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Effects of azide on the S_2 state EPR signals from Photosystem II

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Abstract

The anion azide, N_3^-, has been previously found to be an inhibitor of oxygen evolution by Photosystem II (PS II) of higher plants. With respect to chloride activation, azide acts primarily as a competitive inhibitor but uncompetitive inhibition also occurs [Haddy A, Hatchell JA, Kimel RA and Thomas R (1999) Biochemistry 38: 6104–6110]. In this study, the effects of azide on PS II-enriched thylakoid membranes were characterized by electron paramagnetic resonance (EPR) spectroscopy. Azide showed two distinguishable effects on the S_2 state EPR signals. In the presence of chloride, which prevented competitive binding, azide suppressed the formation of the multiline and g = 4.1 signals concurrently, indicating that the normal S_2 state was not reached. Signal suppression showed an azide concentration dependence that correlated with the fraction of PS II centers calculated to bind azide at the uncompetitive site, based on the previously determined inhibition constant. No evidence was found for an effect of azide on the Fe(II)Q_A^- signals at the concentrations used. This result is consistent with placement of the uncompetitive site on the donor side of PS II as suggested in the previous study. In chloride-depleted PS II-enriched membranes azide and fluoride showed similar effects on the S_2 state EPR signals, including a notable increase and narrowing of the g = 4.1 signal. Comparable effects of other anions have been described previously and apparently take place through the chloride-competitive site. The two azide binding sites described here correlate with the results of other studies of Lewis base inhibitors.

Abbreviations: EPR – electron paramagnetic resonance; OEC – oxygen evolving complex; PPBQ – phenyl-*p*-benzoquinone; PS II – Photosystem II; S_0 through S_4 – oxidation states of the OEC according to the Kok model

Introduction

Oxygen is formed within Photosystem II (PS II) of higher plants at the oxygen evolving complex (OEC) which includes a cluster of manganese ions (Debus 1992; Rutherford et al. 1992; Britt 1996). The catalytic cycle of oxygen production proceeds through a series of oxidation states described in the model of Kok as states S_0 through S_4. Progression through these states involves a single oxidation step per charge separation after light absorption at the reaction center of Photosystem II. Oxygen is evolved upon reaching the highest oxidation state S_4, which is unstable, and the system returns to the lowest oxidation state S_0 to begin the next cycle. Chloride and calcium are recognized as important cofactors of the OEC (Critchley 1985; Coleman 1990).

Numerous studies have investigated the relationship between anion function and the electron paramagnetic resonance (EPR) signals from the OEC. In general chloride is required for normal formation of the signals associated with the manganese cluster in the S_2 state, which include a multiline signal centered at g = 2 and a broad signal centered at g = 4.1. At one

extreme, Br^- functions well in the place of Cl^- both for O_2 evolution activity and S_2 state signal formation (Yachandra et al. 1986; Ono et al. 1987; Lindberg et al. 1993; Lindberg and Andréasson 1996). At the other extreme, substitution with fluoride and other anions in the medium results in poor O_2 evolution activity, suppression of the multiline signal, and an enhancement and narrowing of the signal at $g = 4.1$ (Yachandra et al. 1986; Ono et al. 1987; Haddy et al. 1992; DeRose et al. 1995; Lindberg and Andréasson 1996). Thorough chloride-depletion alone has effects similar to those found in the latter anion substitution studies (Lindberg and Andréasson 1996). In one study, suppression of the multiline signal in chloride-depleted PS II-enriched membranes was reversed by addition of chloride in the dark after flash treatment (Ono et al. 1986), indicating that oxidizing equivalents had been stored even though the multiline signal had not formed.

Previous studies have shown azide to be an inhibitor of oxygen evolution activity. When PS II undergoes catalytic turnovers in the presence of azide, irreversible inhibition of activity results, probably through production of the azidyl radical (Katoh 1972; Kawamoto et al. 1995). Other anion inhibitors of O_2 evolution, most notably I^-, may have similar damaging effects on PS II (Wincencjusz et al. 1999). Azide has been recently shown to inhibit oxygen evolution activity by acting primarily as a competitor of chloride (Haddy et al. 1999), with competitive inhibition constant of about 0.6 mM. In addition an uncompetitive inhibition constant of 11 mM was found, which represents binding to a site not involved in chloride activation.

In this study we have investigated the effects of azide on the EPR signals from PS II, with particular attention to the S_2 state EPR signals. We have found that azide prevented normal progression of the OEC to the S_2 state in two distinct ways. In the presence of chloride azide suppressed the formation of both S_2 state EPR signals, whereas in chloride-depleted PS II membranes azide caused an alteration of the signals similar to that caused by fluoride. These results correlate with the presence of two types of binding sites for azide.

Materials and methods

Preparation and characterization of PS II-enriched thylakoid membranes

PS II-enriched thylakoid membranes were prepared from market spinach by extraction with Triton X-100 as described by Berthold et al. (1981) with modifications (Franzén et al. 1985). Preparations were stored in a final suspension buffer of 20 mM Mes-NaOH (pH 6.3), 0.4 M sucrose, 15 mM NaCl, and 5 mM $MgCl_2$ in a liquid N_2 storage dewar at a concentration of 5–10 mg Chl ml^{-1}.

