EPR Investigation of Water Oxidizing Photosystem II: Detection of New EPR Signals at Cryogenic Temperatures†

Jonathan H. A. Nugent,* Sandra Turconi, and Michael C. W. Evans

Department of Biology, Darwin Building, University College London, Gower Street, London WC1E 6BT, U.K.

Received August 27, 1996; Revised Manuscript Received March 31, 1997⊗

ABSTRACT: Experiments are described which allow the detection and characterization of new EPR signals in photosystem II (PSII). PSII has been extensively studied with the water oxidising complex (WOC) poised in the S_1 and S_2 states. Other stages in the cycle of water oxidation lack characteristic EPR signals for use as probes. In this study, experiments use multiple turnovers of PSII from an initial S_1 state to allow new states of PSII to be studied. The first EPR signal detected, centered at $g = 4.85$ and termed the $g = 5$ signal, is suggested to be a new form of S_2 probably formed by decay of S_3 at cryogenic temperatures, but a novel form of oxidized non-heme iron cannot be fully excluded at present. The second signal is split around $g = 2$ and shows characteristics of signals formed by spin−spin interaction between two paramagnetic species. The split $g = 2$ signal is reversibly formed by illumination at <30 K of a sample containing the $g = 5$ signal. The $g = 2$ signal may be a form of the "S3" EPR signal previously only found in a variety of PSII preparations where oxygen evolution has been inhibited. Those "S3" signals are thought to arise from the interaction of an oxidized amino acid radical and the S_2 state, i.e., S_2X^+. Illumination at higher temperatures or illumination at <30 K, followed by dark-adaptation at 77 K, removes the $g = 5$ signal and prevents subsequent detection of the $g = 2$ signal on illumination at <30 K. The most likely explanation of our data is that illumination at <30 K of centers containing the $g = 5$ species allows accumulation of an oxidized intermediate and that at higher temperatures electron transfer proceeds to re-form an EPR-silent S state equivalent to that initially trapped during sample preparation. Study of these signals should provide an important new insight into the WOC and PSII.

In plants, algae, and cyanobacteria, photosynthetic electron transfer involves two photosystems, termed photosystem I and photosystem II (PSII).[1] PSII is the membrane−protein complex which catalyzes electron transfer from water to plastoquinone [see Renger (1992), Debus (1992), Rutherford et al. (1992), Diner and Babcock (1996), Nugent (1996) for a review]. Many of the PSII cofactors are bound to a core heterodimer of polypeptides termed D1 and D2. Absorption of light leads to photooxidation of the reaction center chlorophylls, P680, and reduction of the bound plastoquinone electron acceptor, Q_A, via a pheophytin intermediate, I. The electron then passes from $Q_A^{\bullet -}$ to another plastoquinone, Q_B. After two turnovers of the reaction center, doubly reduced and protonated Q_BH_2 carries electrons to the membrane plastoquinone pool. Both Q_A and Q_B interact magnetically with a non-heme iron, Fe^{2+}. This non-heme iron can also be oxidized by ferricyanide or certain semiquinones (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987). $P680^+$ is reduced by electrons from the water oxidising complex (WOC) via the intermediate D1 tyrosine 161, Y_Z. A second redox-active tyrosine residue, Y_D, has been identified as tyrosine 161 of D2. $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ can be observed by EPR with almost identical line shapes, but they can be distinguished by their rates of reaction and microwave power saturation behavior. $Y_Z^{\bullet}$ is a transient signal while $Y_D^{\bullet}$ is relatively stable.

The oxidation of water to release molecular oxygen by the WOC is a four-electron transfer process [see Debus (1992), Rutherford et al. (1992), Renger (1993), Yachandra et al. (1993), Klein et al. (1993), and Nugent (1996) for a review]. During its cycle, the WOC passes through different redox states termed S states, S_0 to S_4, electrons being removed from S_0 to S_4 and O_2 being evolved at S_3 to S_0 (Kok et al., 1970). The point in the cycle where water binding occurs is not known. Recent studies strongly suggest that there is a heterogeneity in the binding of the two water molecules. They bind at different sites and/or different S states (Messinger et al., 1995). The S_4 state has a lifetime of about 1 ms, spontaneously decaying to S_0 on oxygen evolution. The S_2 and S_3 states are unstable with short half-lives at room temperature, decaying back to S_1. The dark-stable state is S_1. After a few minutes in the dark, about 75% of PSII is in S_1 and 25% in S_0. On further dark-adaptation, the S_0 is slowly oxidized to S_1 by $Y_D^{\bullet}$ (Vermaas et al., 1984). $Y_D^{\bullet}$ can also be reduced by $Q_A^{\bullet -}$ on storage at cryogenic temperatures (Nugent et al., 1982, 1987). The lifetimes of S_2 and S_3 increase at lower temperatures, decay of these states being very slow at 200 K. S_2 and S_3 states can oxidize Y_D above 200 K (Styring & Rutherford, 1987; Nugent et al., 1987; Kawamori et al., 1987).

† Financial assistance from the U. K. Biotechnology and Biological Sciences Research Council is acknowledged.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: EPR, electron paramagnetic resonance spectrometry; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PPBQ, phenyl-1,4-benzoquinone; DCBQ, 2,5-dichlorobenzoquinone; WOC, water oxidising complex; PSII, photosystem II; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Chl, chlorophyll; Hpp, peak to trough line width of EPR spectrum; EDTA, ethylenediaminetetraacetic acid; Q_A, first PSII plastoquinone electron acceptor; Q_B, second PSII plastoquinone electron acceptor; Y_Z, tyrosine D1 161 ($Y_Z^{\bullet}$ when oxidized); Y_D, tyrosine D2 161 ($Y_D^{\bullet}$ when oxidized); Cyt *b*559, cytochrome *b*559.

S0006-2960(96)02179-4 CCC: $14.00 © 1997 American Chemical Society

At least two, probably four, Mn atoms are actively involved in the WOC. The Mn cluster appears to act both as a catalytic site for water oxidation and as a device for accumulation of oxidising equivalents from the reaction center. The Mn valence states remain to be resolved; however, the S_2 state probably is a mixture of Mn^{3+} and Mn^{4+} states. The oxidation events in S-state turnover have been studied, but there are conflicting interpretations. Two popular models either have Mn oxidized during each of the S-state transitions (Ono et al., 1992) or have some transitions, e.g., S_2 to S_3 (Klein et al., 1993; Yachandra et al., 1993), not involving Mn oxidation. Both Cl^- and Ca^{2+} ions are required for the functional integrity of the WOC and seem to act as cofactors for water oxidation (Yocum, 1991; Debus, 1992; Rutherford et al., 1992; Adelroth et al., 1995). Two EPR signals, termed the "multiline" signal (Dismukes & Siderer, 1980) and the $g = 4.1$ signal (Casey & Sauer, 1984; Zimmerman & Rutherford, 1984), have been identified as arising from the S_2 state, probably in two different structural states. These have been used as one of the main probes of the WOC and its turnover. The production of these EPR signals and X-ray spectroscopy (Yachandra et al., 1993) both support a Mn valence change, to give a Mn^{3+}/Mn^{4+} dimer, on the S_1 to S_2 step.

The EPR properties of dark-adapted PSII membranes (S_1) at cryogenic temperatures have been studied in a number of laboratories for many years and are well established (e.g., De Paula et al., 1985; Nugent et al., 1987). A number of electron transfer reactions are possible, even following illumination at temperatures below 77 K. Below 200 K, it is generally accepted that only a single turnover of the PSII reaction center can occur, the electron transfer forming $Q_A^{\bullet-}$ and one of several oxidized species, either S_2, oxidized cytochrome *b*559 (Cyt *b*559), or a monomeric oxidized Chl. At 77 K and below, the latter two species usually form the major oxidized product. At 200 K, S_2 is the major product, giving either the multiline EPR signal and/or (dependent on the cryoprotectant and sample additions used) the $g = 4.1$ form of S_2 [see Debus (1992) for a review].

Oxidation of the non-heme iron (Fe^{2+} to Fe^{3+}) can provide a second electron acceptor at cryogenic temperatures. There are two ways to oxidize the non-heme iron, either using ferricyanide (Diner & Petrouleas, 1988) or via an exogenous electron acceptor such as phenyl-1,4-benzoquinone (PPBQ) in the Q_B site (Zimmermann & Rutherford, 1986; Diner & Petrouleas, 1988). The natural Q_B plastosemiquinone has a lower redox potential for the $Q_B^{\bullet-}/Q_BH_2$ redox couple and does not appear to oxidize the non-heme iron, while DCBQ gives less oxidation than PPBQ (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987; Diner & Petrouleas, 1988). With PPBQ, Q_A is initially reduced by illumination at <200 K, and $PPBQ^{\bullet-}$ is then formed by annealing the PSII sample in the dark at room temperature, allowing the electron from $Q_A^{\bullet-}$ to go forward to PPBQ. $PPBQ^{\bullet-}$ oxidizes the non-heme iron, being reduced itself to the quinol. The S_2 state formed by 200 K illumination is also stabilized by decreasing the rate of charge recombination. Some centers therefore retain the S_2 state after annealing.

