Articles

EPR Signals from Modified Charge Accumulation States of the Oxygen Evolving Enzyme in Ca^{2+}-Deficient Photosystem II

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ABSTRACT: Photosystem II enriched membranes were depleted of Ca^{2+} and the 17- and 23-kDa polypeptides by treatment with NaCl and EGTA. The 17- and 23-kDa polypeptides were then reconstituted. This preparation was incapable of O_2 evolution until Ca^{2+} was added. An EPR study revealed the presence of two new EPR signals. One of these is a modified S_2 multiline signal with an isotropic g value of 1.96 with at least 26 hyperfine peaks (average spacing 55 G) distributed over approximately 1600 G. The other is a near-Gaussian signal with an isotropic g value of 2.004, which is attributed to a formal S_3 state. Experiments involving the interconversion of these signals and the effect of Ca^{2+} and Sr^{2+} rebinding provide evidence for these assignments. From these results the following conclusions are drawn: (1) These results are consistent with our earlier demonstration that charge accumulation is blocked after formation of S_3 when Ca^{2+} is deficient. (2) Binding of the 17- and 23-kDa polypeptides to photosystem II in the absence of Ca^{2+} results in the perturbation of the Mn cluster. This is taken as a further indication that the Ca^{2+}-binding site is close to or even an integral part of the Mn cluster. (3) The S_3 signal may arise from an organic free radical interacting magnetically with the Mn cluster. However, other possible origins for this signal, including the Mn cluster itself, must also be considered.

Oxygen evolution by plants occurs in photosystem II (PS-II).[1] It seems likely that the reaction center of PS-II is the location of the Mn cluster that acts both as the active site and as the charge-accumulating device of the water-splitting enzyme (Rutherford, 1989, for a review). During the enzyme cycle the donor side of PS-II goes through five different redox states that are denoted S_n, n varying from 0 to 4 according the model of Kok et al. (1970). Three extrinsic polypeptides are bound to the PS-II reaction center on the inside of the thylakoid membrane. Two of these, the 17- and 23-kDa polypeptides, can be removed by washing with salt [see Murata and Miyao (1985) for a review]. In the absence of these polypeptides, the Mn cluster is susceptible to attack by exogenous reducing agents leading to dissociation of the Mn from the enzyme (Ghanotakis et al., 1984a). Removal of these polypeptides results in inhibition of O_2 evolution due to an increased requirement for chloride and calcium ions [reviewed in Homann (1989)].

It has been shown that salt washing resulted in the release of one Ca^{2+} per PS-II center (Cammarata & Cheniae, 1987). Similar results were obtained upon washing with citrate (Ono & Inoue, 1988). Recently, however, Shen et al. (1988) reported that NaCl washing did not result in Ca^{2+} release. It was suggested that the inhibition induced by the salt washing is due to the removal of the 23-kDa polypeptide. However, in earlier work (Ghanotakis et al., 1984b; see also the results reported here) it was shown that reconstitution of this polypeptide does not result in reactivation of the enzyme in the absence of added Ca^{2+}. Although this controversy is obviously of importance, it is not addressed here. The present work was done by assuming that Ca^{2+} depletion actually occurs in treatments that result in inhibitory effects that can be reversed by Ca^{2+} addition.

The dissociation constant of the Ca^{2+} ion required for activity in NaCl-washed membranes was shown to be 50–100 μM and 1–2 mM in, respectively, 70% and 30% of the PS-II centers (Boussac et al., 1985; Cammarata & Cheniae, 1987). A second Ca^{2+} ion with a very high affinity (4 μM for the dissociation constant) has recently been reported in PS-II (Kalosaka et al., 1989). Removal of this very high affinity Ca^{2+} results in the loss of Mn oxidation (Kalosaka et al., 1989). It seems unlikely that this very high affinity Ca^{2+} was extracted in earlier work, especially since, in the majority of studies, the degree of inhibition of oxygen evolution achieved by Ca^{2+} depletion was rather limited [reviewed in Boussac and Rutherford (1988a)].

