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EPR DETECTION OF A CRYOGENICALLY PHOTOGENERATED INTERMEDIATE IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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In Photosystem II preparations at low temperature we were able to generate and trap an intermediate state between the S_1 and S_2 states of the Kok scheme for photosynthetic oxygen evolution. Illumination of dark-adapted, oxygen-evolving Photosystem II preparations at 140 K produces a 320-G-wide EPR signal centered near $g = 4.1$ when observed at 10 K. This signal is superimposed on a 5-fold larger and somewhat narrower background signal; hence, it is best observed in difference spectra. Warming of illuminated samples to 190 K in the dark results in the disappearance of the light-induced $g = 4.1$ feature and the appearance of the multiline EPR signal associated with the S_2 state. Low-temperature illumination of samples prepared in the S_2 state does not produce the $g = 4.1$ signal. Inhibition of oxygen evolution by incubation of PS II preparations in 0.8 M NaCl buffer or by the addition of 400 μM NH_2OH prevents the formation of the $g = 4.1$ signal. Samples in which oxygen evolution is inhibited by replacement of Cl^- with F^- exhibit the $g = 4.1$ signal when illuminated at 140 K, but subsequent warming to 190 K neither depletes the amplitude of this signal nor produces the multiline signal. The broad signal at $g = 4.1$ is typical for a $S = 5/2$ spin system in a rhombic environment, suggesting the involvement of non-heme Fe in photosynthetic oxygen evolution.

Introduction

The oxygen-evolving complex of plant photosytem II carries out the four-step photooxidation of water to molecular oxygen (reviewed in Refs. 1 and 2). This complex can exist in five oxidation states, denoted S_i ($i = 0,\ldots,4$) [3,4]. Photooxidation of the primary electron donor of PS II, P-680, leads to the extraction of electrons from the oxygen-evolving complex through a chain of electron carriers. The removal of one electron advances the system from the state S_i to S_{i+1}. S_3 is advanced to the unstable state S_4, which releases oxygen and returns to the S_0 state. In dark-adapted samples approx. 75% of the PS II centers are in the S_1 state. Although the identities of some of the components involved in the process of photosynthetic water oxidation are known, the chemical nature of most of the species involved, and hence the mechanism of plant oxygen evolution, remain unsolved.

The transition metal manganese appears to be integral to the oxygen-evolving complex, with the number of Mn involved being perhaps as high as four [5]. Either a 3Mn(III)Mn(IV) tetramer [6] or a Mn(III) Mn(IV) dimer [7] appears to be consistent with the multiline EPR signal associated with the

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Abbrevations: PS, Photosystem; Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; cyt b-559$_{HP}$, cytochrome b-559 (high-potential form).

S_2 state of the oxygen-evolving complex [7–9]. The extent of involvement of other metal ions in water oxidation is unknown. A significant amount of iron (approx. 12 per photosynthetic unit) is typically associated with Triton-extracted, oxygen-evolving PS II preparations (Goodin, D. and Blough, N., personal communication); but, except for cytochrome *b*-559 and the single iron associated with the acceptor [10], this iron is not accounted for in terms of identity or function. Cytochrome *b*-559 is associated with the oxidizing side of PS II, but no precise physiological function has been ascribed to it (see Ref. 11); the reduction potential of the cytochrome (approx. 350 mV) is not high enough to indicate a redox species active in water oxidation.

Among the electron carriers between P-680 and the oxygen-evolving complex, a 16 G wide transient EPR signal centered at $g = 2.005$, denoted as Signal $\mathrm{II_{vf}}$, has been identified [12]. This signal has been attributed to a plastosemiquinone anion associated with a divalent cation [13]. Recent studies led to an alternative assignment to a protonated plastosemiquinone radical cation [14]. In any event, it is likely that Signal $\mathrm{II_{vf}}$ reflects the oxidized direct donor to P-680^+ [15]. There is indirect evidence which is suggestive that more than one intermediate electron carrier is involved between the oxygen-evolving complex and P-680 (see Ref. 2).