Other EPR signals attributed to the WOC have been detected at cryogenic temperatures in inhibited PSII, where Ca^{2+} or Cl^- have either been depleted or been displaced/replaced by treatment with ammonia, acetate, or fluoride (Boussac et al., 1989, 1990a,b; Sivaraja et al., 1989; Ono & Inoue, 1990; Baumgarten et al., 1990; Hallahan et al., 1992; Andreasson & Lindberg, 1992; MacLachlan & Nugent, 1993; van Vliet et al., 1994; DeRose et al., 1995; van Vliet & Rutherford, 1996; Szalai & Brudvig, 1996). In some of these preparations, a modified multiline signal is observed. This is assigned to a different form of the S_2 state and is dependent on the presence of chelators (Boussac et al., 1990a; Ono & Inoue, 1990) such as EDTA, EGTA, or citrate. In other cases, following chloride depletion or acetate treatment, an EPR-silent S_2 state appears to be formed (MacLachlan & Nugent, 1993; van Vliet et al., 1994; Szalai & Brudvig, 1996). Illumination at 273 K and freezing to 77 K of inhibited preparations result in the the generation of a broad signal split about $g = 2$ which was initially thought to be an "S3" state (Boussac et al., 1989). The signal is now better assigned to an S_2X^+ interaction state, where X has been suggested to be histidine (Boussac et al., 1990b; Boussac & Rutherford, 1995), tyrosine (Y_Z) (Hallahan et al., 1992), or a radical formed from water (Kusunoki, 1995). A recent study has indicated that X is Y_Z in calcium-depleted PSII (Gilchrist et al., 1995). This allows the position of the Mn cluster to be suggested, from the interaction of Y_Z and S_2, as within 10 Å of Y_Z (Hallahan et al., 1992). This has lead to new models suggesting how Y_Z and the Mn cluster may interact in the mechanism of water oxidation (Gilchrist et al., 1995; Tommos et al., 1995; Hoganson et al., 1995). The S_2X^+ interpretation of the "S3" signal has also been used to support the lack of oxidation of Mn on the S_2 to S_3 step, but the relationship to normal turnover of the WOC and native S_3 state is not known. The "S3" EPR signal has not been detected in the native S_3 state produced during flash turnover of oxygen-evolving PSII. It is therefore uncertain whether Mn, a Mn ligand, or another amino acid is oxidized when S_3 is formed *in vivo*.

Information on the higher S states is required to help understand both the structure of the WOC and the mechanism of water oxidation. The S_1 and S_2 states are the most easily studied, as the S_3 and S_4 states are difficult to obtain in high yield. Illumination at physiological temperatures in the presence of exogenous electron acceptors leads to a mixture of S states. Even illumination methods using a decreased temperature during illumination to control S-state turnover still lead to scrambling of S states, due to the similar temperature dependence of various S-state transitions and the Q_A to Q_B reactions (Styring & Rutherford, 1988; De Paula et al., 1985). The best method for multiple S-state turnover uses saturating laser flashes to advance the S states sequentially from S_1, but this is not possible at the high PSII concentrations required for most EPR experiments. Experiments using chemical treatments to increase the yield of S_3, by allowing a double turnover of the WOC from S_1, have been used. These have used either oxidation of the non-heme iron (Guiles et al., 1990) or a redox-active herbicide bound in the Q_B site (Bocarsly & Brudvig, 1992). In these experiments, advancement beyond S_2 could only be monitored by measurements of the decrease in the yield of the multiline S_2 signal, as no EPR marker for S_3 was available.

In this study, a series of EPR experiments have been used to investigate the effect of multiple turnovers of PSII. The aim was to obtain information on higher S states and to detect new signals which may be useful in further experiments on oxygen-evolving PSII. "S3" type $g = 2$ split signals were detected in native oxygen-evolving preparations, and a new EPR signal near $g = 5$ was also observed.

A preliminary report of this study has been published (Nugent & Turconi, 1995).

MATERIALS AND METHODS

PSII membranes (also termed BBY's; Berthold et al., 1981) were prepared from market spinach or freshly cropped 10−14-day-old pea seedlings according to the method of Berthold et al. (1981) using Triton X-100, with the modifications of Ford and Evans (1983). Reagents used were all analytical grade. Control rates of oxygen evolution for PSII membranes were 400−800 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} using ferricyanide and dimethylbenzoquinone as electron acceptors and measured in a Clark-type oxygen electrode at 298 K. The membranes were stored at 77 K in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 15 mM NaCl, 5 mM $MgCl_2$, and 0.4 M sucrose, pH 6.5 (buffer A). Before preparation of EPR samples, the PSII membranes were (except where stated) washed in buffer A containing 2 mM EDTA to remove adventitiously bound Mn^{2+}, followed by centrifugation and resuspension in 20 mM MES, 15 mM NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.4 M sucrose, pH 6.5. Removal of the WOC was achieved by incubating PSII membranes in 0.8 M tris(hydroxymethyl)aminomethane (Tris), 2 mM EDTA, pH 9, for 1 h under room light followed by centrifugation to recover the membranes which were resuspended finally in buffer A. This is termed Tris-washed PSII.

For EPR, 0.3−0.4 mL samples (approximately 10 mg of Chl/mL; 40−50 μM PSII) were placed in calibrated 3 mm quartz EPR tubes. They were given a brief illumination to turn over the PSII reaction center and restore $Y_D^\bullet$ lost on storage. Further procedures were carried out in the dark or under a dim green light. Samples were dark-adapted for 3 h at 273 K and then treated as described in the text and figure legends. Additions of 0.5 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 0.5−1 mM 2,5-dichlorobenzoquinone (DCBQ), and PPBQ were made from freshly made concentrated stock solutions in dimethyl sulfoxide (maximum final concentration 1%). Ferricyanide was dissolved in water. Treated or untreated samples were then dark-adapted for a further 30 min on ice before being frozen to 77 K in the dark (total dark-adaptation 4 h). This produced samples initially in the S_1 state, as indicated by the absence of the S_2 EPR markers, the multiline signal, or the $g = 4.1$ signal.

During experiments, samples were illuminated at a variety of temperatures from 4 to 295 K as indicated below. Illumination in the EPR cavity at <30 K used a 150 W light source and fiber-optic light guide, while other illumination used a 1000 W light source, protecting the sample from heating where necessary by a 5 cm water filter. Temperatures within the EPR cryostat were measured with a calibrated thermocouple beneath the sample. At <10 K, slight changes caused by heating during illumination were reversed by the helium flow in less than 1 min. Heating artifacts were monitored or eliminated by suitable controls as discussed in the text. In many cases, spectra from illuminated samples were recorded a short time (usually 1 min) after illumination, to avoid heat-induced effects. Samples for 77 K dark-adaptation or illumination were placed in liquid nitrogen in a silvered dewar, while samples for 200−250 K dark-adaptation or illumination were incubated using an ethanol/dry ice bath in a clear glass dewar. The temperature was measured by a thermometer.

Scheme 1

dark adaptation (total 4h)

⇓

illumination at 200 K (15 min) ⇐

⇓ (repeat) ⇑

annealing at 290 K (in the dark, 1 min) ⇒

⇓

illumination at 235 K (5 min)

⇓

storage at 77 K (> 1 week)

g = 5 signal observed

⇓

illumination at < 30 K

g = 2 signal observed

The initial treatment given to samples in the S_1 state was illumination at 200 K for 15 min followed by thawing to 290 K for 30−45 s in the dark and refreezing to 77 K (total time from frozen to refrozen, 60−75 s). The latter annealing process allows electron transfer from $Q_A^{\bullet-}$ produced by the illumination to added electron acceptors. In the case of samples containing PPBQ, the $PPBQ^{\bullet-}$ formed is then able to oxidize the non-heme iron Fe^{2+} to Fe^{3+} (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987). In the case of other samples, $Q_B^{\bullet-}$ is formed in some centers.

Two cycles of the 200 K illumination/annealing produced a slightly higher subsequent yield of the new signals, probably due to the increased percentage of PSII reaction centers turned over. After one or two cycles of this illumination and annealing, the refrozen sample was then illuminated at 235 K. For simplicity, we shall refer to samples given the 200 K illumination, 290 K annealing, and 235 K illumination treatment as method A. The samples were then stored at 77 K in the dark. An outline of the protocol is shown in Scheme 1; the EPR signals observed are described under Results.

Samples were examined by EPR at cryogenic temperatures using a Jeol RE1X spectrometer fitted with an Oxford Instruments cryostat. Large sample size and careful wiping of the tubes prior to insertion into the cryostat help prevent frozen oxygen from condensing on the tube or sample surface and thereby distorting spectra. EPR conditions are given in the figure legends. Spectra were recorded and manipulated using a Dell microcomputer running Asyst software. No filtering, smoothing, fitting, or background subtractions were used. Difference spectra were obtained only from subtraction of spectra from the same sample. The vertical scale in figures showing first-derivative EPR spectra is arbitrary, with spectra at the same instrument gain unless stated in the figure legend. Identical sets of samples in calibrated EPR tubes were made for each experiment, using the same preparation and chlorophyll concentration. The signals present in control samples (Cyt *b*559 etc.) were directly compared in experiments which involved EPR data collection on different days. This confirmed the reproducibility of spectra so that no normalization was required or performed. S_2 multiline signal intensities were determined as peak to trough heights, using six peaks above and below $g = 2$, away from interference

caused by overlap of other PSII signals. The S_2 yield was compared to that achieved by illumination of an S_1 sample at 200 K for 15 min. The amplitude of split $g = 2$ signals was measured as the peak to trough height from the light on minus dark difference spectrum.