In the literature there is a good deal of controversy over the effectiveness of NaCl washing and the nature of the inhibition induced by Ca^{2+} release [reviewed in Boussac and Rutherford (1988a)]. Our results indicate that in NaCl-washed material inhibition of the enzyme cycle occurs at the S_3 to S_0 transition (Boussac & Rutherford, 1988a). To reconcile these data with those of the earlier contradictory studies, we proposed that depletion of Ca^{2+} from its site gave different kinds of inhibition depending upon the S state. In the majority of centers S_3 to S_0 is blocked, while, in a small fraction of centers, no S-state turnover occurs. A model was proposed in which a single Ca^{2+}-binding site is associated with O_2 evolution but the Ca^{2+}-binding affinity of the site varies with the intermediate state of the enzyme cycle (i.e., the S state) (Boussac & Rutherford, 1988a). In support of this model it was found that the effectiveness of Ca^{2+} depletion by salt washing done

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[1] Abbreviations: PS-II, photosystem II; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PPBQ, phenyl-p-benzoquinone; Tris, tris(hydroxymethyl)aminomethane; Tyr_D, a tyrosine residue that acts as a side-path electron donor of PS-II responsible for the EPR signal II_{slow}.

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in each of the S states was as follows: $S_3 > S_0 \approx S_2 > S_1$ (Boussac & Rutherford, 1988b).

Sr^{2+} was shown by Ghanotakis et al. (1984c) to reconstitute enzyme activity up to 40% in Ca^{2+}-deficient PS-II. We demonstrated that Sr^{2+} replaced Ca^{2+} in all of the centers but that O_2 evolution activity was diminished due to less efficient enzyme cycling (Boussac & Rutherford, 1988c). Sr^{2+}-reconstituted NaCl-washed membranes gave rise to an EPR signal from the Mn cluster in the S_2 state which was markedly modified and also resulted in a stabilization of the $g = 4.1$ EPR form of the S_2 state. This indicates that Sr^{2+} (and hence Ca^{2+}) is probably located close to the Mn cluster (Boussac & Rutherford, 1988a).

We have performed a Ca^{2+} depletion of PS-II by NaCl washing followed by reconstitution with the 17- and 23-kDa polypeptides. S-state advancement in these membranes was studied by EPR and oxygen evolution. Such membranes exhibit two new EPR signals which can be attributed to modified forms of the S_2 and S_3 states. In addition, the results are consistent with the conclusion that the enzyme cycle is blocked after formation of the S_3 state in Ca^{2+}-deficient membranes. Some of the data reported here have been published in a preliminary form elsewhere (Boussac & Rutherford, 1989).

Materials and Methods

Photosystem II particles from spinach chloroplasts were prepared according to the method of Berthold et al. (1981) with the modifications of Ford and Evans (1983). The activity of these membranes was 400–600 μM O_2 (mg of Chl·h)$^{-1}$. All further procedures used acid-rinsed vessels. The media containing MES and sucrose were passed through a Chelex (50–100 mesh) column. Highly pure NaCl (less than 0.1 ppm of Ca^{2+} contamination) was added after the Chelex step.

The following protocol is a modification of that of Ghanotakis et al. (1984b). The freshly prepared PS-II membranes were washed in 0.3 M sucrose, 10 mM NaCl, 20 mM MES, pH 6.5, and 2 mM EGTA and then collected by centrifugation (35000g). The pellet (approximately 250 mg of Chl) was resuspended in 50 mL of a medium containing 0.3 M sucrose, 20 mM MES, pH 6.5, 1.2 M NaCl, and 20 mM EGTA and rapidly transferred into a dialysis membrane (cutoff 8000–10 000 dalton). Then the membranes were dialyzed for 2 h, in daylight at 4 °C, against 2 L of a medium containing 0.3 M sucrose, 1.2 M NaCl, and 20 mM MES, pH 6.5. A second dialysis was performed for 3 h in the dark against a medium without salt containing 0.3 M sucrose and 20 mM MES, pH 6.5. After this step, the membranes were collected by centrifugation, resuspended in 0.3 M sucrose, 20 mM MES, pH 6.5, and 10 mM NaCl in daylight, and stored at −80 °C until used. For EPR measurements the samples were put into calibrated quartz tubes, and after a 30-min dark adaptation on ice, PPBQ dissolved in DMSO was added as an electron acceptor to a final concentration of 1 mM.

SDS–polyacrylamide gel electrophoresis was done with the system of Chua (1980) in a 15% acrylamide gel with 6 M urea added. The samples were solubilized in the presence of dithiothreitol and 2 M urea for 2 h at 37 °C. The gel was stained with Coomassie brilliant blue R-250. Removal of the 17-, 23-, and 33-kDa polypeptides by Tris washing was done as in Boussac et al. (1986).

Oxygen evolution in continuous white light was measured by using a Clark-type electrode at 23 °C. Samples were diluted to 20 μg of Chl/mL in 0.3 M sucrose, 30 mM NaCl, and 20 mM MES, pH 6.5, in the presence of 0.5 mM PPBQ.