O_2 evolution activity was measured as described previously (Haddy et al. 1999) at 25 °C using a Clark-type O_2 electrode (Yellow Springs Instruments, model 5331) and phenyl-*p*-benzoquinone (PPBQ) (Eastman Kodak Chemicals) as electron acceptor. O_2 evolution rates of control samples in the final suspension buffer were typically 300–400 μmol O_2 mg Chl^{-1} hr^{-1} using unpurified PPBQ or 400–500 μmol O_2 mg Chl^{-1} hr^{-1} using PPBQ that had been recrystallized from ethanol.

Treatment with azide in the presence of chloride

PS II-enriched membranes were treated with azide under average room light by suspending to about 0.5 mg Chl ml^{-1} in cold (4 °C) final suspension buffer containing 25 mM Cl^- (described above) and then adding the indicated concentrations of NaN_3 from a stock solution. After incubating on ice for 30 minutes, the membranes were centrifuged at 48,000 $\times$ *g* for 7–10 min. Pellets were resuspended in the same medium, including both azide and 25 mM Cl^-, to 5–10 mg Chl ml^{-1} and transferred to 4 mm outer diameter, clear fused quartz EPR tubes (Wilmad Glass). EPR samples were routinely prepared with 3.0 cm height of sample in the tubes. EPR samples were dark adapted on ice for 3–4 hours before freezing in liquid N_2. A portion of each sample was reserved for concentration measurement and O_2 evolution assay in the presence of the same concentration of azide as used for treatment.

For experiments on the reversibility of signal suppression and O_2 evolution activity, PS II-enriched membranes were incubated in the final suspension buffer (with 25 mM Cl^-) and the indicated concentration of NaN_3 as described above. After centrifuging, the pellets were resuspended in the same buffer either with azide (for azide treatment) or without azide (for removal of azide). Membranes were centrifuged and the wash procedure was repeated. Control membranes were prepared in parallel using the final suspension buffer containing 25 mM Cl^- but no NaN_3. EPR samples were prepared as described above.

Chloride depletion/anion repletion

A low chloride buffer containing 20 mM Mes-NaOH (pH 6.3), 0.40 M sucrose, and 5 mM $CaSO_4$ was used for Cl^- depletion. We estimate that the concentration of chloride was $\leq$80 μM based on the quoted purity of the reagents. PS II-enriched membranes were suspended in the buffer to a concentration of about 0.5 mg Chl ml^{-1} and centrifuged to pellet. This procedure was repeated twice to ensure complete replacement of the buffer medium. The membranes were then resuspended to a concentration of 1.3 mg Chl ml^{-1} and placed in dialysis tubing (Sigma Chemical, 12 kD cutoff). The membranes were dialyzed against the low chloride buffer at 4 °C for 21–22 hours in the dark. These PS II membranes will be referred to as Cl^--depleted. For addition of anions, the membranes were split up and diluted to about 0.3 mg Chl ml^{-1} in the low chloride buffer. To each part was made either no addition or NaCl, NaN_3, or NaF was added from a stock solution to a final concentration of 20 mM. After incubating on ice in the dark for about 30 min, the membranes were centrifuged to pellet and resuspended in the same medium (containing either 20 mM Cl^-, 20 mM $N_3{}^-$, 20 mM F^-, or no anion) to 9.5–10.5 mg Chl ml^{-1}. EPR samples were prepared and dark-adapted as above using precision, clear fused quartz EPR tubes (Wilmad Glass).

Time course of S_2 state signal formation

For the experiments involving the dependence of S_2 state signal formation on illumination time, PS II-enriched membranes (8–9 mg Chl ml^{-1} each) were prepared in the final suspension buffer (with 25 mM Cl^-) in the presence or absence of 10 mM NaN_3 in 4 mm outer diameter, precision bore, clear fused quartz EPR tubes (Wilmad Glass) as described above. EPR samples were illuminated repeatedly at 195 K using the illumination system described below and spectra were taken after each illumination. For data analysis, signals from both control and azide-containing samples were first corrected for sample concentration, then normalized to the maximum signal intensity of the control samples. For these experiments, error bars for signal heights were estimated from the noise in the EPR spectrum. Non-linear regression analyses of the data were performed using SigmaPlot, version 4.0 (SPSS Incorporated), using a function that describes first order kinetics of signal formation: $I = I_{max}\ (1 - e^{-kt})$, where I is signal intensity at time t, I_{max} is the maximum signal formed, and k is the first order kinetic rate constant.

EPR spectroscopy

EPR spectroscopy was performed using a Bruker Instruments EMX 6/1 EPR spectrometer operating at 9.5 GHz. Temperature was controlled with an Oxford Instruments ESR 900 cryostat. Conversion of spectra from Bruker binary to ASCII format was accomplished using EWPlot (Scientific Software Services) written by Philip D. Morse of Illinois State University.

The S_2 state was achieved by illuminating EPR samples in a dry ice/methanol bath (195 K) for 6 min using a Dolan-Jenner model 180 Fiber-lite illumination system similar to that described for O_2 evolution assays. The dual light beams were each passed through about 6 cm of 5 mM $CuCl_2$ solution in cylindrical glass containers before reaching the sample. Difference spectra were produced by subtracting a spectrum of the dark-adapted sample from that of the sample after illumination. Signals from the S_2 state were observed at 9.5–10 K using EPR settings that included microwave power of 20 mW, modulation frequency of 100 kHz, and modulation amplitude of 2.0 mT.