A difference between pea and spinach PSII was that spinach had a higher concentration of the cytochrome *b*/*f* complex, as detected by the EPR signal of the Rieske iron−sulfur center. This increased cytochrome *b*/*f* content was associated with EPR signals near $g = 6$ which showed characteristic line shape changes after addition of exogenous quinones (for example, see Figure 6). As these signals caused some interference with measurements, the spectra shown are mostly pea PSII, although the characteristics reported below were seen in both species. The absence of photosystem I was confirmed by the lack of signals from oxidized P700 or reduced iron−sulfur centers following illumination at <30 K.

$P_{1/2}$ (the microwave power for half-saturation) was determined graphically from a plot of log $(I/\sqrt{P})$ vs log $\sqrt{P}$, where P is the microwave power and I is the signal amplitude. $P_{1/2}$ is found by extrapolating the initial ($P \ll P_{1/2}$) and final ($P \gg P_{1/2}$) linear sections of the curves to their point of intersection, which is the $P_{1/2}$ value.

RESULTS

The oxidation of the non-heme iron to provide an additional electron acceptor in PSII has been used previously in attempts to form S_3 (Guiles et al., 1990) and was the starting point of this study. Following the Guiles et al. (1990) protocol, initially a 77 K illumination and thawing cycle, we see a similar pattern of oxidation of electron donors, monitored by EPR, as in Guiles et al. (1990).

Figure 1 shows a sample given a treatment modified to replace the 77 K illumination by 200 K illumination (method A) using in this experiment PPBQ as the exogenous electron acceptor. Figure 1A shows the EPR spectrum of a sample which had been dark-adapted (4 h) and then frozen, followed by two cycles of illumination at 200 K and annealing at 290 K. The $g = 8.1$ and $g = 5.5$ signals (feature 1) show the EPR signal of oxidized non-heme iron. These signals were absent in samples without PPBQ addition [not shown, but see Zimmermann and Rutherford (1986) and Diner and Petrouleas (1988)]. The sharp feature (2) at $g = 4.3$ originates from rhombic Fe^{3+} in the membrane preparation and is not affected by illumination. Oxidized Cyt *b*559 is present, having visible features near $g = 2.2$ (here overlaid by the multiline S_2 signal, feature 4) and $g = 3$ (feature 3). The multiline S_2 signal (feature 4) from a proportion of centers in S_2 (approximately 40% compared to the maximum size obtained in S_2 before annealing) is clearly seen around $g = 2$. The intense $g = 2$ radical from $Y_D^\bullet$ has been deleted for clarity. In summary, at this step, S_1- and S_2-containing centers are present. In some centers, an additional electron acceptor, the oxidized non-heme iron, is present. Iron oxidation following electron transfer from $Q_A^{\bullet-}$ to $PPBQ_B$ would increase the stability of S_2, by preventing the back-reaction with $Q_A^{\bullet-}$. The oxidized non-heme iron is therefore more likely to be in centers which contain S_2.

Following illumination of this sample at 235 K for 4 min, the spectrum in Figure 1B is observed. Figure 1C gives the post 235 K illumination minus pre 235 K illumination difference spectrum (1B − 1A), showing that the major

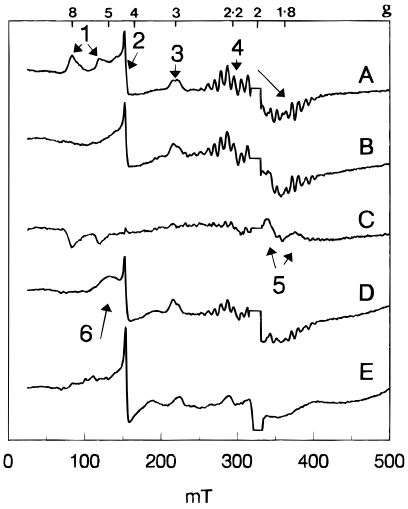

FIGURE 1: EPR spectra showing the changes occurring during sample preparation. (A) Pea PSII containing 0.75 mM PPBQ which had been dark-adapted (4 h) and then frozen, followed by two cycles of illumination at 200 K and annealing at 290 K (Scheme 1). The $g = 8.1$ and $g = 5.5$ signals (arrowed 1) show the EPR signal of oxidized non-heme iron. The sharp resonance at $g = 4.3$ (2) originates from rhombic Fe^{3+}. Cyt *b*559 has resonances near $g = 2.2$ and $g = 3$ (3). The multiline S_2 signal (4) is also present. (B) Spectrum following illumination of sample in (A) at 235 K for 4 min. (C) Difference spectrum B − A. The interaction of $Q_A^{\bullet-}$ and the non-heme Fe^{2+} is indicated as 5. (D) Sample as shown in spectrum B, after storage in the dark at 77 K for 2 weeks. The signal termed the $g = 5$ signal (see text) is shown arrowed as 6. (E) Sample of Tris-washed PSII treated as in spectra A and B and then stored for 1 month at 77 K. The intense $g = 2$ radical from $Y_D^\bullet$ has been deleted. EPR conditions: microwave power, 10 mW; modulation, 2 mT; temperature, 7 K. No smoothing or other manipulation of spectra was performed.

changes are a reduction of the non-heme iron and reduction of Q_A (indicating one or more electrons transferred). The EPR signal characteristic of $Q_A^{\bullet-}$, from the interaction of $Q_A^{\bullet-}$ and the non-heme Fe^{2+}, is near $g = 1.9$ (feature 5). As shown in Figure 1C, only minor changes in the multiline amplitude were observed when 200 K illumination cycles were used as pretreatment before 235 K illumination. The final yield of the S_2 multiline following 235 K illumination, measured using individual peak heights in several preparations, was 40−60% of the maximum size and similar to that observed by Guiles et al. (1990) following 240 K illumination. An increase in the S_2 multiline occurred on 235 K illumination if no exogenous quinone was present or the pretreatment used only 77 K preillumination [see Guiles et al. (1990)]. This was due to the lower yield of S_2 following annealing in these samples. Only very small changes in other electron donors, Cyt *b*559 and oxidized Chl, were observed. It is therefore assumed that about 50% of centers are not in S_2. Guiles et al. (1990) suggested that the other centers had advanced to an EPR-silent S_3 state. This is difficult to prove as both their (240 K) and our (235 K) illumination conditions could have allowed multiple turnovers of the WOC, as both Q_A to Q_B electron transfer and S-state turnover are possible at this temperature (Styring & Rutherford, 1988). This will be discussed below.

Following the 235 K illumination step, further illumination of the sample in the EPR cavity at <30 K produced no changes to the spectrum. This is expected as the potential electron acceptors, Q_A and oxidized non-heme iron, are both in the reduced state following the 235 K illumination.

After illumination at 235 K, samples were stored in the dark at 77 K. It has been shown previously that electron transfer in PSII can occur during dark storage at 77 K following illumination (Nugent & Evans, 1979; Nugent et al., 1982, 1987). For example, either Q_A or non-heme iron reduced by illumination at 77 K can be slowly reoxidized by reactions during storage at 77 K. This involves oxidized PSII electron donors; usually $Y_D^{\bullet}$ is preferred, but a slight loss of the multiline signal is also observed (Nugent et al., 1982, 1987; Kawamori et al., 1987). The Y_D formed can then be reoxidized by illumination at 77 K or below, rereducing the non-heme iron or Q_A (Nugent & Evans, 1979; Nugent et al., 1982, 1987). Y_D or the non-heme iron cannot normally be oxidized at cryogenic temperatures, so that it is concluded that the reduced forms created in these experiments by illumination and dark storage at 77 K are trapped in "oxidized" conformations [see also Davydov et al. (1994)]. In both cases, this probably means that structural changes such as proton movements normally associated with those redox state changes are prevented at 77 K.

After 2 weeks storage at 77 K, the spectrum in Figure 1D was obtained. A new signal centered near $g = 5$ has formed. This will be termed the $g = 5$ signal (feature 6), and samples prepared in this way and containing the signal will be termed $g = 5$ samples. Feature 5, $Q_A^{\bullet-}$, has been lost, presumably by reoxidation, together with a small percentage reduction of the multiline signal. No significant changes in either the rhombic iron signal near $g = 4.3$ or Cyt *b*559 were observed, but a weak and variable background signal due to oxygen trapped during storage was present.

In summary, no $g = 5$ signal is observed immediately following the 235 K illumination, but it forms gradually during several days storage at 77 K. This is characteristic for the formation of a new state by slow electron transfer/charge recombination following illumination as outlined previously. The yield of the $g = 5$ signal varied between preparations and was observed to be larger in samples where the S_2 multiline yield after 235 K illumination was small and vice versa. The $g = 5$ signal was reproducibly produced to the same yield in multiple samples made with the same batch of PSII. In a series of samples to investigate the effect of illumination at 235 K in producing the $g = 5$ state, 5 min illumination (sample 10 mg of Chl/mL) was required to produce the maximum signal size while longer illumination (up to 10 min) maintains this yield.