EPR spectra were recorded at helium temperatures with a Bruker ESP 300 X-band spectrometer equipped with an Oxford Instruments cryostat. The samples were illuminated in a nonsilvered Dewar flask in a solid CO_2–ethanol bath at 198 K or in an ethanol bath cooled to 0 °C with liquid nitrogen. Illumination was done with an 800-W projector through infrared filters.

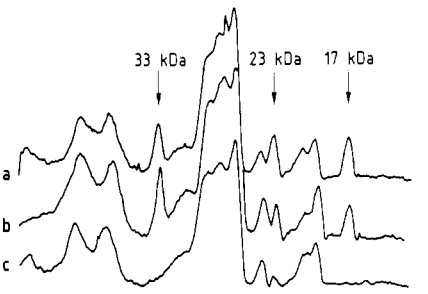

FIGURE 1: Gel densitogram of PS-II membranes. (a) Untreated membranes; (b) NaCl/EGTA-treated membranes reconstituted with the 17- and 23-kDa polypeptides; (c) Tris-washed membranes. Traces were scaled to the amount of chlorophyll loaded on the gel.

Experiments at different X-band frequencies were done by using an optical EPR cavity as in Hansson et al. (1987), the inherent frequency of which was 9.429 GHz. Insertion of quartz rods through the optical paths gave rise to a shift of the natural frequency down to the minimal available frequency from the klystron, which was 9.000 GHz. Insertion of a polished Al rod into the cavity was used to perform measurements up to 9.51 GHz.

Results

The activity of the PS-II membranes that had been NaCl treated and then reconstituted with the 17- and 23-kDa polypeptides as reported above was measured. Oxygen evolution in the presence of 20 mM Ca^{2+} or the absence of Ca^{2+} was, respectively, 390 and 0 μM O_2/(mg of Chl·h). Figure 1 shows the gel densitogram of the reconstituted membranes (lane b) compared with untreated (lane a) and Tris-washed (lane c) PS-II membranes. The level of reconstitution of the 17- and 23-kDa proteins reached approximately 80–85% when compared with the untreated membranes.

EPR studies of the polypeptide-reconstituted PS-II showed that these membranes exhibited a signal which was stable in the dark at room temperature (spectrum a, Figure 2A). This signal differs considerably from the normal multiline signal arising from the S_2 state [see Brudvig et al. (1989) for a review]. It possesses at least 26 lines with an average hyperfine line spacing of 55 G. This signal disappeared in the dark after the addition of Ca^{2+} (spectrum b, Figure 2A) or Sr^{2+}. The kinetics of the loss of the stable multiline signal after addition of 40 mM Ca^{2+} or Sr^{2+} is shown in Figure 2B. The change is apparently biphasic and is half-complete after $\approx$5 min. During the course of the kinetics, no change in the amplitudes of the EPR signal from Tyr_D^+ or cytochrome b_{559}^+ occurred (not shown). The normal multiline signal was not detected in the dark after the addition of Ca^{2+} (or Sr^{2+}). However, an illumination at 198 K, performed when the disappearance of the stable multiline signal was almost complete, resulted in the formation of the normal S_2 multiline signal (spectrum c, Figure 2A). When Sr^{2+} was added instead of Ca^{2+}, the 198 K illumination induced signal was typical of the Sr^{2+}-modified multiline signal reported earlier (spectrum d, Figure 2A) (Boussac & Rutherford, 1988a). The signal arising from the reduced primary acceptor, $Q_A^-Fe^{2+}$, can be seen between 360 and 380 mT ($g = 1.82$). Since we attribute the stable multiline signal to a modified S_2 state (see below), the data of Figure 2 are explained as follows; Ca^{2+} binding to the modified S_2 state results in the formation of the normal S_2 state but no