Studies at low temperature have proven useful in examining the mechanisms of PS II reactions (reviewed in Refs. 16 and 17). Recently, such studies have focused on the S states [9,18,19]. It has been shown that the threshold temperature for the formation of the S_2 state from S_1 is about 170 K, while the threshold temperature for advancement of S_2 to S_3 is about 220 K [9]. In the present study we have investigated the consequences of illumination at temperatures below the threshold temperatures in an attempt to detect the formation of intermediate species which are kinetically inert at these low temperatures.

Materials and Methods

Photosystem II preparations were obtained by modifications of two Triton X-100 fractionation procedures [20,21]. Broken chloroplasts were prepared from market spinach. Destemmed leaves were ground at 4°C in a Waring blender for 10 s in a medium containing 0.2 M sucrose, 5 mM $MgCl_2$, 15 mM NaCl and 0.05 M Hepes at pH 7.5. The homogenate was strained through three layers of cheesecloth and centrifuged at $5000 \times g$ for 8 min. The pellet was suspended in buffer containing 0.15 M NaCl, 5 mM $MgCl_2$, 5 mM EDTA, and 0.05 M Hepes at pH 7.5. The loose pellet from a 5 s spin at $100 \times g$ was discarded, and the broken chloroplasts were pelleted by a 8 min $5000 \times g$ spin. The pellet was washed once with suspension buffer which contained 15 mM NaCl, 5 mM $MgCl_2$ and 50 mM Mes at pH 6.0 and spun again at $5000 \times g$ for 8 min. The pellet was suspended in the same buffer, with 5 mM sodium ascorbate and 25 : 1 (w : w) Triton X-100 per chlorophyll added. The pellet from a 2 min spin at $1000 \times g$ was discarded before spinning the supernatant for 10 min at $35\,000 \times g$. The pellet from this spin was taken up in the Mes suspension medium and spun again at $35\,000 \times g$ for 10 min after the pellet from a $2000 \times g$ spin was discarded. Control samples were suspended in the same buffer. For hydroxylamine treatment, the suspension buffer contained 400 μM $NH_2OH \cdot HCl$. High-salt treatment was accomplished by suspending samples in buffer containing 0.8 M NaCl at 0°C for 90 min in the dark. These samples were pelleted and washed once with Mes suspension buffer before final suspension. Where indicated, fluoride was substituted for chloride by suspending the PS II samples in buffer containing 20 mM NaF and 50 mM Mes at pH 6.0. These samples were washed twice before final suspension in the same buffer. Where indicated, samples were oxidized with 5 mM $K_3W(CN)_8$ and 5 mM $K_4W(CN)_8$. $K_3W(CN)_8$ was prepared as in Ref. 22, $K_4W(CN)_8$ as in Ref. 23. All samples were suspended at a final concentration of 3–5 mg Chl/ml and contained approx. 50% glycerol in the final suspension medium. Oxygen-evolution rates of 300–500 μmol O_2 per mg Chl per h were obtained when control preparations were assayed in suspension buffer containing 2,5-dichloro-*p*-benzoquinone.

For EPR measurements samples were placed in quartz tubes (3 mm inner diameter) and kept in the dark for 15 min before freezing in liquid nitrogen. Sample handling and measurement were completed within 48 h. Continuous illumination was for 30 s from a 400 W tungsten source. The sample temperature was maintained with a continuous stream of liquid-nitrogen-cooled nitrogen gas or a solid CO_2 methanol bath.

EPR spectra were recorded with a Varian E 109 spectrometer equipped with a Liquid Transfer Heli-Tran Refrigerator (Air Products, Model LTR). Spectra were recorded on a digital oscilloscope (Nicolet Eplorer IIIa) and stored in a signal averager. Signal-averaged spectra were transferred to a VAX 11/780 computer for manipulation and display. Spectra were typically obtained by averaging four or more sweeps of the magnetic field.