It is possible for dioxygen to condense on the sample or sample tube during long-term storage at 77 K. Dioxygen gives rise to several broad peaks across the X-band spectrum. To eliminate this explanation for the $g = 5$ signal, a Tris-washed sample was treated by the method A protocol and is shown in Figure 1E. Following 235 K illumination, this sample had been stored for 1 month, longer than the sample in Figure 1D. No signal with characteristics of the $g = 5$ signal was observed although oxygen contamination levels were higher as monitored by the underlying signals. No multiline signal is observed as the Mn complex is removed by the Tris-washing procedure.

The $g = 5$ signal, which has general characteristics that suggest involvement of a transition metal, has not previously been observed from PSII samples. As the normal line shape and yield of signals from Cyt *b*559 are still seen, this gives two possible sources for this signal in PSII, involving either the non-heme iron or the manganese cluster of the WOC. The EPR signals arising from interaction of the non-heme iron Fe^{2+} and the reduced electron acceptors I^-, $Q_A^{\bullet-}$, and $Q_B^{\bullet-}$ are found near $g = 2$ while the oxidized non-heme iron has characteristic signals near $g = 6$ as shown in Figure 1. Therefore, if the signal originates from the non-heme iron, a novel oxidized form would have to be involved.

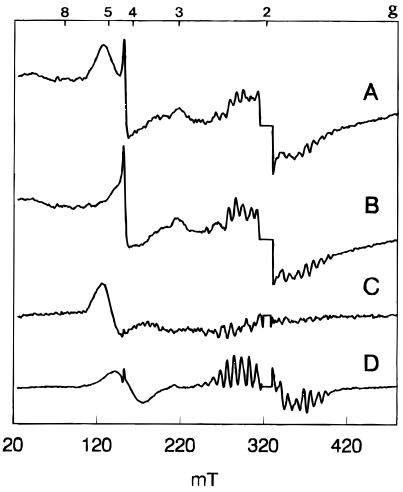

FIGURE 2: EPR spectra showing the effect of warming a sample containing the $g = 5$ signal to 235 K for 2 min. (A) Pea PSII sample (containing 0.5 mM PPBQ) prepared as Figure 1A−D, containing the $g = 5$ signal. (B) Sample warmed to 235 K for 2 min. (C) Difference spectrum A − B. (D) 200 K illuminated minus dark difference spectrum of a pea PSII sample containing 0.5 mM DCBQ, which was dark-adapted for 4 h before freezing to 77 K. EPR conditions: microwave power, 10 mW; modulation, 2 mT; temperature (A−C), 4.5 K, (D) 8 K. Other details as in Figure 1.

The $g = 5$ signal remains stable to further long term storage at 77 K but disappears upon warming in the dark to 200 K or above, allowing the line shape of the signal to be observed in the difference spectrum. Figure 2 shows the effect of warming a sample with the $g = 5$ signal for 2 min at 235 K in the dark. The $g = 5$ signal (monitored at 5 K which increases the signal size compared to Figure 1) shown in Figure 2A disappears on warming (Figure 2B), giving the difference spectrum shown in Figure 2C. The $g = 5$ signal has a $g = 4.85 \pm 0.1$ with a H_{pp} width of approximately 25 mT. The line shape and characteristics are unlike other known PSII components and clearly different to possible contaminants such as dioxygen. Following this warming procedure, the $g = 5$ signal is not restored by further storage of the sample in the dark at 77 K.

The illuminated minus dark difference spectrum in Figure 2D shows for comparison the characteristic S_2 $g = 4.1$ and multiline line shapes, obtained when a dark-adapted S_1 sample was illuminated at 200 K.

If the $g = 5$ signal was a new form of oxidized non-heme iron, formed slowly during 77 K storage, then the characteristic oxidized non-heme iron spectrum (Figure 1A) may be expected to form at higher temperatures where structural relaxation could occur. As indicated in Figure 2, no signals near $g = 6$ similar to those from the oxidized non-heme iron (Figure 1) are observed following the warming to 235 K, or even to room temperature, 293 K (not shown). This evidence suggests that the $g = 5$ signal originates from the WOC and not the non-heme iron of PSII.

Figure 3 shows the multiline signal before (3A) and after (3B) warming a $g = 5$ sample to 200 K for 2 min. The $g = 5$ signal was lost as in Figure 2. Figure 3C shows the net effect on the multiline signal. Warming to 200 K is

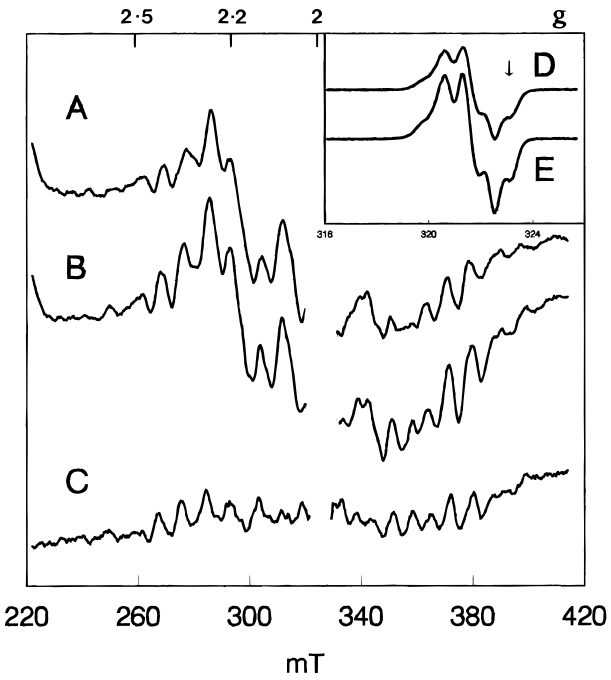

FIGURE 3: EPR spectra showing the effect on the multiline signal and Y_D of warming a sample containing the $g = 5$ signal to 200 K for 2 min. (A) Pea PSII sample (containing 0.75 mM PPBQ) prepared as in Scheme 1 and Figure 1A−D, containing the $g = 5$ signal. (B) Sample warmed to 200 K for 2 min. (C) Difference spectrum B − A. Inset: (D) $g = 2$ region in (A) ($g = 2$ is marked by an arrow); (E) $g = 2$ region in (B). EPR conditions: microwave power, 10 mW; modulation, 2 mT; temperature, 9 K. Inset: microwave power, 10 μW; modulation, 0.2 mT.

accompanied by a slight line shape change, resulting in an unusual difference spectrum (Figure 3C). The multiline changes are small and require further investigation. They may be due to subtle structural changes which may slightly alter the microwave power saturation behavior of the signal or other characteristics and not reflect any electron transfer. For the discussion below, it is important to note that no decrease in the yield of multiline occurs on warming.

Organic Radicals at g = 2. If a PSII sample is illuminated at 200 K to form S_2, then during the following 77 K dark storage some decay of $Y_D^\bullet$ occurs due to recombination with $Q_A^{\bullet-}$. Subsequent thawing of the sample in the dark allows the S_2 state to reoxidize Y_D. This is monitored using EPR as an increase in $Y_D^\bullet$ and loss of multiline S_2 (Styring & Rutherford, 1987; Nugent et al., 1987; Kawamori et al., 1987) and has been shown to occur down to 210 K (Kawamori et al., 1987). $g = 5$ samples contain $Y_D^\bullet$ and generally only a very small yield of the Chl^+ signal. This is as expected from the 235 K illumination which is at a higher temperature than required for Chl^+ generation, but allows formation of $Y_D^\bullet$ in a high percentage of centers. On 77 K storage following the 235 K illumination, the $Y_D^\bullet$ concentration is reduced slowly over a period of days by recombination reactions in some centers. $Y_D^\bullet$ can be restored by <10 K illumination. The inset to Figure 3 shows that if a $g = 5$ sample is warmed to 200 K in the dark, then in addition to the loss of the $g = 5$ signal and the multiline changes (Figure 3A−C), an increase in $Y_D^\bullet$ is observed. $Y_D^\bullet$ and $Y_Z^\bullet$ have similar EPR line shapes, and also changes in the power saturation characteristics of these signals occur with S-state changes. However, it is unlikely that $Y_Z^\bullet$, which is difficult to trap, would be present. The increase observed does not result from a change in the power saturation characteristics of the

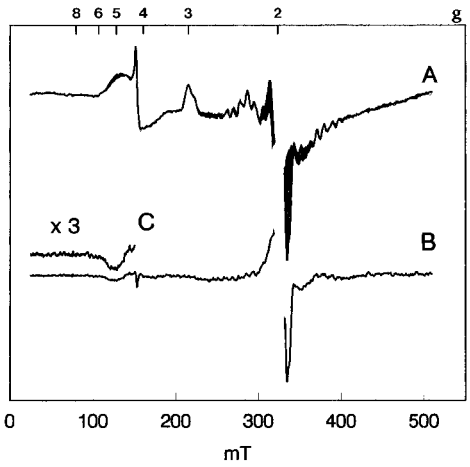

FIGURE 4: EPR spectra showing the effect of illumination at <30 K on a sample containing the $g = 5$ signal. (A) Pea PSII sample (containing 0.5 mM PPBQ) prepared as in Scheme 1 and Figure 1A−D, containing the $g = 5$ signal, and then illuminated at 10 K. Shading shows the effect of illumination. (B) Difference spectrum showing changes induced by 10 K illumination. The sharp line at $g = 4.2$ is a subtraction artifact and is not reproducible. (C) Part of (B) amplified × 3. EPR conditions: microwave power, 10 mW; modulation, 2 mT; temperature, 8 K.