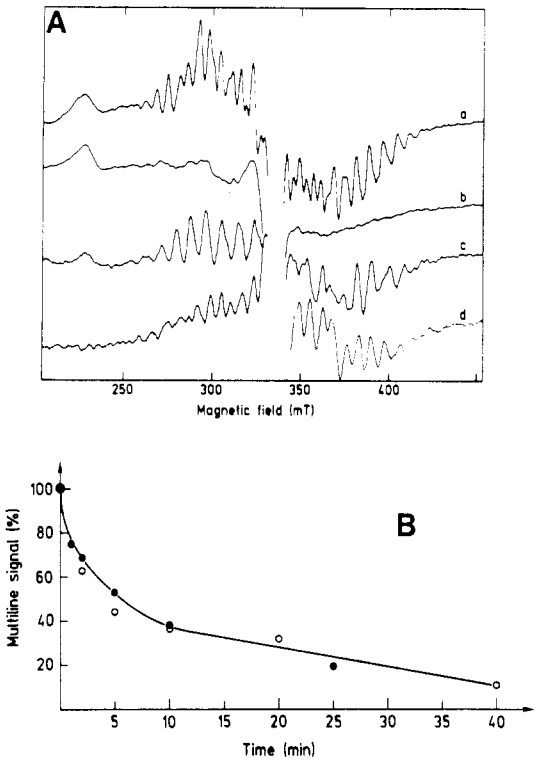

FIGURE 2: EPR spectra from NaCl-treated/polypeptide-reconstituted PS-II membranes and the effect of Ca^{2+} and Sr^{2+} addition. (A) (Spectrum a) NaCl-treated/polypeptide-reconstituted PS-II membranes that had been dark adapted on ice for 60 min; (spectrum b) NaCl-treated/polypeptide-reconstituted PS-II membranes that had been incubated in the dark for 60 min in the presence of 10 mM Ca^{2+}; (spectrum c) after 198 K illumination minus dark spectrum recorded following incubation with Ca^{2+}; (spectrum c) after 198 K illumination minus dark spectrum recorded following incubation with Sr^{2+} for 60 min on ice in the dark. (B) Kinetics of the disappearance of the dark-stabe multiline signal in the dark, on ice, in the presence of 40 mM Ca^{2+} (●) or 40 mM Sr^{2+} (○). Instrument settings: microwave frequency 9.44 GHz; modulation amplitude 16 G; temperature 10 K; microwave power 20 mW. The chlorophyll concentrations (≈6–8 mg/mL) are slightly different in (a)–(d).

S_2 multiline signal is detectable because its decay to S_1 occurs more rapidly than its formation due to slow Ca^{2+}-binding kinetics.

Figure 3A shows that illumination at 0 °C resulted in the almost complete loss of the stable multiline signal (spectrum b). At the same time a large signal appeared in the $g = 2$ region. Figure 3B shows the same experiment recorded by using EPR parameters more appropriate for resolution of the new signal. In parts A and B of Figure 3 spectrum a corresponds to a dark-adapted sample and spectrum b to a sample rapidly frozen after an illumination at 0 °C. The light-induced signal has a near-Gaussian line shape and a width of 164 G. When a dark incubation was given after illumination but prior to the freezing of the sample, the 164 G wide signal disappeared (curve ●, Figure 3C) and the stable multiline signal reappeared (curve ○, Figure 3C). These phenomena occurred with matching kinetics. This result is interpreted as showing that illumination at 0 °C of Ca^{2+}-deficient, polypeptide-reconstituted membranes allows one, and only one, further positive equivalent to be accumulated. The stable multiline signal can be formed by deactivation ($t_{1/2} \approx 4.5$ min) of this new state which is observed as the 164 G wide signal. We have shown previously in salt-washed membranes lacking the 17- and 23-kDa polypeptides that Ca^{2+} depletion results in an electron-transfer block after the formation of the S_3 state. This is consistent with the assignment of the 164 G wide signal as a formal S_3 state and with the stable multiline signal arising from a modified S_2 state. It is of note that illumination of