EPR signals from the Fe(II)Q_A center were observed after illumination in a liquid nitrogen bath for 10 min using the illumination system described above. Observations of the signals were made at 4 K, using EPR settings that included microwave power of 32 mW, modulation frequency of 100 kHz, and modulation amplitude of 2.0 mT.

The intensities of signals, except for the g = 4.1 signal in Cl^--depleted/anion-repleted PS II membranes, were assessed by measuring the peak-to-trough heights of the signals in baseline-corrected spectra. The use of signal height as a measure of relative intensity is valid as long as the shapes of the signals do not change. For the multiline signal the heights of the second, third, and fourth main peaks from the left of the signal center were averaged. Except where otherwise noted, error bars for the signal heights were estimated by propagation of the errors estimated in EPR spectral noise, tube volume, and sample concentration. The intensity of the g = 4.1 signals from PS II membranes that had been Cl^- depleted and anion repleted were assessed by double integration using the Bruker Instruments Win-EPR software.

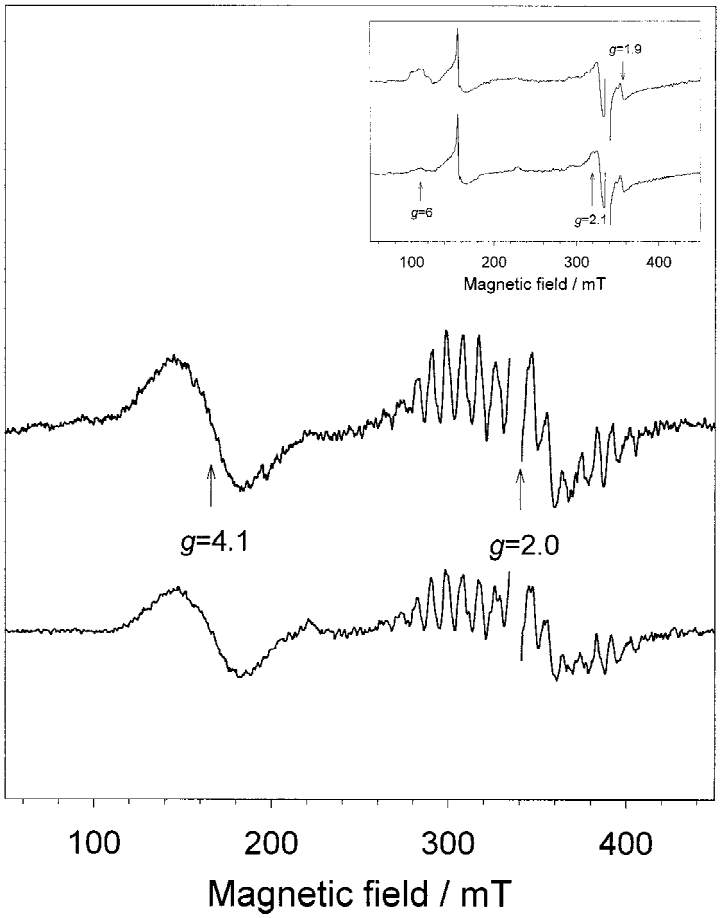

Figure 1. Effect of azide on the S_2 state EPR signals of PS II-enriched thylakoid membranes: top, control; bottom, 8 mM NaN_3. Samples were prepared in buffer containing 25 mM Cl^- and EPR spectroscopy was carried out as described in 'Materials and methods'. Signal heights were rescaled to correct for differences in chlorophyll concentrations. Each spectrum shown is a difference spectrum made by subtracting the spectrum of the dark-adapted sample from that of the sample after illumination. EPR instrument settings included: microwave frequency, 9.48 GHz; microwave power, 20 mW; modulation amplitude, 2.0 mT, and temperature, 10 K. *Inset*: Effect of azide on the EPR spectrum of dark-adapted PS II-enriched thylakoid membranes: top, control; bottom, 8 mM NaN_3. The spectra presented correspond to the dark-adapted spectra used for the difference spectra of Figure 1. A signal at g = 2.05 arose from the quartz of the EPR tube and cryostat insert.

Results

Effects of azide on the S_2 state EPR signals in the presence of chloride

The effects of NaN_3 on the multiline and g = 4.1 EPR signals from the S_2 state of PS II-enriched thylakoid membranes were examined. When millimolar concentrations of NaN_3 were added to the final suspension buffer, which contained 25 mM chloride, the S_2 state signals were suppressed. For example, treatment with 8 mM NaN_3 resulted in a decrease of both the multiline signal at g = 2.0 and the signal at g = 4.1 by about 45% relative to the control (Figure 1). No changes in signal appearance or line width relative to the control were evident.