Results

Fig. 1. shows the EPR spectra of a PS II preparation recorded at 10 K. The spectrum of the dark-adapted sample (spectrum a) is included as a reference. Spectrum b shows The EPR obtained at 10 K after illumination at 140 K. As reported previously for chloroplasts [9], upon illumination below the threshold temperature of 160 K, we see very little of the multiline signal associated with the S_2 state. The spectrum c is obtained after warming this sample to 190 K for 90 s in the dark. The multiline signal that appears between 2600 and 4000 G was produced by the process of warming the sample above the threshold temperature in the dark, after illumination below the threshold temperature. This result is suggestive that an intermediate between the S_1 and S_2 states was generated and trapped by the illumination at 140 K.

Subtraction of the dark-adapted spectrum from the illuminated sample spectrum facilitates a more careful inspection of the changes produced by illumination and then by warming. Fig. 2a shows the difference spectrum obtained by subtraction of the dark-adapted spectrum from the spectrum of a PS II preparation which was illuminated at 140 K. The prominent features produced by the low temperature illumination were a broad derivative-shaped signal centered at $g = 4.1$, a peak at $g = 3.1$, another derivative-shaped signal at $g = 2.16$, and a negative peak at $g = 1.9$; there was only a small amount of the multiline signal formed. The $g = 3.1$ and $g = 2.16$ features are due to oxidized cytochrome *b*-559 high potential (cyt *b*-559_{HP}) [24]; the negative feature at $g = 1.9$ is possibly due to the reduced PS II acceptor [10]. The light-induced $g = 4.1$ feature has not been previously reported; this feature appeared on top of a 5-fold larger

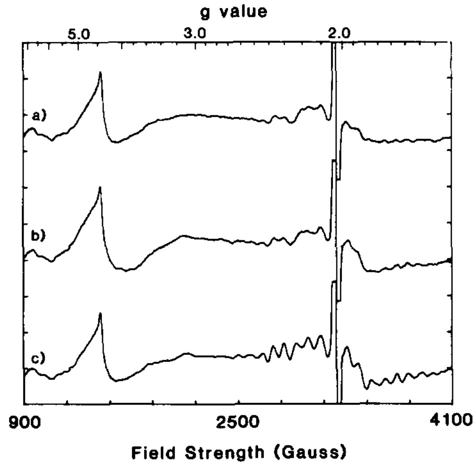


Fig. 1. EPR spectra of a PS II preparation recorded at 10 K. (a) Dark-adapted; (b) illuminated at 140 K for 30 s; (c) warmed to 190 K in the dark for 90 s after illumination at 140 K for 30 s. Spectrometer conditions: microwave power, 50 mW; microwave frequency, 9.2 GHz; field modulation, 32 G; modulation frequency, 100 kHz; sweep rate, 2500 G/min; time constant, 64 ms.

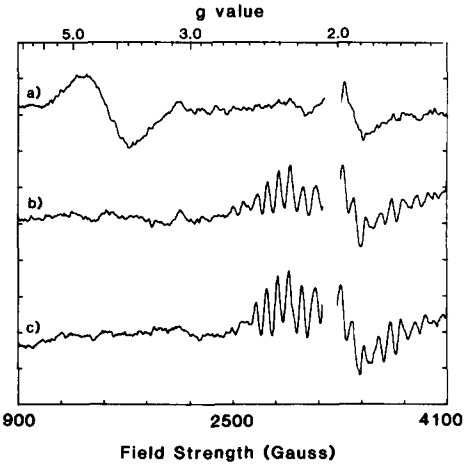


Fig. 2. Effects of illumination temperature and warming on the EPR spectrum of a PS II preparation. Spectra of the dark-adapted samples have been subtracted in each case. (a) Illuminated at 140 K for 30 s; (b) warmed to 190 K in the dark for 90 s after illumination at 140 K; (c) illuminated at 190 K. Spectrometer conditions as in Fig. 1. Spectra are expanded 4-fold relative to Fig. 1.

background signal centered at $g = 4.1$ (see Fig. 1) which made detection of the light-induced signal difficult in the absence of subtraction. Quantitation of the $g = 4.1$ feature by double integration with ferric EDTA as an external standard gives an estimation which is consistent with the involvement of this species in PS II electron transfer.