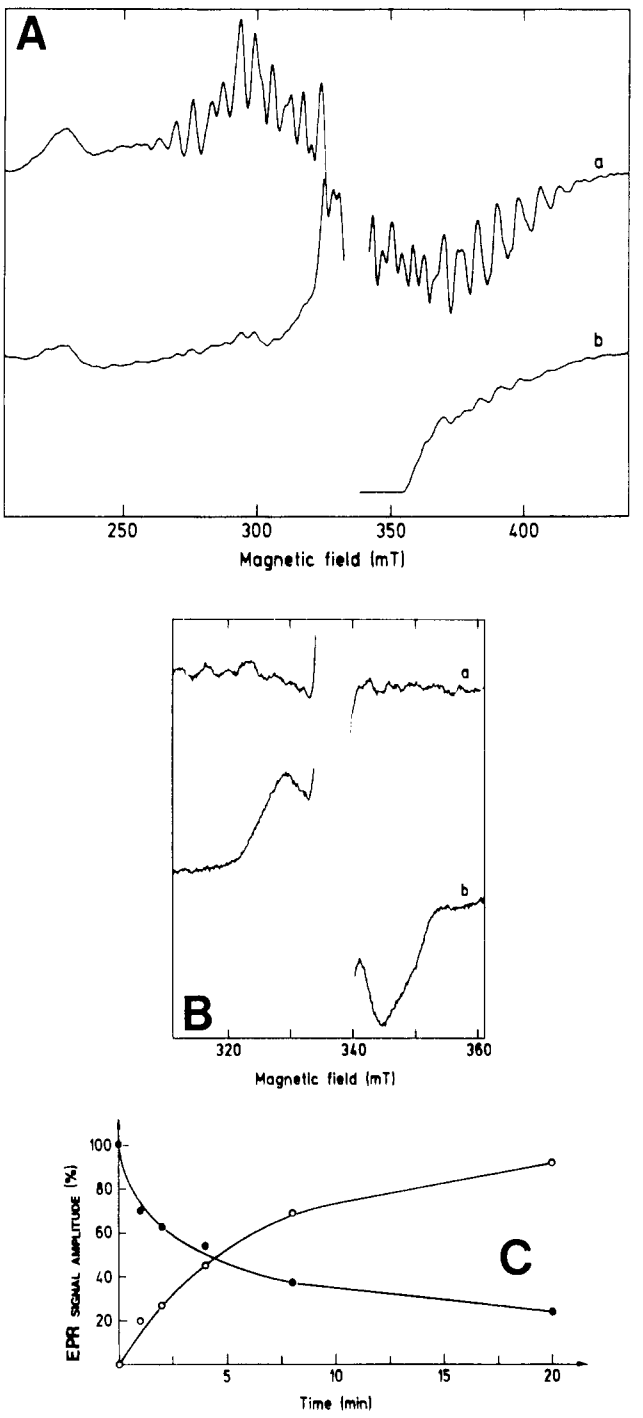

FIGURE 3: EPR spectra from NaCl-treated/polypeptide-reconstituted PS-II membrane and the effect of illumination. (A) (Spectrum a) NaCl-treated/polypeptide-reconstituted PS-II membranes recorded after dark adaptation on ice; (spectrum b) after illumination for 2 min at 0 °C in the presence of PPBQ followed by rapid freezing (≈2 s), in the dark, in a 198 K bath. Instrument settings as in Figure 2. (B) (Spectrum a) NaCl-treated/polypeptide-reconstituted PS-II membranes after dark adaptation on ice (same sample as for spectrum a in part A); (spectrum b) after illumination for 2 min at 0 °C in the presence of PPBQ followed by rapid freezing, in the dark, in a 198 K bath (same sample as for spectrum b in part A). Instrument settings: microwave frequency 9.44 GHz; modulation amplitude 3 G; temperature 10 K; microwave power 2 mW. (C) Kinetics of the disappearance of the 164 G wide signal (spectrum b of part B) (curve ● recorded in the dark, on ice, with the EPR settings of Figure 3B). Kinetics of the reappearance of the stable multiline signal (curve ○ recorded with the EPR settings used in Figure 2).

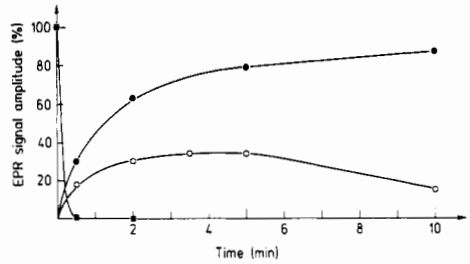

FIGURE 4: Effect of Ca^{2+} addition immediately after illumination of NaCl-treated/polypeptide-reconstituted PS-II membranes monitored by EPR. The samples were illuminated 2 min at 0 °C in the presence of PPBQ. Then 100 mM Ca^{2+} was rapidly added in the dark, and the samples were incubated in the dark, on ice, for various times. Curve ■ shows the kinetics of the disappearance of the 164 G wide signal. Curve O represents the kinetics of the appearance of the normal multiline signal. After the points of curve O were recorded, the samples were submitted to a 198 K illumination, and the total amplitude of the normal multiline was plotted in curve ●. The 100% value for the amplitude of the normal multiline signal is obtained from a sample illuminated at 198 K after incubation with Ca^{2+} for 1 h in the dark at 0 °C. Each signal was recorded with the appropriate instrument settings described in Figures 2 and 3B.