The effects of azide on the EPR spectra of dark-adapted PS II samples were also examined, since azide is known to ligate to metals such as iron. In dark-adapted samples, the presence of millimolar concentrations of NaN_3 suppressed the signals from axial and near-axial high spin Fe(III) around g = 6 (Figure 1, inset). These resonances, which are dark stable, are probably due to oxidized cytochromes or other heme proteins or possibly to oxidized non-heme iron on the acceptor side of PS II. High spin heme may be converted to the low spin form by azide, as is observed for N_3^--myoglobin which shows principle g-factors

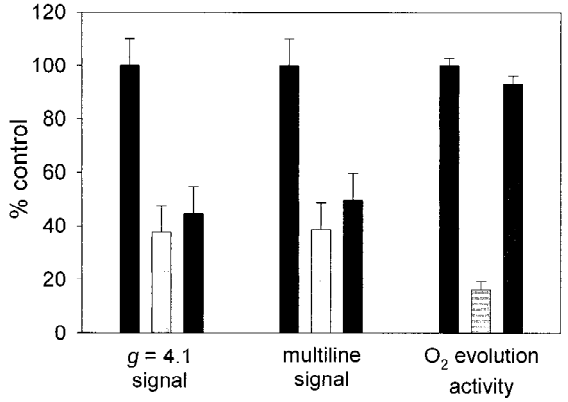

Figure 2. Effect of azide removal on the S_2 state signals and O_2 evolution activity: black, control; light gray, 15 mM NaN_3; dark gray, washed in N_3^--free buffer after treatment with 15 mM NaN_3. Samples were prepared in the presence of 25 mM Cl^- and EPR difference spectra were taken as described in Materials and methods. O_2 evolution activity was measured using the same samples as were used for EPR spectroscopy.

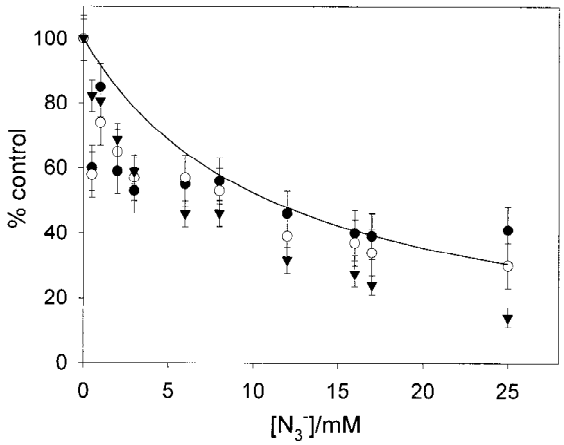

Figure 3. Azide concentration dependence of the intensities of the S_2 state EPR signals and O_2 evolution activity: ●, g = 4.1 signal; ○, multiline signal; and ▼, O_2 evolution activity. The solid line shows the calculated percentage of PS II centers without azide bound to the uncompetitive site, based on $K_i' = 11$ mM. Sample preparation and EPR spectroscopy were carried out as described in Figure 1. The signal heights and O_2 evolution activities were plotted relative to those in the absence of NaN_3.

of 2.77, 2.22 and 1.73. This likelihood is supported by the appearance of a signal at about $g = 2.12$ after azide treatment.

Recovery of the OEC after removal of azide

When PS II-enriched membranes were treated with azide in the presence of 25 mM Cl^- as described above and then washed with azide-free buffer, nearly complete recovery of O_2 evolution activity and partial recovery of the ability to form the S_2 state signals was found. A typical result is shown in Figure 2, in which a sample treated with 15 mM NaN_3 was subsequently washed with N_3^--free buffer. In the presence of 15 mM NaN_3, the S_2 state $g = 4.1$ and multiline signals were reduced to 38% and 39% of the control signals, respectively. Parallel samples that were washed with N_3^--free buffer after azide treatment showed $g = 4.1$ and multiline signals that were still only 45% and 50% of the control signals, respectively. O_2 evolution activity of the same samples was assayed. The sample treated with 15 mM NaN_3 showed 16% of the control activity (in the presence of 15 mM NaN_3), while the sample that had been washed after azide treatment showed 93% of the control activity.

Similar experiments were performed on several occasions, using various concentrations of azide. The S_2 state signals were found to partially recover to variable degrees, which did not appear to depend on the concentration of azide used. However the recovery of the O_2 evolution activity was always at least 90% and usually close to 100%. The recovery of O_2 evolution activity indicates that the effect of azide on the S_2 state signals was not due to the loss of manganese from the OEC.

Azide concentration dependence of S_2 state signal intensity and O_2 evolution activity

The dependence of the S_2 state signal intensity on azide concentration was examined. A series of PS II-enriched membrane samples was treated with concentrations of NaN_3 ranging from 0.5 to 25 mM in the presence of 25 mM Cl^- and the EPR signals from the S_2 state were examined (Figure 3). The intensities of both S_2 state signals decreased with increasing NaN_3 concentration, showing the same dependence within experimental error. The concentration dependence of the signal intensity appeared to be biphasic with a pronounced decrease at lower concentrations of N_3^- and a more gradual decrease at the higher concentrations.

The O_2 evolution activity of each sample prepared for EPR analysis was assayed, using the same azide concentration as was used for sample preparation. The O_2 evolution activity showed an azide concentration dependence which, when analyzed assuming a simple binding model, was characterized by 50% inhibition at an azide concentration, $[N_3^-]_{50\%}$, of 4.5 mM. In a previous study using samples that had not been pre-treated with azide, O_2 evolution activity of PS II

membranes fell to half of the control activity when $[N_3^-]$ = 7.8 mM using the same buffer conditions (Haddy et al. 1999). The similarity of these values indicates that the majority of the effects of azide occurred within minutes of exposure to azide, although some further inhibitory effects probably occurred during the longer time period used for preparation of the EPR samples.