$Y_D^\bullet$ radical. An increase in $Y_D^\bullet$ therefore shows that warming a $g = 5$ sample results in the loss of a non-multiline S_2 or a higher than S_2 state, as the $Y_D^\bullet$ increase occurs without significant loss in the overall yield of the S_2 multiline.

The Split Signal at g = 2. If a sample containing the $g = 5$ signal is illuminated in the EPR cavity at <30 K, then a second new signal is observed near $g = 2$ (Figure 4). The signal is split around $g = 2$ and shows similar characteristics to those of signals formed by spin−spin interaction between two paramagnetic species (Ruzicka et al., 1975). Two types of interaction can occur: a dipolar interaction and/or an electronic exchange interaction. In either case, two sets of lines with a common center can be observed, with weaker outer lines and an approximately equal separation of lines. However, this depends on the strength of the coupling and the *g*-values of the individual components. The formation of the split signal is accompanied by a small decrease in the $g = 5$ signal shown in Figure 4B,C. Another change following illumination at <30 K is a weak, broad feature near $g = 2.5-3$. Cyt *b*559 is in the oxidized state, giving the peak near $g = 3$, and therefore is not available for electron donation.

The yield of the $g = 2$ split signal was proportional to the size of the $g = 5$ signal present in the dark. The results suggest either (1) that electron transfer occurs to or from the $g = 5$ species or (2) that the components interacting to form the split $g = 2$ signal include that giving rise to the $g = 5$ signal and that the $g = 5$ decrease results from the interaction. Illumination at 77 K of a $g = 5$ sample results in loss of both the $g = 5$ signal and the ability to form the $g = 2$ split signal by subsequent illumination at 10 K. This suggests that <30 K illumination allows an intermediate state to accumulate, giving the split signal. At 77 K, the equilibrium is altered as the electron transfer proceeds further, thereby eliminating the $g = 5$ and split signal. Following illumination at 77 K, a further period of storage at 77 K in the dark for several days allows recovery of the $g = 5$ signal and the ability to re-form the split signal. The effects of illumination at 77 K are therefore slowly reversible (not shown).

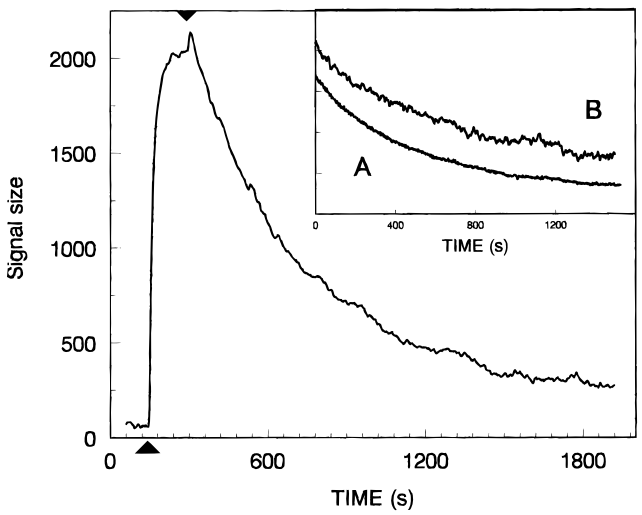

FIGURE 5: Decay of the $g = 2$ split signal following illumination at <10 K. Spinach PSII sample prepared to form the $g = 5$ signal (method A, Scheme 1) was illuminated (bottom arrow) to form the $g = 2$ split signal. Illumination was stopped (top arrow), and the decay of the signal was recorded with time. EPR conditions as for Figure 4 but response time 3 s, field position $g = 2.077$. Inset: (A) decay of the $g = 2$ split signal (at $g = 1.925$); (B) restoration of the $g = 5$ signal (at $g = 4.5$, plotted as $1 -$ data for comparison of curves) after illumination at 6 K using successive illuminations of the same sample.

Both the split $g = 2$ and $g = 5$ signals show apparent Curie behavior, increasing in size down to 4.2 K at nonsaturating microwave powers. The temperature dependence of the $g = 2$ signal is difficult to determine accurately as the temperature also affects the rates of formation and decay of the signal (see below). The $P_{1/2}$ microwave power saturation values were $g = 5$ (at 4.5 K), 20 mW, split $g = 2$ (at 10 K, formed by illumination at 10 K), 10 mW, and Cyt *b*559 as control (at 10 K), 0.5 mW.

Decay of Split g = 2 Signal. The $g = 2$ split signal decayed completely following cessation of illumination at 8 K with a half-life of about 420 s at 8 K (measured on the low-field side of the signal, $g = 2.077$, Figure 5, main panel). The signal could then be restored by reillumination at 8 K. This behavior was mirrored by the portion (involving about 25% of the total $g = 5$ signal) of the $g = 5$ signal amplitude removed by illumination (see inset B and Figure 6), confirming the relationship between the two signals. The inset shows that the decay at 6 K of the $g = 2$ split signal and the restoration of the $g = 5$ signal in the same sample (plotted as the inverse of the data for easy comparison) have very similar kinetics. This rapid recombination reaction contrasts with the very slow (days) recombination of $Q_A^{\bullet-}$ with $Y_D^{\bullet}$, the multiline and $g = 4.1$ forms of S_2, Cyt *b*559, or Chl^+.

Figure 6 shows more clearly the small change in the $g = 5$ region during 5 K illumination and its subsequent restoration on cessation of illumination. The twin peaks near $g = 6$ show no light-induced changes and were observed in spinach but not pea samples as mentioned under Materials and Methods. The $g = 5$ spectrum obtained by this treatment is similar to that obtained by warming the sample (Figure 2). The absence of similar light-induced changes in surrounding peaks suggests that this is not a heating artifact.

Figure 7B−D shows the changes caused by illumination in the region below $g = 2$ in a $g = 5$ sample prepared by method A. Figure 7A shows, as a control, the effect of

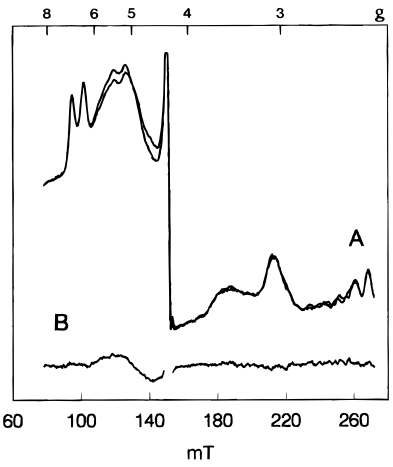

FIGURE 6: EPR spectra showing the $g = 5$ changes following illumination at 8 K in a spinach PSII sample containing 0.67 mM PPBQ, prepared by method A as in Figure 1A−D. (A) spectrum recorded first during illumination at 8 K; the sample was then kept in the dark at 8 K for 45 min and the spectrum rerecorded. (B) Difference spectrum showing restoration of the $g = 5$ signal during the 45 min dark period. EPR conditions as in Figure 4.

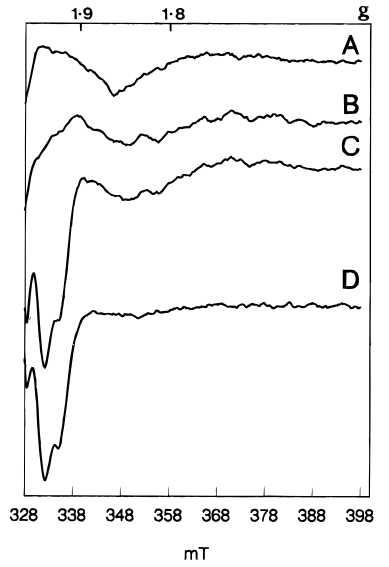

FIGURE 7: EPR spectra showing the Q_A region of pea PSII samples. (A) Sample poised in the S_1 state by 4 h dark-adaptation. Trace shows the "under illumination at 9 K minus the dark" difference spectrum containing the "$g = 1.9$" form of Q_A. (B) Sample poised in the $g = 5$ state as in method A (Scheme 1). Trace shows a difference spectrum obtained by subtraction of the dark spectrum from one started 40 min after illumination at 9 K. (C) Sample as (B). Difference spectrum obtained by subtraction of the dark spectrum from one started 1 min (2 min scan time) after a period of illumination at 9 K. (D) Sample as (B). Difference spectrum obtained by subtraction of a spectrum taken 40 min after illumination from one started 1 min after illumination at 9 K. EPR conditions as in Figure 4 except for a temperature 9 K.

illumination at 9 K on a sample poised in S_1. Only the $g = 1.9$ form of $Q_A^{\bullet-}$ is formed. As this sample was recorded under illumination, it also shows that no significant changes due to heating were seen in these experiments. Figure 7B shows the difference spectrum of spectra taken before illumination and 40 min following illumination, i.e., after most of the $g = 2$ split signal has decayed. It shows the $Q_A^{\bullet-}$ formed by illumination and some residual split signal. Figure 7C shows the formation of the $g = 2$ split signal and $Q_A^{\bullet-}$ in the difference spectrum of a sample before and immediately after illumination. Figure 7D, the difference spectrum of spectra taken immediately after illumination and