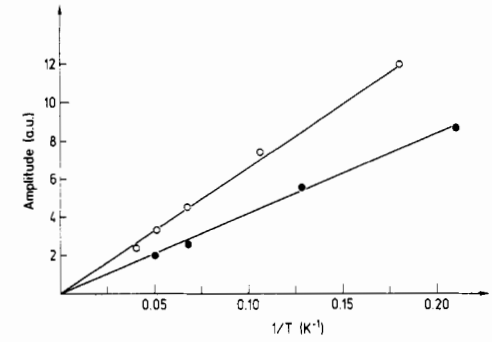

FIGURE 5: Temperature dependence of the amplitude of the EPR signals. (●) Stable multiline signal; (O) 164 G wide signal. The signals were recorded with the appropriate sample and instrument settings as described in Figures 2 and 3B, respectively, except that the microwave power was 20 nW in both cases.

samples at 198 K resulted in only a very small fraction of the 164 G wide signal (Boussac & Rutherford, 1989). Illumination of a sample exhibiting the 164 G wide signal results in no further change in the signal. In both cases the signal arising from $Q_A^-Fe^{2+}$ is photoinduced with an amplitude identical with that seen in control membranes.

Figure 4 shows the amplitude of the EPR signals recorded when Ca^{2+} was rapidly added in the dark after the illumination at 0 °C and then incubated for various times at 0 °C. After the addition of 100 mM Ca^{2+}, the 164 G wide signal totally disappeared during the time of the mixing (curve ■, Figure 4). This kinetic depended strongly on the Ca^{2+} concentration; for example, the half-time was approximately 30–40 s with 50 mM Ca^{2+} (not shown). At the same time the normal multiline appeared but its rise time was much slower than the decay time of the 164 G wide signal and its amplitude reached 30% before it began to decrease with the usual deactivation kinetics (curve O, Figure 4). A 198 K illumination produced an increase in the amplitude of the normal multiline signal. This increase represents the centers in the S_1 state prior to 198 K illumination. In Figure 4, the incubation time dependent changes in the multiline signal observed before and after the 198 K illumination were practically unaffected by variations in the Ca^{2+} concentration (not shown). These results indicate that the Ca^{2+}-induced decay of the 164 G wide signal results in the formation of a state which is not detected by EPR. This state decays in the dark, with kinetics that are Ca^{2+} independent, to form the normal S_2 multiline signal which decays with the usual kinetics. This is a strong indication that the 164 G wide signal arises from a formal S_3 state. We have previously shown that Ca^{2+} is more easily lost (Boussac & Rutherford, 1988b) and rebound (unpublished) in the S_3 state than in the other S states. The rapid binding of Ca^{2+} to the modified S_3 state is thus expected. This then gives rise to normal S_3 state which decays to form the S_2 state. The decay time of the S_3 state appears somewhat faster ($t_{1/2} \approx 1$ min at 0 °C) than seen previously in untreated material ($t_{1/2} = 4$ min at room temperature) (Styring & Rutherford, 1988a), but this is not a serious objection to the assignment since the factors that influence S_3 decay have not been extensively studied at 0 °C.

Some of the spectroscopic properties of the two new EPR signals have been determined. Measurements of the stable multiline signal at different X-band frequencies were performed and the shifts of the hyperfine lines determined as a function of microwave frequency. As observed for the S_2 multiline signal by Hansson et al. (1987), who used a similar method, the stable multiline signal does not show appreciable g anisotropy. In addition, plots of the hyperfine line positions versus frequency yielded straight lines, the slope of which could be fitted with $g = 1.96 \pm 0.02$ to first order. A similar study on the 164 G wide EPR signal yielded $g = 2.004 \pm 0.001$.

It was shown previously that in untreated PS-II membranes the relaxation properties of Tyr_D^+ were influenced by the redox state of the Mn cluster (Styring & Rutherford, 1988b). Here the $P_{1/2}$ of Tyr_D^+ was measured at 15 K in the presence of the stable multiline signal ($P_{1/2} = 0.25$ mW), the 164 G wide signal ($P_{1/2} = 0.25$ mW), and the S_1 state formed after incubation with Ca^{2+} ($P_{1/2} = 0.125$ mW). The ratios of the values of $P_{1/2}$ were similar to those reported earlier for the S_2, S_3, and S_1 states in untreated membranes (Styring & Rutherford, 1988b) and are thus consistent with the assignment of the new signals to S_2 and S_3. The $P_{1/2}$ values of the new signals themselves were also determined. At 5.9 K, the stable multiline signal had a $P_{1/2}$ of 1.8 mW, and the 164 G wide signal had a $P_{1/2}$ of 0.8 mW. The amplitudes of both signals recorded under nonsaturating conditions behave linearly with temperature and follow the Curie law, indicating that both states are ground states (Figure 5).

