer & Cheniae, 1977) by which Mn^{2+} is incorporated in the enzyme site. Similar reactions between D^+ and Mn^{2+} may occur also in the earlier steps of photoactivation.

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