Fig. 2b shows the difference spectrum obtained by subtraction of the dark-adapted spectrum from the spectrum of the illuminated sample after warming to 190 K in the dark. There are two major changes produced in the spectrum by the warming process. The light-induced $g = 4.1$ feature disappears and, as also shown in Fig. 1c, the multiline signal appears. The features at $g = 3.1$, 2.16 and 1.9 are still evident, although the latter two are somewhat obscured by the presence of the multiline signal. The ability to produce the $g = 4.1$ signal by illumination below the threshold temperature for advancement of S_1 to S_2, and the concommitant disappearance of this signal and rise of the multiline signal upon warming in the dark above the threshold temperature are suggestive that the light-induced $g = 4.1$ signal is due to an intermediate state S_1' between the S_1 and S_2 states. In support of this hypothesis, the difference spectrum in Fig. 2c shows that little or none of the light-induced $g = 4.1$ signal is trapped by illumination at 190 K, which is above the threshold temperature. Illumination at an intermediate temperature such as 170 K produces approx. half the maximum amount of both the $g = 4.1$ and multiline signals (data not shown).

We attempted to observe the analogous behavior for the S_2 to S_3 transition (Fig. 3). For this experiment, cyt *b*-559 was chemically oxidized by the addition of 5 mM $K_3W(CN)_8$ and 5mM $K_4W(CN)_8$ to the PS II preparation to prevent competitive donation. The sample was illuminated at 190 K, then warmed in the dark to 260 K. The illumination placed the oxygen-evolving complex in the S_2 state, and the warming allowed the PS II acceptor to become reoxidized [25]. A second illumination was then given at 190 K, which is below the threshold temperature of 220 K for S_2 to S_3 advancement [9]. The effect of the second illumination on the EPR spectrum is shown in Fig. 3a. No changes were evident in the $g = 4.1$ region of the spectrum of the oxidized sample after the second illumination. There was a small increase in the multiline signal amplitude, which is probably due to regeneration of a small amount of S_2 which had been lost upon warming to 260 K. The multiline signal amplitude did not decrease, because the second illumination was carried out at 190 K, which is below the S_2 to S_3 threshold temperature. The effect on the EPR spectrum of warming the sample in the dark to 260 K after the second illumination is shown in Fig. 3b. No change in the spectrum is produced by the warming process.

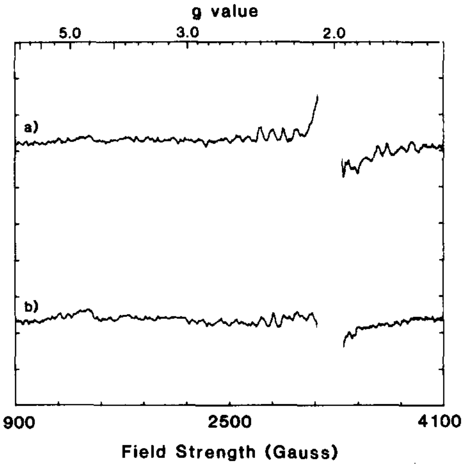


Fig. 3. Illumination and warming of the S_2 state. Samples were prepared initially in S_2 as described in the text. Spectra of samples in S_2 have been subtracted. (a) Illuminated at 190 K for 30 s; (b) warmed to 260 K in the dark for 90 s after illumination at 190 K. Spectrometer conditions as in Fig. 1. Spectra are expanded 4-fold relative to Fig. 1.

To assess further the relation between the light-induced $g = 4.1$ signal and oxygen evolution, the effects of several inhibitory treatments specific for the oxidizing side of PS II were observed. Treatment of PS II preparations with 0.8 M NaCl [26] or addition of 400 μM NH_2OH [27] reversibly decreased the oxygen-evolution rates to less than 30% of control and eliminated the ability to generate the multiline signal by illumination at 190 K (data not shown). The difference spectra in Fig. 4 show that, in samples in which oxygen evolution was inhibited by these two methods, the ability to generate and trap the $g = 4.1$ feature by illumination at 140 K is also lost. The small amount of the $g = 4.1$ feature evident in Fig. 4b may be a consequence of the residual activity in the salt-treated sample.