Discussion

In this work we report two new EPR signals, a stable multiline signal and a 164 G wide signal, which we attribute to a modified S_2 state and a modified S_3 state, respectively. The key observation that favors this assignment is found in Figure 4, which shows the formation of an EPR-invisible intermediate state prior to the appearance of normal S_2 state when Ca^{2+} is added to the state that exhibits the 164 G wide signal. It is most likely that this intermediate state is the normal S_3 state which is formed by rapid binding of Ca^{2+} to a modified S_3 state. The normal S_3 state formed in this way then undergoes its usual decay reactions [see Styring and Rutherford (1988a)].

$$\begin{array}{ccccc} \text{modified } S_3 & \xrightarrow[\text{dark}]{+Ca^{2+}} & \text{normal } S_3 & \xrightarrow{\text{dark}} & \\ \text{164 G wide signal} & & \text{not detected by EPR} & & \\ & & \text{normal } S_2 & \xrightarrow{\text{dark}} & \text{normal } S_1 \\ & & \text{normal multiline} & & \text{not detected by EPR} \end{array}$$

Other models (e.g., if the 164 G wide signal is an abnormal S_2 state) require ad hoc explanations of the EPR-invisible intermediate state.

Having identified the 164 G wide signal as arising from a modified S_3 state, the data in Figure 2, showing complete interconversion of the two new signals without formation of intermediate states, lead directly to the conclusion that the stable multiline signal is a modified S_2 state.

$$\underset{\text{stable mutliline}}{\text{modified } S_2} \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} \underset{\text{164 G wide signal}}{\text{modified } S_3}$$

The modified S_2 state is presumably formed by photooxidation during sample preparation.

There are several less direct lines of evidence that are consistent with these signal assignments. (1) Conclusive evidence has been presented previously that Ca^{2+} depletion in salt-washed materials results in a block in the enzyme cycle after S_3 formation (Boussac et al., 1985a; Boussac & Rutherford, 1988a). According to the signal assignments, inhibition occurs at the same point in the new preparation. (2) The relaxation properties of the Tyr_D^+ free radical measured in the modified S_2 and S_3 states compared with the normal S_1 state are very similar to those reported for the normal S_2 and S_3 states (Styring & Rutherford, 1988b). (3) Faster Ca^{2+} binding occurs in the modified S_3 state than in the modified S_2 state in accordance with our expectations from Ca^{2+} release (Boussac & Rutherford, 1988b) and Sr^{2+}-binding studies (unpublished) done on salt-washed material. (4) The observation that the modified S_2-state to modified S_3-state transition does not occur at 198 K but does occur at 0 °C is consistent with the known temperature dependence of the normal S_2 to S_3 transition (Styring & Rutherford, 1988a).

In previous work, Ca^{2+} depletion in polypeptide depleted PS-II showed a normal S_2 multiline signal. In addition, no unusual stability was noted for the S_2 state monitored by EPR (Boussac & Rutherford, 1988a) and by thermoluminescence (Ono & Inoue, 1986) in PS-II membranes depleted of Ca^{2+} and the 23- and 17-kDa polypeptides. It seems likely that the modified EPR properties and the unusual stability of the S_2 state (probably a negative shift in the E_m of the S_1/S_2 couple) are due to the perturbation of the Mn cluster which is caused by the polypeptide reconstitution treatment. Polypeptide rebinding itself is known to protect the Mn from external reductants (Ghanotakis et al., 1984a); however, there is no evidence indicating that this influences the inherent S_2 lifetime in the absence of added reductants.

If the 23- and 17-kDa polypeptides induce changes in the protein that result in extra ligands to the Ca^{2+} (or even provide direct ligands to the Ca^{2+} itself), it is possible that such changes occurring when the Ca^{2+} site is vacant could result in a perturbation of the Mn cluster. Alternatively, the Ca^{2+} site may be occupied by another ion of a different size (e.g. Na^+; Waggoner et al., 1989) or which does not have the flexibility of ligand geometries characteristic of Ca^{2+}; thus, the extra structural constraints imposed by the binding of the 23-kDa (and/or the 17-kDa) polypeptide could result in distorsions of the Mn cluster. We have shown previously (Boussac & Rutherford, 1988a), and in the present work, that replacement of Ca^{2+} with Sr^{2+} results in a perturbation of the Mn cluster. These observations indicate that the Ca^{2+}-binding site is close to or even an integral part of the functional Mn cluster.

Since the submission of this paper, Ono and Inoue (1989a) reported a stable multiline signal in salt-washed material, lacking the 17- and 23-kDa polypeptides, but which were treated with a high concentration of EDTA in the light. In the current work, the polypeptide-reconstituted material has also encountered high concentrations of chelator (in this case EGTA) and room light. Indeed, during the course of the work reported here we observed that high concentrations were a prerequisite for generation of the new signals. Thus, we consider that EGTA/light effects could be involved in the perturbation of the Mn cluster reported here. A further alternative, then, to explain the phenomena reported here could be that salt washing/Ca^{2+} depletion exposes the Mn cluster to direct liganding by the chelator, the effects of which could be modulated by the structural constraints induced by polypeptide rebinding. The relative influence of the chelator versus the rebinding of the polypeptides must be assessed experimentally in future studies.