The EPR signal intensities were compared with the fraction of PS II centers expected to bind azide to the site that is uncompetitive with chloride, based on the inhibition constant $K_i' = 11$ mM (Haddy et al. 1999). The effects of azide at the site that is competitive with Cl^- are expected to be only minor under the conditions of high Cl^- concentration. The O_2 evolution activity decreased with increasing N_3^- concentration more markedly than the intensity of the S_2 state EPR signals, particularly at the higher N_3^- concentrations. This suggests that the decrease in O_2 evolution activity was mostly but not completely accounted for by the inhibition of azide at the uncompetitive site. With the exception of some data points taken at low N_3^- concentration, the correlation between the S_2 state signal intensities and the fraction of centers free from azide at the uncompetitive site was within experimental error (Figure 3).

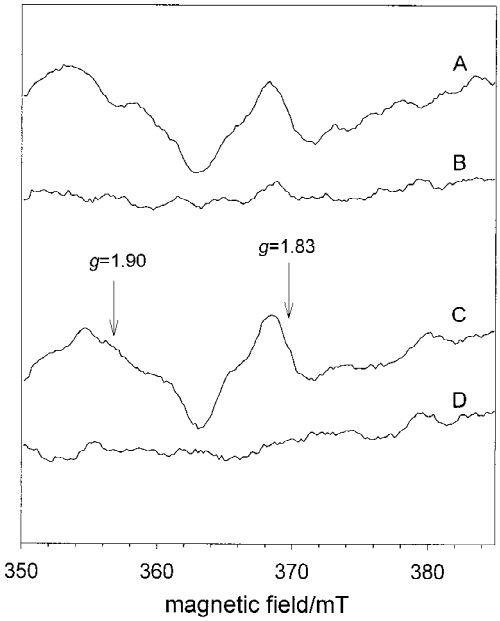

Figure 4. Formation of EPR signals from the Fe(II)Q_A^- site: A, control PS II-enriched membranes, after illumination at 77 K and, B, before illumination; C, PS II-enriched membranes treated with 17 mM NaN_3, after illumination and, D, before illumination. Sample preparation in the presence of 25 mM Cl^- and EPR spectroscopy were carried out as described in 'Materials and methods'.

Effect of azide on signals from Fe(II)Q_A^-

Signals from the reduced Fe(II)Q_A^- center on the acceptor side of PS II were observed after illumination of PS II-enriched membranes at 77 K. Two EPR signals are associated with this center, one appearing at g = 1.90 and the other at g = 1.83. These signals are optimally observed at higher microwave power and lower temperature (4 K) than the S_2 state signals and these conditions also serve to suppress the Rieske center signal at g = 1.90. The Fe(II)Q_A^- signal at g = 1.90 is more evident at higher pH and is identified with ligation of bicarbonate to Fe(II). The signal at g = 1.83 is more evident at lower pH and is identified with the absence of bicarbonate ligated to Fe(II) (Rutherford and Zimmermann 1984). If azide bound to the Fe(II)Q_A^- site, the signal at g = 1.83 may increase in intensity, as has been observed for formate, due to displacement of bicarbonate (Vermass and Rutherford 1984; Diner and Petrouleas 1990). Alternatively, if electron transfer from the reaction center to the quinone acceptor were prevented by azide, the signals would decrease in intensity.

In Figure 1 (and Figure 6), the spectrum in the field region above g = 2 appeared to show a decrease in intensity in the presence of azide that may have arisen from a decrease in the Fe(II)Q_A^- signals. However, closer examination under more optimal conditions indicated that treatment of PS II-enriched membranes with up to 25 mM N_3^- in the presence of Cl^- resulted in no observable difference in the signals at g = 1.83 and g = 1.90 (Figure 4). These signals were examined in several separate experiments without observation of a clear change due to azide in the concentration range for which effects on the uncompetitive site were expected. One complicating factor in these spectra (and in the spectra of the S_2 state as well) was the appearance of a light-induced peak at g = 1.87 with the characteristics of a g_x turning point, just between the two Fe(II)Q_A^- signals. The peak at g = 1.87 was probably associated with a light-induced signal at g = 1.94, which was observed in all spectra after illumination, and a sharp peak at g = 2.05, which became apparent when the multiline signal was suppressed (as in Figure 6). These g-factors correlate with those of the iron-sulfur center F_A (and F_B) of Photosystem I, which must be present in small amounts. In the S_2

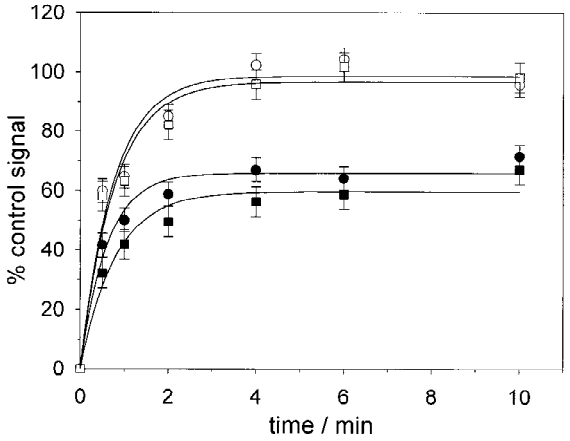

Figure 5. Time course of S_2 state signal formation for control PS II-enriched membranes: □, multiline signal; ○, g = 4.1 signal; and for the sample containing 10 mM NaN_3: ■, multiline signal; ●, g = 4.1 signal. EPR samples prepared with 25 mM Cl^- were repeatedly illuminated at 195 K for the periods shown to a total of ten minutes. The data for each signal are presented relative to the maximum signal intensity found in the absence of NaN_3. The lines represent fits found by regression analysis based on pseudo-first order kinetics of signal formation as described in Materials and methods. EPR instrument settings were as given in the legend of Figure 1.