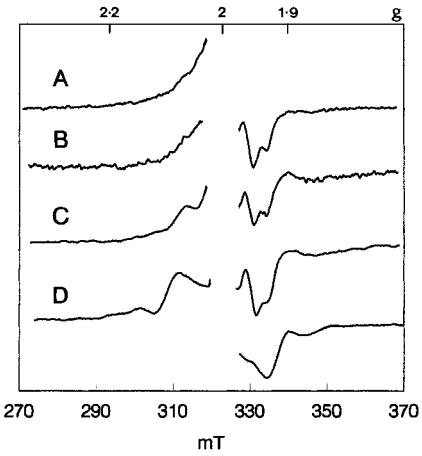

FIGURE 8: EPR spectra showing the line shape of the $g = 2$ split signals. Spectra A−C are the difference between a sample illuminated at 9 K and the dark state. Spectrum D is the difference spectrum of a sample frozen under illumination minus the unilluminated spectrum. All samples contain 0.67 mM PPBQ except spectrum C which had no additions. (A) Spinach PSII sample poised in the $g = 5$ state as in Figure 5. (B) Pea PSII sample poised in the $g = 5$ state as in Figure 1A−D and Scheme 1. (C) Pea PSII with no additions, poised in the $g = 5$ state as in (B). (D) Acetate-treated PSII, spectrum as in MacLachlan and Nugent (1993), Figure 4. EPR conditions: microwave power, (A) 1 mW, (B) 0.4 mW, (C) 10 mW, (D) 5 mW; modulation, (A,C) 1 mT, (B,D) 1.6 mT; temperature, 9 K. The large signal from $Y_D^{\bullet}$ at $g = 2$ has been removed.

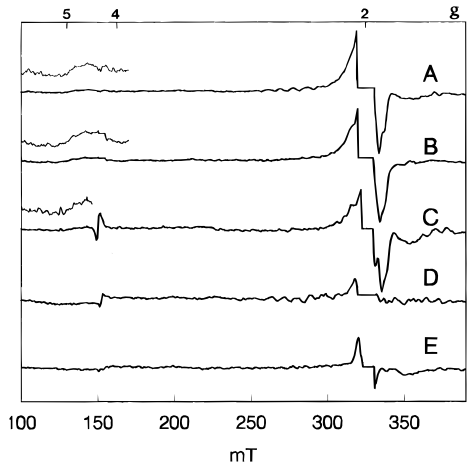

FIGURE 9: EPR spectra showing the changes on illumination at 9 K in samples with different additions. Samples of pea PSII were prepared by method A (Scheme 1) with the following additions: (A) 0.67 mM PPBQ; (B) 0.67 mM DCBQ; (C) no additions; (D) 0.67 mM DCBQ, 4% ethanol; (E) 0.5 mM DCMU. Spectra are the difference of samples in the dark (following 235 K illumination and 77 K storage etc.) and the sample illuminated at 9 K. For (A−C), the low-field region has been expanded to show the difference more clearly. EPR conditions as in Figure 4 but with a temperature of 9 K. The spectra in (C−E) were recorded at twice the instrument gain of spectra A and B.

40 min after illumination, shows that only a small proportion of $Q_A^{\bullet-}$ decays on the time scale of the split $g = 2$ decay. This suggests either that $Q_A^{\bullet-}$ is not involved in the decay of the $g = 2$ split signal or that only a small proportion of centers are involved.

Line Shape of g = 2 Split Signal. Figure 8 illustrates more clearly the line shape of the $g = 2$ split signal obtained in this study. All spectra are the difference of a spectrum recorded during illumination at <10 K minus the dark spectrum of a $g = 5$ sample. Figure 8,B shows the line shape of the split signal at 1 and 0.4 mW (nonsaturating microwave power) obtained by illumination of a $g = 5$ sample containing PPBQ. The anisotropic line shape is unusual for this type of interaction signal and may indicate for example that more than one signal is present, that it arises from a mixture of dipolar and exchange coupling, or that one or both of the interacting species have unusual EPR properties. The line shape of the $g = 2$ split signal seen in a sample with no addition of exogenous quinone (Figure 8C) is similar, the resolution of the shoulder on the high field side being variable. Figure 8D shows the more symmetrical S_2X^+ split signal observed in acetate-treated PSII (MacLachlan & Nugent, 1993).

Characteristics of Signals and Experimental Conditions for Optimum Yield. An investigation of the effect of varying the 235 K illumination temperature on the subsequent yield of the $g = 5$ and $g = 2$ split signals after 77 K storage was carried out. This showed that maximum yield in 0.67 mM PPBQ-treated pea PSII samples was obtained by a 5 min illumination at 230−240 K, following the 200 K preillumination and annealing (method A). The size of the $g = 5$ and split $g = 2$ signals subsequently obtained after 77 K storage, as a function of illumination temperature prior to 77 K storage, fell sharply from a maximum around 230−240 K to low levels at 210 and 273 K. Replacing the 200 K preillumination step by 77 K preillumination prior to 235 K illumination consistently produced a lower yield of both signals. This suggests that the requirement for optimum yield of 200 K preillumination and annealing plus exogenous quinones such as PPBQ is due to a need for (a) oxidation of secondary electron donors such as cytochrome *b*559, thereby reducing competition among electron donors, and (b) formation of the S_2 state in centers where oxidized electron acceptors are available for electron transfer at cryogenic temperatures.

A variety of electron acceptor conditions using PPBQ, DCBQ, ferricyanide, or no additions were used in experiments to eliminate the possibility that the exogenous acceptor was involved in the formation of both the $g = 5$ and split $g = 2$ signals. Each treatment, using the method A protocol, allowed the split $g = 2$ signal to form on 10 K illumination with similar line shapes and properties (Figure 9), but different yield. The $g = 5$ signal change is also clearly observed in both PPBQ- and DCBQ-treated samples (Figure 9A,B). In ferricyanide-treated (not shown) and samples with no additions (Figure 9C), formation of the $g = 5$ signal is observed but together with some $g = 4.1$ S_2 signal. Only the $g = 5$ signal changes on illumination at <30 K. These results confirm that the signals originate from PSII components and not exogenous additions.

Addition of 4% ethanol (Figure 9D) or the Q_b site inhibitor DCMU (Figure 9E) inhibits formation of both $g = 5$ and $g = 2$ split signals, and they are also not present in samples lacking the Mn cluster (Tris-washed samples, not shown). No $g = 2$ split signal is detected in PSII membranes upon freezing under illumination with no 77 K storage, conditions required for production of "S3" (S_2X^+) signals in samples with inhibited water oxidation (Boussac et al., 1989; MacLachlan & Nugent, 1993). Cryoprotectants have been shown to affect PSII EPR [see Debus (1992) for a review]. Sucrose was generally used as cryoprotectant in these experiments; use of 20% (v/v) glycerol also allowed formation of $g = 5$ and split $g = 2$ signals, but use of 30% (v/v) ethylene glycol inhibited signal formation. This may be due

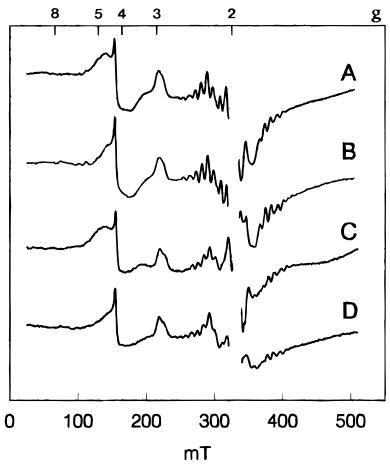

FIGURE 10: EPR spectra showing the loss of the $g = 5$ signal and split $g = 2$ signal during incubation at 77 K for 2.5 min in the dark following illumination at 9 K. Pea PSII samples prepared by method A (Scheme 1). The sample shown in (A) and (B) contained no additions while the sample in (C) and (D) contains 0.75 mM PPBQ. EPR conditions as in Figure 4. (A, C) Sample during illumination at 9 K. (B, D) Sample following illumination at 9 K and 2.5 min incubation at 77 K.

to alteration of the temperature dependencies of reactions by the cryoprotectant, but other reasons such as a dependence on the size of the cryoprotectant molecule cannot be ruled out at present. Omission of the EDTA wash stage during sample preparation did not affect the yield of $g = 5$ or split $g = 2$ signals. These results show that the signals do not arise from centers damaged or modified during sample preparation.

Warming following Illumination at Cryogenic Temperatures. The hypothesis that the $g = 2$ split signal results from interaction with the component giving rise to the $g = 5$ signal was investigated further by illumination of a $g = 5$ sample at 9 K, and then warming to 77 K in the dark for 2.5 min (Figure 10). The illumination reproduces the effect shown in Figure 4, removing part of the $g = 5$ signal and inducing the split $g = 2$ signal, while the 77 K dark step causes loss of most of the remaining $g = 5$ signal and removes the ability to form the split $g = 2$ signal on reillumination at 9 K. No significant changes in the Cyt *b*559, $Q_A^{\bullet-}$, or multiline species were observed during the 77 K dark step. In the sample with no additions (Figure 10 A,B) some $g = 4.1$ S_2 signal is present, but is unchanged by the 9 K illumination or 77 K dark step. This adds to the data above in suggesting that the split signal is an intermediate stage in electron transfer, trapped by the very low temperature, and that at 77 K the electron can travel further. At 77 K, most structural changes would be inhibited. A further storage period of several days at 77 K following the illumination at 9 K and 2.5 min dark at 77 K restores the $g = 5$ signal and the ability to form the split $g = 2$ signal by illumination at <30 K.