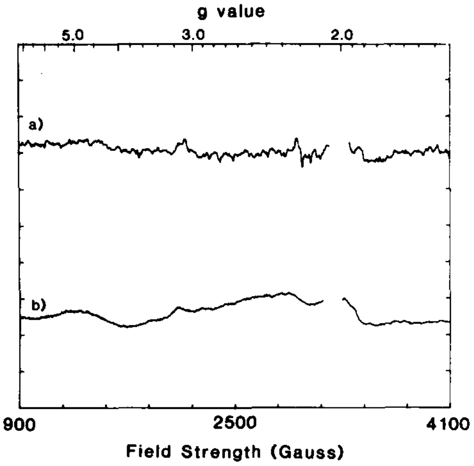


Fig. 4. Effects of inhibitory treatments on the low temperature photochemistry. Illumination at 140 K for 30 s. Spectra of the dark-adapted samples have been subtracted. (a) 400 μM NH_2OH in suspension buffer; (b) incubation with 0.8 M NaCl. Spectrometer conditions as in Fig. 1. Spectra are expanded 4-fold relative to Fig. 1.

A third inhibitory treatment gave different results. Substitution of F^- for Cl^- [28] reversibly inhibits oxygen-evolution rates by about 75%, and prevents photoproduction of the multiline signal at 190 K to same degree. The spectrum in Fig. 5a shows that it is possible to generate and trap the $g = 4.1$ feature by illumination at 140 K of a sample in which F^- was substituted for Cl^-. The $g = 4.1$ signal produced in this case is approx. 10% narrower than that produced in non-inhibited samples. In contrast to the behavior of control samples, approx. two-thirds of the light-induced signal at $g = 4.1$ remains after warming to 190 K. Virtually no multiline signal is produced by warming (Fig. 5b). Illumination of a F^- substituted sample at 190 K produces approximately the same amount of the $g = 4.1$ feature as is produced at 150 K, together with a small amount of multiline signal (data not shown).

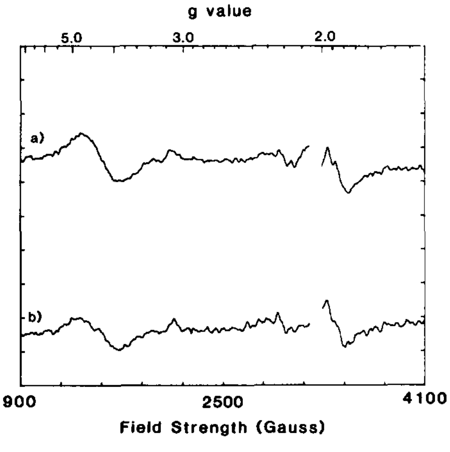


Fig. 5. Effects of substituting F^- for Cl^-. Spectra of the dark-adapted samples have been subtracted. (a) Illuminated at 140 K for 30 s; (b) warmed to 190 K for 90 s in the dark after illumination at 140 K. Spectrometer conditions as in Fig. 1. Spectra are expanded 4-fold relative to Fig. 1.

Discussion

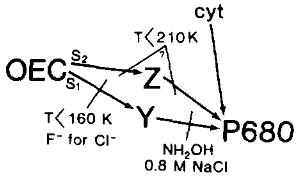
The results shown in Figs. 1 and 2 demonstrate the ability to trap an intermediate state S_1' between the S_1 and S_2 states of the Kok scheme by illumination of dark-adapted PS II preparations below the threshold temperature for advancement of S_1 to S_2. This procedure generated a 320 G wide EPR signal centered at $g = 4.1$ when observed at 10 K. When the intermediate state was permitted to relax in the dark by warming the sample above the threshold temperature for S_1 to S_2 advancement, the light-induced $g = 4.1$ signal disappeared and the multiline signal associated with the S_2 state appeared. Illumination above the threshold temperature produced the multiline signal associated with the S_2 state but not the $g = 4.1$ signal. The light-induced signal at $g = 4.1$ is similar to that which would be produced by a $S = 5/2$ species in a rhombic environment [29], and suggests the involvement of non-heme ferric iron in the photochemistry leading to oxygen evolution. The nature of the advancement of S_1' to S_2 will be discussed further below.