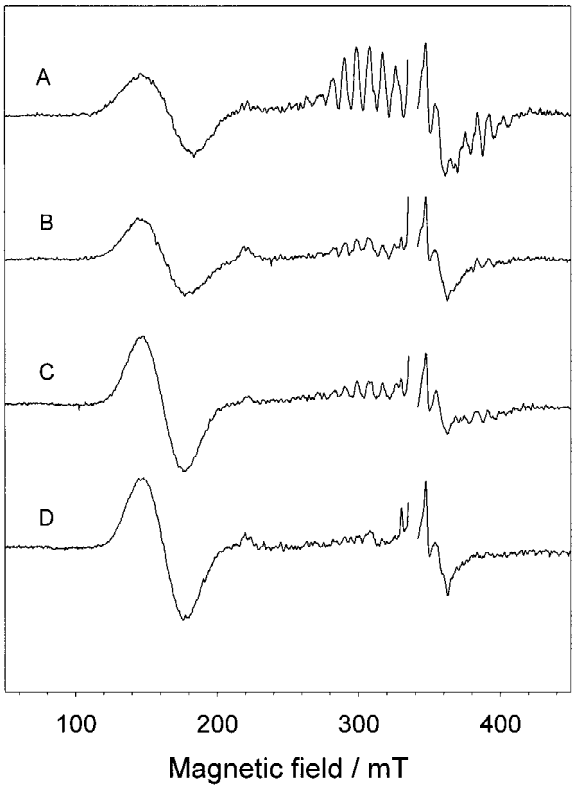

Figure 6. The S_2 state EPR signals in PS II-enriched membranes after Cl^- depletion and addition of: A, 20 mM NaCl; B, no addition; C, 20 mM NaN_3; or D, 20 mM NaF. Chloride depletion was carried out in a buffer containing 20 mM Mes-NaOH (pH 6.3), 0.40 M sucrose, and 5 mM $CaSO_4$ as described in 'Materials and methods'. Signal heights were rescaled to correct for differences in chlorophyll concentrations. EPR instrument settings were as given in the legend of Figure 1.

state spectra, the presence of these signals as well as the multiline signal complicates an evaluation of the $Fe(II)Q_A^-$ signals. Under the more optimal conditions of Figure 4, the multiline signal is absent and the relative interference of the signal at g = 1.87 is decreased. Here the signals from $Fe(II)Q_A^-$ are easier to evaluate, although the signal at g = 1.87 may have masked small changes in them.

Time course of S_2 state signal formation

The dependence of S_2 state signal formation on illumination time was examined, to ensure that the reduction in signal height in the presence of azide was not due to a slowing of signal formation. A decrease in the rate of S_2 state signal formation in the presence of azide would indicate a change in the activation energy barrier for progression to the S_2 state. EPR samples in the presence and absence of 10 mM NaN_3 (both containing 25 mM Cl^-) were illuminated repeatedly at 195 K for brief periods totaling ten minutes and the EPR signals were observed after each illumination (Figure 5).

The time course of signal formation was analyzed assuming a pseudo-first order dependence on PS II concentration. This assumption is relevant under saturating light intensity. The half-time for signal formation in the control PS II sample was found to be 0.52–0.53 min for both the multiline and g = 4.1 signals. In the presence of azide, the half-time for multiline signal formation was about 0.55 min, while the half-time for g = 4.1 signal formation was about 0.42 min. The rate of g = 4.1 signal formation appeared to be somewhat faster in the presence of azide, however this half-time may not be significantly different from that of the others since the experimental error was estimated to be $\pm$0.1 min in each case. In general, the decrease in S_2 state signal intensity in the presence of azide cannot be attributed to a slowing of signal formation.

Chloride depletion and anion repletion

The effects of azide on the S_2 state signals in the absence of chloride were investigated and compared with the effects of fluoride. PS II-enriched membranes were chloride-depleted by dialysis in low chloride ($\leq$80

Table 1. Effects of N_3^- and F^- on the S_2-state EPR signals and O_2 evolution activity of Cl^--depleted PS II-enriched membranes. PS II-enriched membranes were chloride depleted as described in the legend of Figure 6 and the indicated anion was added in the form of the sodium salt. All values are given as percentages of the Cl^--containing control sample. Signal heights and intensities were averaged from either three (for Cl^--depleted and Cl^--containing samples) or two (for N_3^--containing and F^--containing samples) separate spectra. O_2 evolution activity was assayed as described in 'Materials and methods' in the buffer used for Cl^--depletion, but with the addition of 20 mM of the indicated anion. O_2 evolution activity of the Cl^--containing sample was 390 μmol O_2 mg Chl^{-1} hr^{-1}. Activities given are the averages of four separate determinations. Errors were estimated to be ±5% for multiline signal height and O_2 evolution activity and ±10% for the integrated g = 4.1 signal intensity

Anion added	Multiline signal height[a]	g = 4.1 signal intensity	O_2 evolution activity (μmol O_2 mg Chl^{-1} hr^{-1})
20 mM Cl^-	100%	100%	100%
None	26	92	14
20 mM N_3^-	32	132	<2
20 mM F^-	16	119	22

[a] The height given was the average of the second, third, and fourth peaks from the left of center of the spectrum.