DISCUSSION

Formation of the New Signals. The complex experimental results can be rationalized as follows. The sample pretreatment by 200 K illumination followed by annealing gives a mixture of states. Some centers relax back to S_1 while others, especially those with native or exogenous Q_B, are relatively stabilized in S_2. The slightly higher yield of S_2 obtained from the addition of exogenous quinones may be significant in increasing the eventual yield of the new EPR states. In the case of PPBQ as exogenous electron acceptor, the electron transfer from $Q_A^{\bullet-}$ to Q_B during the pretreatment leads to oxidation of the non-heme iron. Therefore, during illumination at 235 K, one (Q_A) or two (Q_A and Fe^{3+}) acceptors are available. In centers containing native or exogenous Q_B (such as DCBQ), it may be possible for two electrons to be slowly transferred from Q_A to Q_B. At 235 K, exchange of quinone/quinol at the Q_B site is likely to be slow, restricting further turnovers. Protonation reactions involved with oxidation of the non-heme iron (Diner & Petrouleas, 1988) and reduction of Q_B will also be slow. In the WOC, the limiting reaction is the formation of S_4 which will be severely retarded at 235 K (Warden et al., 1976; Styring & Rutherford, 1988).

The combined effect of the restrictions on the electron donor and acceptor sides of PSII probably means that only one or two turnovers can occur during the 235 K illumination. From the initial mixture of S_1 and S_2 states, this would form S_2, S_3, perhaps S_2X^+ or S_3X^+ (where X would be Y_Z or an intermediate between the WOC and Y_Z), or even S_4 in a proportion of centers during the 235 K illumination. This is in line with the analysis of Guiles et al. (1990), and as shown in Figure 1, about 50% of centers are not in S_2 (or S_1 as S_1 to S_2 formation would have occurred on 235 K illumination). The lack of EPR signals from the WOC of some centers immediately after 235 K illumination (Figure 1B) indicates either integral spin state systems are formed or interactions between nearby components broaden the signals so that they are not observed. An EPR-silent S_3 state has been indicated by previous studies and is likely to be present in these samples following 235 K illumination. This seems distinct from the S_2 X^+ state which forms split $g = 2$ signals in inhibited samples (Boussac et al., 1989, 1990a,b; Sivaraja et al., 1989; Ono & Inoue, 1990; Baumgarten et al., 1990; Hallahan et al., 1992; Andreasson & Lindberg, 1992; MacLachlan & Nugent, 1993; van Vliet et al., 1994; DeRose et al., 1995; van Vliet & Rutherford, 1996; Szalai & Brudvig, 1996).

During 77 K storage in the dark following the 235 K illumination, reactions occur forming the $g = 5$ signal. The recombination reactions during 77 K storage are probably limited to one electron returning from $Q_A^{\bullet-}$, but with the possibility of reoxidation of the non-heme iron, $Q_B^{\bullet-}$, or Q_B^{2-} species also occurring. Reduced Q_B species are not known to recombine with oxidized electron donors at cryogenic temperatures and are usually thought to be stabilized by Bohr protonation (Diner et al., 1991). However, less stable, more reducing forms of Q_B species may be formed by the 235 K illumination if the protonation reactions are prevented.

A further period of illumination at <30 K then forms a split signal at $g = 2$. The yield of the $g = 2$ split signal is linked to the amount of $g = 5$ signal present, and a decrease in the amplitude of the $g = 5$ signal is seen upon formation of the split signal. This suggests a relationship between the two and is demonstrated in Figure 5. This could be an electron donor/electron acceptor pair or that the split $g = 2$ signal involves an interaction between components including that giving the $g = 5$ signal. The latter would place the origin of the $g = 5$ and split $g = 2$ signals on components close in the structure, either on electron acceptor or an electron donor pathways. Quantitation of the new signals, counting the number and location of electrons and estimating the percent centers, is difficult without more information about their origin but must only involve a maximum of about

50% of centers as the rest have the multiline S_2 state. The competing formation and decay reactions, in addition to competition from other electron transfer reactions, will make higher yields of the $g = 5$ signal difficult to obtain.

Origin of the g = 5 Signal. The fact that identical signals are observed in both samples with no additions, following additions of various electron acceptors and in different preparations, shows that the new EPR signals originate from endogenous PSII electron carriers. Dioxygen contamination during storage of samples at 77 K is also discounted by the controls shown. Oxygen-evolving PSII contains many redox-active cofactors, and a large variety of interactions are therefore possible.

The $g = 5$ and split $g = 2$ signals have characteristics (*g*-value, line shape, microwave power saturation, etc.) which indicate the involvement of a transition metal complex. High-spin iron signals near $g = 6$ which were attributed to Cyt *b*559 have recently been reported [see Hulsebosch et al. (1996)], but these are observed at alkaline pH values and have *g*-value and line shape characteristics distinct from the $g = 5$ signal. With normal yields and EPR line shapes of PSII components such as low-spin Cyt *b*559 and iron−quinone $Q_A^{\bullet-}$ being obtained, this leaves two possibilities, the non-heme iron and the manganese cluster. EPR signals from the oxidized non-heme iron are well characterized in situations where the Q_B site is empty or contains quinone or quinol (Diner & Petrouleas, 1988). Three results suggest that the non-heme iron is not involved: (1) the usual non-heme iron signal is not formed directly from the $g = 5$ signal on thawing in the dark (Figure 2); (2) the $g = 5$ signal is present in a sample with no exogenous electron acceptor, where non-heme iron oxidation should be minimal or absent; (3) the line shape and *g*-value are quite unlike any other signal attributed to the non-heme iron in PSII. These points strongly suggest that the $g = 5$ signal is not a novel trapped form of the oxidized non-heme iron. It is therefore more likely that it arises from the Mn complex of the WOC.

The $g = 5$ signal has similar characteristics to the S_2 $g = 4.1$ signal. Its *g*-value and line shape suggest it originates from a $S' = {}^3/_2$ spin system or the middle Kramer's doublet of a $S' = {}^5/_2$ spin system. Support for the presence of a new S_2 or higher state is given by the annealing experiments which result in loss of the $g = 5$ signal and oxidation of Y_D, with no net loss of the multiline S_2 state. The heterogeneity present complicates explanation of this experiment, but in some centers, the S_2 multiline may be lost, forming S_1 when Y_D is oxidized while in others the multiline S_2 may be formed from other S_2 or higher S states. Recent work has lead to proposals that two forms of the $g = 4.1$ S_2 signal exist, one being an excited state of the multiline signal and the other being a ground state form assigned to an interaction between an oxidized amino acid and the Mn cluster (Smith & Pace, 1996). If the $g = 5$ and $g = 4.1$ S_2 signals involved a trapped amino acid radical, they may be expected to decay slowly by recombination with $Q_A^{\bullet-}$ in analogy to the behavior of $Y_D^{\bullet}$ and the "S3" split signals found in inhibited samples. This is not observed, and as shown, the $g = 5$ signal only appears on long-term dark storage. It is unlikely that the $g = 5$ signal involves a trapped oxidized amino acid.

Conformational changes in the WOC upon the S_2 to S_3 transition have been suggested, based on the effects of exogenous reductants (Messinger et al., 1991) and the high reorganizational energy (Renger & Hanssum, 1992). These may involve deprotonation to maintain overall charge neutrality. Analysis of the conditions required to obtain the S_2 $g = 4.1$ signal suggests that changes in the ligands to Mn, perhaps at the water/chloride binding site, control the appearance of this form of S_2. It is tempting, therefore, to suggest that the $g = 5$ signal represents the Mn cluster in a redox state equivalent to S_2 but with a changed ligand arrangement compared to the known forms of S_2. This would be due to the S_3 state being trapped and then reduced at cryogenic temperatures where structural changes are inhibited. This could result, for example, in an S_2 state with a decreased positive charge as protonation of the complex on reduction is prevented by the low temperature. S_3 appears to be EPR-silent and could be the state produced when the $g = 5$ sample is illuminated at 77 K as well as that being formed initially on 235 K illumination. The decreased positive charge may also make oxidation of Y_Z more favorable at cryogenic temperatures.

Split g = 2 Signal. Illumination below 200 K is known to restrict turnover of PSII to one or two turnovers with Q_A or Q_A plus Fe^{3+} acting as electron acceptors. The relatively rapid decay of the $g = 2$ split signal on cessation of illumination at <10 K, restoring the state before illumination, suggests a recombination reaction occurs. This means that the electron is returning from I^-, Fe^{2+}, reduced Q_A, or Q_B.