Illumination at 140 K produced several signals in addition to the $g = 4.1$ signal mentioned above. The features at $g = 3.1$ and 2.16 (Fig. 2) are due to cyt *b*-559_{HP} which has been photooxidized. As was the case for the light-induced $g = 4.1$, signal, no cyt *b*-559_{HP} signal was produced by illumination at 190 K. This behavior of the cytochrome agrees with that reported previously for chloroplasts based on optical measurements [30]. Unlike the light-induced $g = 4.1$ feature, the cytochrome signal produced by illumination at 140 K did not decrease in amplitude after warming to 190 K. This observation, aside from reduction potential arguments, rules out the oxidized cytochrome *b*-559 as the molecular species associated with the intermediate state. The signal at $g = 1.9$ which is produced by illumination at 140 or 190 K is possibly due to the reduced acceptor of PS II. The difference in g value, $g = 1.9$ vs. $g = 1.82$, from that reported [10] make this assignment tentative. This signal does not seem to be involved in the relaxation of the intermediate state, because it is not affected by the warming process.

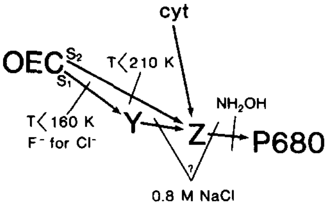
Samples prepared in the S_2 state did not exhibit photogeneration of the $g = 4.1$ signal when given further illumination at 190 K, which is below the S_2 to S_3 advancement threshold temperature. The lack of change in the multiline signal amplitude upon warming to 260 K indicates that warming above the S_2 to S_3 threshold after the illumination did not advance the samples to the S_3 state. It is reasonable to conclude that no intermediate, $g = 4.1$ species or otherwise, was generated in these experiments by illumination of the S_2 state below the threshold temperature.

Low temperature studies and room temperature kinetic measurements of the photochemistry of the S_1 and S_2 states have led to suggestions (see Ref. 2) that the donation path of S_1 to P-680 differs from that of S_2 to P-680. The results of our study support the proposal that there are different donation paths. The intermediate donor which gives rise to the light-induced $g = 4.1$ signal operates when the system is in the S_1 state, but not when it is in the S_2 state.

Two models (Fig. 6) are presented which can account for the data. The species which gives the $g = 4.1$ signal when oxidized is denoted by Y. Based on the g value of this signal the oxidized donor Y^+ is most likely non-heme Fe(III). This species acts as an electron carrier between the state S_1 and P-680. Centers in the S_2 state donate to Z, the Signal II_{vf} species. The models differ only in the location of Z. In the first model, both Y and Z donate directly to P-680 (Scheme 1 in Fig. 6). In the second, Z is the primary donor to P-680 for all S states, and Y lies between the S_1 state and Z (Scheme 2 in Fig. 6). The temperature threshold of 160 K for advancement of S_1 to S_2 is due to the inability of S_1 to reduce Y^+ below this temperature. The 220 K threshold temperature for advancement of S_2 to S_3 could be explained by a freezing out of the electron transfer either from Z to P-680 (Scheme 1) or from S_2 to Z (Scheme 1 or 2). The behavior of the system in the S_0 and S_3 states is not directly addressed by the data in this study. Although previous investigations have paired S_0 with S_1 and S_3 with S_2, further study of the low temperature behavior of these S states will need to be done to incorporate them into the models.



SCHEME 1



SCHEME 2

Fig. 6. Models for the donor side of PS II. OEC, manganese-containing oxygen-evolving complex; cyt, cytochrome *b*-559; P-680, primary donor of Photosystem II.