μM) buffer containing 5 mM $CaSO_4$ for 21–22 h. These were either left in the Cl^--depleted state or NaCl, NaN_3, or NaF was added to a final concentration of 20 mM. Cl^--depleted PS II membranes to which Cl^- had been added showed normal formation of the S_2-state signals (Figure 6A). Very little formation of the S_2-state multiline signal was observed in Cl^--depleted PS II membranes (Figure 6B), and addition of either N_3^- or F^- resulted in little further change (Figure 6C and D), except for more complete suppression in the presence of F^-. However, repletion with either N_3^- or F^- led to alteration of the g = 4.1 signal relative to both the Cl^--containing and Cl^--depleted PS II membranes, seen as a concurrent narrowing and increase in signal height. The integrated intensities of the g = 4.1 signals from F^--containing and N_3^--containing PS II membranes increased by about 19% and 32%, respectively, relative to the Cl^--containing control (Table 1). These results indicate that N_3^- affected the S_2 state signals from Cl^--depleted PS II membranes in a manner similar to F^-. O_2 evolution assays of the same PS II samples (in the presence of the inhibitory anion) revealed about 14% and 22% activity for the Cl^--depleted and F^--containing samples, respectively, whereas the N_3^--containing sample showed essentially no activity at all. This latter observation is undoubtably accounted for by the previously reported irreversible inhibition of PS II that occurs when catalytic turnovers take place in the presence of N_3^- (Kawamoto et al. 1995).

Discussion

We have investigated the effects of azide on the S_2 state EPR signals from PS II-enriched thylakoid membranes under two different conditions: in the presence of 25 mM Cl^- and after Cl^- depletion. In a previous study of N_3^- inhibition of O_2 evolution two types of sites were found, one that was competitive with chloride activation, with K_i = 0.6 mM, and another that was uncompetitive, with K_i' = 11 mM (Haddy et al. 1999), during catalytic turnover. Assuming a simple binding model in which the dissociation constant equals the uncompetitive constant K_i', the uncompetitive site would be significantly occupied in the presence of mM concentrations of azide, with about 50% occupation at 10 mM N_3^- in a typical EPR sample. The degree of occupation of the N_3^- site that is competitive with Cl^- activation, however, depends on both the dissociation constant for azide, taken to be equal to the competitive inhibition constant K_i = 0.6 mM, and on the binding affinity for Cl^-. The dissociation constant for Cl^- has been measured directly in the absence of catalytic turnovers and was found to be K_d = 20 μM for the high affinity, low exchange site described by Lindberg and Andréasson (1996). Using these constants, almost all Cl^--competitive sites would be occupied by Cl^-, with a few percent occupied by N_3^- at the concentrations used for typical EPR samples. The opposite situation would occur in Cl^--depleted PS II membranes, for which a great majority of Cl^--competitive sites would be occupied by N_3^- at mM concentrations of the inhibitor.

In Cl^--depleted PS II membranes, we found that N_3^- had effects on the S_2 state EPR signals that were similar to those observed for fluoride. Chloride-depletion or replacement of chloride with fluoride or other anions is known to result in suppression of the multiline signal and an increase in the g = 4.1 signal (Yachandra et al. 1986; Ono et al. 1987; Haddy et al. 1992; DeRose et al. 1995; Lindberg and Andréasson 1996). F^- and ammonia also cause a decrease in the line width of the g = 4.1 signal, which has been shown to be due to an increase in the zero field splitting of the magnetic center giving rise to the signal (Haddy et al. 1992). In the absence of Cl^-, N_3^- was found to have these effects as well. The multiline signal was nearly absent, indicating that binding at the competitive site resulted in loss of signal beyond that observed for binding at the uncompetitive site. The g = 4.1 signal showed a notable increase in intensity (as well as line width narrowing), which is the opposite of what was observed in the presence of Cl^-. We therefore observed two apparently opposite effects on this signal from the two N_3^- binding sites.

Although azide and fluoride binding in place of chloride had very similar effects on the S_2-state EPR signals, there were major differences in their effects on catalysis from this site. The fluoride-induced form of the g = 4.1 signal has been shown to go through catalytic turnovers as represented by a damped oscillating signal in response to flashes (van Vliet et al. 1996). This would explain the observation made here that fluoride supported a low level of O_2 evolution activity that exceeded that of the Cl^--depleted PS II membranes. After replacement of chloride with azide, however, we found a complete absence of catalytic activity. This observation correlates with the previous report that azide causes irreversible damaging effects on PS II during catalytic turnovers as a result of the formation of azidyl radical (Kawamoto et al. 1995). We can therefore conclude that the effects due to azide described by Kawamoto and coworkers occur from the chloride competitive site.