Secondary electron donors usually are unable to compete at cryogenic temperatures with the very fast $P680^+I^-$ back-reaction allowing I^- to form. If it were possible to form I^- in addition to $Q_A^{\bullet-}$ on illumination at <30 K, then a split $g = 2$ from the weak interaction $I^-Q_A^{\bullet-}$ would occur. This was observed following chemical reduction of Q_A and illumination at cryogenic temperatures (Klimov et al., 1980). However, I^- $Q_A^{\bullet-}$ formation in nonchemically-reduced samples has not been observed in EPR studies of oxygen-evolving PSII, and it does not have a similar line width or line shape to the signal shown in this study. It is not observed for example in Figure 7A. $Q_A^{\bullet-}$ is formed by <30 K illumination of a $g = 5$ sample, but there are only small changes (<20%) to the amplitude of the iron−semiquinone signal during decay of the split $g = 2$ signal (Figure 7). Therefore, if the electron acceptor is Q_A, then the split $g = 2$ signal is formed in low yield.

The $g = 5$ signal does recover in parallel to the decay of the split $g = 2$ signal (Figure 5) and would appear to give us a suitable electron acceptor/donor pair to account for the effects of illumination at cryogenic temperatures. The result revealing that the $g = 5$ state is lost in the dark at 77 K following illumination at <30 K (Figure 10) shows that a further electron transfer probably occurs on warming to 77 K. A recombination reaction from the electron acceptor to the electron donor side on warming to 77 K in the dark would result in a rapid return to the initial state before illumination, which does not occur as the $g = 5$ state is only restored by 77 K storage. The fast decay of the split $g = 2$ signal and concomitant restoration of the $g = 5$ signal in the dark following 10 K illumination plus the slow recovery of the $g = 5$ signal following 77 K illumination therefore indicate that the $g = 5$ change during illumination at 10 K is due to interaction rather than electron transfer, i.e., that the split $g = 2$ signal involves an interaction with the component giving rise to the $g = 5$ signal. The second component of the electron donor/acceptor pair involved in the formation of the split $g = 2$ signal remains uncertain.

The split $g = 2$ signal is therefore likely to originate from an electron donor X^+ species formed by illumination which

interacts with the manganese cluster to give S_2X^+. EPR-silent S_3 could then be re-formed by electron transfer at 77 K.

CONCLUSION

Two new EPR signals have been detected in PSII. Experiments to trace their origin are presented and discussed in detail. We cannot completely rule out involvement of a novel state of the electron acceptors of PSII involving oxidized non-heme iron. However, the evidence does strongly favor the explanation that the signals involve components of the water oxidation system.

ACKNOWLEDGMENT

We thank Dr. Johannes Messinger for his critical reading of the manuscript.

REFERENCES

Adelroth, P., Lindberg, K., & Andreasson, L. E. (1995) *Biochemistry 34*, 9021−9027.
Andreasson, L. E., & Lindberg, K. (1992) *Biochim. Biophys. Acta 1100*, 177−183.
Baumgarten, M., Philo, J. S., & Dismukes, G. C. (1990) *Biochemistry 29*, 10814−10822.
Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
Bocarsly, J. R., & Brudvig, G. W. (1992) *J. Am. Chem. Soc. 114*, 9762−9767.
Boussac, A., & Rutherford, A. W. (1995) *Biochim. Biophys. Acta 1230*, 195−201.
Boussac, A., Zimmermann, J. L., & Rutherford, A. W. (1989) *Biochemistry 28*, 8984−8989.
Boussac, A., Zimmermann, J. L., & Rutherford, A. W. (1990a) *FEBS Lett. 277*, 69−74.
Boussac, A., Zimmermann, J. L., Rutherford, A. W., & Lavergne, J. (1990b) *Nature 347*, 303−306.
Davydov, R., Kuprin, S., Graslund, A., & Ehrenberg, A. (1994) *J. Am. Chem. Soc. 116*, 11120−11128.
Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
De Paula, J. C., Innes J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114−8120.
DeRose, V. J., Latimer, M. J., Zimmermann, J.-L., Mukerji, I., Yachandra, V. K., Sauer, K., & Klein, M. (1995) *Chem. Phys. 194*, 443−459.
Diner, B. A., & Petrouleas, V. (1988) *Biochim. Biophys. Acta 895*, 107−125.
Diner, B. A., & Babcock, G. T. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D., & Yocum, C., Eds.) Kluwer, Dordrecht, The Netherlands.
Diner, B. A., Petrouleas, V., & Wendoloski, J. J. (1991) *Physiol. Plant. 81*, 423−436.
Dismukes, G. C., & Siderer, Y. (1980) *FEBS Lett. 121*, 78−80.
Ford, R. C., & Evans, M. C. W. (1983) *FEBS Lett. 160*, 159−163.
Gilchrist, M. L., Ball, J. A., Randall, D. W., & Britt, R. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9545−9549.
Guiles, R. D., Zimmermann, J. L., McDermott, A. E., Yachandra, V. K., Cole, J. L., Dexheimer, S. L., Britt, R. D., Wieghardt, K., Bossek, U., Sauer, K., & Klein, M. P. (1990) *Biochemistry 29*, 471−485.
Hallahan, B. J., Nugent, J. H. A., Warden, J. T., & Evans, M. C. W. (1992) *Biochemistry 31*, 4562−4573.
Hoganson, C. W., Lydakis-Simantiris, N., Tang, X.-S., Tommos, C., Warncke, K., Babcock, G. T., Diner, B. A., McCracken, J., & Styring, S. (1995) *Photosynth. Res. 46*, 177−184.
Hulsebosch, R. J., Hoff, A. J., & Shuvalov, V. A. (1996) *Biochim. Biophys. Acta 1277*, 103−106.
Kawamori, A., Satoh, J., Inui, T., & Satoh, K. (1987) *FEBS Lett. 217*, 134−138.
Klein, M. P., Sauer, K., & Yachandra, V. K. (1993) *Photosynth. Res. 38*, 265−277.
Klimov, V. V., Dolan, E., Shaw, E. R., & Ke, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7227−7231.
Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457−475.
Kusunoki, M. (1995) *Chem. Phys. Lett. 239*, 148−157.
MacLachlan, D. J., & Nugent, J. H. A. (1993) *Biochemistry 32*, 9772−9780.
Messinger, J., Wacker, U., & Renger, G. (1991) *Biochemistry 30*, 7852−7862.
Messinger, J., Badger, M., & Wydrzynski, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3209−3213.
Nugent, J. H. A. (1996) *Eur. J. Biochem. 237*, 519−531.
Nugent, J. H. A., & Evans, M. C. W. (1979) *FEBS Lett. 101*, 101−104.
Nugent, J. H. A., & Turconi, S. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp 455−458, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Nugent, J. H. A., Diner, B. A., & Evans, M. C. W. (1982) *Biochim. Biophys. Acta 682*, 106−114.
Nugent, J. H. A., Demetriou, C., & Lockett, C. J. (1987) *Biochim. Biophys. Acta 894* 534−542.
Ono, T., & Inoue, Y. (1990) *Biochim. Biophys. Acta 1020*, 269−277.
Ono, T., Noguchi, T., Inoue, Y., Kusunoki, M., Matsushita, T., & Oyanagi, H. (1992) *Science 258*, 1335−1337.
Petrouleas, V., & Diner, B. A. (1987) *Biochim. Biophys. Acta 893*, 126−137.
Renger, G. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 45−99, Elsevier Science Publishers, Amsterdam.
Renger, G. (1993) *Photosynth. Res. 38*, 229−247.
Renger, G., & Hanssum, B. (1992) *FEBS Lett. 299*, 28−32.
Rutherford, A. W., Zimmermann, J. L., & Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 179−229, Elsevier Science Publishers, Amsterdam.
Ruzicka, F. J., Beinert, H., Schepler, K. L., Dunham, W. R., & Sands, R. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2886−2890.
Sivaraja, M., Tso, J., & Dismukes, G. C. (1989) *Biochemistry 28*, 9459−9464.
Smith, P. J., & Pace, R. J. (1996) *Biochim. Biophys. Acta 1275*, 213−220.
Styring, S. A., & Rutherford, A. W. (1987) *Biochemistry 26*, 2401−2405.
Styring, S. A., & Rutherford, A. W. (1988) *Biochim. Biophys. Acta 933*, 378−387.
Szalai, V. A., & Brudvig, G. W. (1996) *Biochemistry 35*, 1946−1953.
Tommos, C., Tang, X.-S., Warncke, K., Hoganson, C. W., Styring, S., McCracken, J., Diner, B. A., & Babcock, G. T. (1995) *J. Am. Chem. Soc. 117*, 10325−10335.
van Vliet, P., & Rutherford, A. W. (1996) *Biochemistry 35*, 1829−1839.
van Vliet, P., Boussac, A., & Rutherford, A. W. (1994) *Biochemistry 33*, 12998−13004.
Vermaas, W. F. J., Renger, G., & Dohnt, G. (1984) *Biochim. Biophys. Acta 764*, 194−202.
Warden, J. T., Blankenship, R. E., & Sauer, K. (1976) *Biochim. Biophys. Acta 423*, 462−478.
Yachandra, V. K., DeRose, V. J., Latimer, M. J., Mukerji, I., Sauer, K., & Klein, M. P. (1993) *Science 260*, 675−679.
Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1−15.
Zimmermann, J. L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160−167.
Zimmermann, J. L., & Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 416−423.

BI962179P