A reasonable explanation for the fact that the donor Y functions when the oxygen-evolving complex is in the S_1 state but not in the S_2 state is that advancement from S_1' to S_2 involves transfer of a ligand from the iron center Y to the manganese complex.

$$S_1\, L - Y \xrightarrow{h\nu} S_1\, L - Y^+ \rightarrow S_2 - L\, Y \qquad (1)$$

In this postulated, event, the iron center in the S_2 state would differ from the iron center in the S_1 state by the loss of a ligand. The absence of the ligand could easily change the reduction potential of the center and/or the rate at which it donates to P-680 (Scheme 1) or Z (Scheme 2). Such a ligand transfer could involve an inner sphere mechanism for the electron transfer from S_1 to Y^+. If the ligand is an anion, it would provide a counterion for an S state advancement in which no proton is released from the oxygen-evolving complex. Chloride or hydroxide derived from water are likely candidates for a ligand in this situation, but the actual identity is a matter of speculation.

The effects on the behavior of the light-inducible $g = 4.1$ signal of treatments which specifically inhibit oxygen evolution in PS II preparations confirm the involvement of the $g = 4.1$ species in oxygen evolution. The models in Fig. 6 place the effects of the inhibitory treatments relative to the location of the elements in the chain between water and P-680. At concentrations similar to those used in this study hydroxylamine has been shown to inhibit oxygen evolution reversibly by preventing electron transfer between the primary donor P-680 and Z [27], the species which gives the room temperature EPR transient signal II_{vf} and has been suggested to be the immediate donor to P-680. That the light-induced $g = 4.1$ signal could not be generated in the presence of 400 μM NH_2OH (Fig. 4) is consistent with that finding. The total inhibition of the light-induced $g = 4.1$ feature formation by NH_2OH concentrations which leave approx. 30% of control oxygen evolution activity is not necessarily inconsistent; oxygen-evolution measurements monitor the system in the steady state, while low-temperature illuminations are akin to single-flash conditions.

The exact location of the effect of peptide release upon treatment with 0.8 M NaCl is not known, but the inhibition of the formation of the light-induced $g = 4.1$ signal places the effect of this treatment between Y and P-680. A more detailed assignment awaits the determination of the behavior of Z and P-680 after high salt treatment.

The ability to generate the light-induced $g = 4.1$ signal, but not the multiline signal, when chloride was replaced by fluoride places the effect of this treatment between the species Y and the manganese of the oxygen evolving complex; S_1' cannot be advanced to S_2. On the other hand, Muallem et al. [31] have proposed that Cl^- depletion inhibits O_2 evolution by blocking the advancement of S_3 to S_4. The relation between the effects of F^- substitution reported here and the effects of Cl^- depletion are not clear. Perhaps the effects on the oxygen-evolving complex of F^- substitution are different from those of Cl^- depletion. The fact that F^- substitution changes the lineshape of the light-induced $g = 4.1$ signal is suggestive that fluoride inhibits by binding to the species Y. Alternatively, the apparent differences in behavior could be ascribed to different methods of observation. In our experiments, the state S_2 is monitored directly by the presence of the multiline EPR signal. It is conceivable that Cl^- depletion and F^- substitution change the nature of the S-states in such a way that electrons can be removed from PS II, but the chemical nature of the stored oxidizing equivalents is different from those in Cl^--sufficient preparations; these modified S-states would be incapable of fully oxidizing water to oxygen. Indeed, evidence has been presented that the S states in Cl^--depleted chloroplasts are unusual; the deactivations of the S_2 and S_3 states are considerably slower in Cl^--depleted chloroplasts [31], and it has been shown that the ability to generate the multiline signal associated with the S_2 state is lost in Cl^--depleted chloroplasts (Yachandra, V., personal communication). Further investigations of F^--substituted and Cl^--depleted preparations are necessary to resolve fully the nature of the halide effect in oxygen evolution.

Acknowledgements

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