The controversial conclusion reached earlier, that Ca^{2+} depletion results in inhibition of the enzyme cycle after S_3 formation (Boussac & Rutherford, 1988a), is in accord with the interpretation of the current work. An important mechanistic role for Ca^{2+}, the O_2 evolving step, is implied. Roles for Ca^{2+} in deprotonation reactions (Boussac & Rutherford, 1988b) and as a water/Cl^- binding site close to the Mn (Rutherford, 1989) have been recently suggested.

Using a citrate treatment, Ono and Inoue (1988) obtained a Ca^{2+}-deficient preparation that retained the 17- and 23-kDa polypeptides. From a recent thermoluminescence study of this preparation, Ono and Inoue (1989b) concluded that the S-state cycle was blocked after S_2 formation, in contrast to the conclusion here and earlier (Boussac et al., 1985a; Boussac & Rutherford, 1988a) that inhibition occurs after S_3 formation. Because a different biochemical procedure was used, it is difficult to judge the significance of this result. However, if the Ca^{2+}-depleted state is comparable to that characterized in the present work, we would predict that a single S-state advancement would occur, i.e., S_2 to S_3, since S_2 is stable in the dark. Thus, the thermoluminescence data would be reconciled with the current interpretation. Alternatively, the citrate treatment may produce a preparation that is similar to the salt-washed material which we have used in our earlier studies [e.g., Boussac and Rutherford 1988a)]. In this case the S_2 state exhibits its usual stability; however, the S_3 state is unusually unstable (Boussac et al., 1985a). This could result in the absence of a room temperature S_3 band in the thermoluminescence study.

The spectral properties of the modified S_2 state should provide information on the structure of the Mn complex in this state. The g value ($g = 1.96$), its isotropic nature, and the approximate width of the signal and that it arises from a spin $^1/_2$ ground state are all properties similar to the normal S_2 multiline signal (Hansson et al., 1987). These characteristics could occur in a mixed-valence Mn dimer, but clusters with higher nuclearity are at least as likely. The increased number of lines seen in the new stable S_2 signal may be relevant to this question; however, further work is required before any conclusion can be drawn.

The chemical origin of the 164 G width signal is unknown. One possibility is that it may arise from an organic free radical interacting magnetically with the Mn complex. The proposed weak magnetic interaction must explain the disappearance of the multiline signal, the unusually large width of the signal of the putative free radical, and the fast relaxation properties exhibited by the signal. In addition, the modified S_3 signal seems to show some poorly resolved wings which might be predicted from such an interaction. This explanation for the origin of the S_3 signal is attractive since almost this exact situation was put forward earlier to explain the apparent lack of Mn oxidation upon S_3 formation (Styring & Rutherford, 1988b; Guiles et al., 1989) despite the loss of the S_2 multiline signal upon S_3 formation.

The origin of the putative organic free radical could be an oxidized amino acid. It has already been demonstrated that tyrosine can be oxidized in PS-II (Barry & Babcock, 1987). Other candidates include oxidized chlorophyll, carotenoid, and quinol. If, however, the new signal represents a true S_3 state, then electronic absorption measurements of S_3 probably rule out chlorophyll, carotenoid, and perhaps quinol oxidation at this step [see Lavergne (1987) and citations therein].

It is also worth considering the possibility that the 164 G wide signal could arise from the Mn cluster itself. However, a satsifactory model has yet to be imagined that can explain (1) how the Mn cluster could give the spectral properties of the signal and (2) how such a state could be produced upon oxidation of S_2 which itself has a spin $^1/_2$ ground state.[2]

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[2] While characterization of the two new EPR signals was in progress, a broad $g = 2$ signal was observed by M. Latimer (University of California—Berkeley) in salt-washed PS-II preparations (personal communication). Since the paper was submitted, Latimer (personal communication), Ono and Inoue (1989b), and Baumgarten et al. (1989) have reported a stable multiline signal in salt-washed and citrate-washed PS-II membranes. The latter group has also observed a 200 G wide EPR signal around $g = 2$. In addition, we have reported some preliminary orientation data on the modified multiline signal which led us to suggest that the modified S_2 signal must arise from a cluster of Mn which is bigger than a dimer (Boussac et al., 1989b).

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