In the presence of Cl^-, where a large fraction of azide binding was to the uncompetitive site, we found that azide prevented progression of the oxygen evolving complex to the normal S_2 state, as shown by the concurrent suppression of both S_2 state EPR signals (Figure 1). The loss of S_2 state multiline and g = 4.1 signal intensity was not due to the loss of manganese during azide treatment (under non-catalytic conditions), since oxygen evolution activity recovered almost completely after the removal of azide from the medium (Figure 2). Reduction of signal intensity was found to be unrelated to a general slowing of signal formation (Figure 5), indicating that azide bound at the uncompetitive site stopped formation of the S_2 state signals. Suppression of the S_2 state signal intensities correlated fairly well with the percentage of PS II centers free from N_3^- bound to the uncompetitive site (Figure 3). Signal suppression did not correlate as well with O_2 evolution activity and the additional suppression of catalytic activity was probably due to additional inhibitory effects of azide at the chloride-competitive site and/or a higher overall affinity for N_3^- during catalytic turnover.

In some experiments, azide that had bound in the presence of chloride was removed from PS II by washing in azide-free buffer. After this treatment, the intensity of the S_2 state signals did not consistently recover (Figure 2). This was as if removal of azide from the medium was not sufficient to ensure its removal from PS II or the reversal of its effects. O_2 evolution activity of the same samples, on the other hand, recovered to nearly control levels after washing in azide-free buffer. The lack of complete recovery of the intensity of the S_2 state signals therefore appeared to be related to the lack of catalytic turnovers during sample preparation. One possibility is that azide must overcome a conformational barrier to exchange at the uncompetitive sites under non-catalytic conditions. Anions may have freer access to the site while passing through the higher S-states during catalytic turnovers. This possibility may also be related to the greater suppression by azide of O_2 evolution activity relative to suppression of the S_2 state signal intensities. Another possible explanation for the difficulty in reversing azide's effects may be that azide binding was followed by a chemical change that resulted in poor release of azide. For example, bound azide may have become oxidized to the azidyl radical (which has reduction potential of 1.3 V) at the uncompetitive site, then reacted to form a covalent bond. Another possibility may be that if the uncompetitive site was at the manganese cluster, azide caused a Mn(III) or Mn(IV) ion to shift from high to low spin state, by analogy with azide's effect on Fe(III) of metmyoglobin, thereby altering the binding properties of the metal ion and reducing the ability for azide to exchange. Knowledge about the location of the uncompetitive azide binding site would facilitate an assessment of the likelihood of these explanations.

A possible site that was considered for identification with the uncompetitive binding of azide was

the non-heme iron of the Fe(II)Q_A complex on the acceptor side of Photosystem II, since there is precedence for the binding of anions such as formate (Vermass and Rutherford 1984; Diner and Petrouleas 1990), cyanide (Koulougliotis et al. 1993; Sanakis et al. 1994), and acetate (Kühne et al. 1999) at this site. In the present study, no evidence was found for an effect of azide on the EPR resonances from Fe(II)Q_A^- when azide concentrations of up to 25 mM were used (Figure 4). The lack of an increase in intensity of the signals at $g = 1.83$ indicates that azide did not significantly displace bicarbonate ligated to Fe(III) at the Fe(II)Q_A site. Also the lack of a decrease in Fe(II)Q_A^- signal intensity indicates that azide did not significantly interfere with electron transfer between the reaction center and the quinone acceptor site. Thus the location of the uncompetitive azide site appeared not to be at the non-heme Fe(II) site nor to prevent electron transfer to Fe(II)Q_A. While no effect of azide at this site was observed here, it may be that given higher concentrations (i.e. >100 mM) of azide, such as has been used for other anions, an effect on the Fe(II)Q_A^- signals would be observed. This possibility is suggested in the results of a study of chlorophyll fluorescence decay in the presence of azide and other anions (Cao and Govindjee 1990).

The lack of an observable effect of azide on the acceptor side signals of Photosystem II suggests that the uncompetitive site was located between the site of electron donation from water and the reaction center. This was consistent with the results of the previous study, in which removal of the 17 and 23 kD polypeptides of PS II was found to lower the value of K_i' for uncompetitive inhibition to 2 mM (Haddy et al. 1999), suggesting an association with the donor side of PS II. Two sites for the effects of other anions or Lewis bases on the donor side of PS II have been proposed in other studies. Fluoride and several amines were found using assays of chloride-activated O_2 evolution to show primarily competitive inhibition, but also uncompetitive inhibition (Sandusky and Yocum 1984; Sandusky and Yocum 1986), similar to the results found for azide (Haddy et al. 1999). Two sites for anion (Br^-, NO_3^-, NO_2^-, I^-) binding have also been proposed in a UV absorption difference spectroscopy study of the oxidation states of the OEC (Wincencjusz et al. 1999): when bound at the Cl^- site, these anions affected the rate of the final step of the water oxidation cycle, but when bound at the second site, these anions appeared to attack higher oxidation states of the OEC. The data presented here give the first indication that anion binding to a second site, which is uncompetitive with chloride, has effects on the S_2 state EPR signals.

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