Betz, A., Hofsteenge, J., & Stone, S. R. (1991) *Biochem. J. 275*, 801-803.
Betz, A., Hofsteenge, J., & Stone, S. R. (1992) *Biochemistry 31*, 1168-1172.
Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467-3475.
Bode, W., Turk, D., & Stürzebecher, J. (1990) *Eur. J. Biochem. 193*, 175-182.
Braun, P. J., Dennis, S., Hofsteenge, J., & Stone, S. R. (1988) *Biochemistry 27*, 6517-6522.
Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835-840.
Cornish-Bowden, A., & Endrenyi, L. (1981) *Biochem. J. 193*, 1005-1008.
Fersht, A. R. (1987) *Trends Biochem. Sci. 12*, 301-304.
Grütter, M. G., Priestly, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., & Stone, S. R. (1990) *EMBO J. 9*, 2361-2365.
Horovitz, A., Serrano, L., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *J. Mol. Biol. 216*, 1031-1044.
Karshikov, A., Bode, W., Tulinsky, A., & Stone, S. R. *Protein Sci.* (in press).
Knecht, R., & Chang, J.-Y. (1987) *Anal. Chem. 58*, 2375-2379.
Kunkel, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488-492.
Lazar, J. B., Winant, R. C., & Johnson, P. H. (1991) *J. Biol. Chem. 266*, 685-688.
Maschler, R., Steiner, V., Grütter, M. G., & Fritz, R. (1988) Eur. Patent Application 89810414.6, EP-AP0347376.
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 249*, 277-280.
Rydel, T. J., Tulinsky, A., Bode, W., & Huber, R. (1991) *J. Mol. Biol. 221*, 583-601.
Scharf, M., Engels, J., & Tripier, D., (1989) *FEBS Lett. 255*, 105-110.
Segel, I. H. (1975) *Enzyme Kinetics*, Chapter 6, Wiley, New York.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622-4628.
Stone, S. R., Braun, P. J., & Hofsteenge, J. (1987) *Biochemistry 26*, 4617-4624.
Stone, S. R., Dennis, S., & Hofsteenge, J. (1989) *Biochemistry 28*, 6857-6863.
Turk, D., Stürzebecher, J., & Bode, W. (1991) *FEBS Lett. 287*, 133-138.
Wallace, A., Dennis, S., Hofsteenge, J., & Stone, S. R. (1989) *Biochemistry 28*, 10079-10084.
Wells, J. A. (1990) *Biochemistry 29*, 8509-8517.
Winant, R. C., Lazar, J. B., & Johnson, P. H. (1991) *Biochemistry 30*, 1271-1277.

Investigation of the Origin of the "S3" EPR Signal from the Oxygen-Evolving Complex of Photosystem 2: The Role of Tyrosine Z†

Beverly J. Hallahan,‡ Jonathan H. A. Nugent,*,‡ Joseph T. Warden,§ and Michael C. W. Evans‡
Department of Biology, Darwin Building, University College London, Gower Street, London WC1E 6BT, U.K., and Department of Chemistry, Rensselaer Polytechnic Institute, Troy, New York 12180-3590
Received October 31, 1991; Revised Manuscript Received February 21, 1992

ABSTRACT: The origin of the "S3" EPR signal from calcium-depleted photosystem 2 samples has been investigated. This signal is observed after freezing samples under illumination and has been assigned to an interaction between the manganese cluster and an oxidized histidine radical [Boussac et al. (1990) *Nature 347*, 303-306]. In calcium-depleted samples prepared by three different methods, we observed the trapping of the tyrosine radical Y_Z^+ under conditions which also formed the "S3" signal. An "S3"-type signal and Y_Z^+ were also formed in PS2 samples treated with the water analogue ammonia. Following illumination at 277 K, the "S3" and Y_Z^+ signals decayed at the same rate at 273 K in the dark. Both the Y_Z^+ and "S3" signals decayed on storage at 77 K and could be subsequently regenerated by illumination at 8-77 K. No evidence to support histidine oxidation was found. The effects of DCMU, chelators, and alkaline pH on the dark-stable multiline S_2 and the "S3" signals from calcium-depleted samples were determined. Both signals required the presence of EGTA or citrate for maximum yield. The addition of DCMU caused a reduction in the yield of "S3" generated by freezing under illumination. Incubation at pH 7.5 resulted in the loss of both signals. We propose that a variety of treatments which affect calcium and chloride binding cause a stabilization of the S_2 state and slow the reduction of Y_Z^+. This allows the trapping of Y_Z^+, the interaction with the manganese cluster (probably in the S_2 state) resulting in the "S3" signal. The data allow the position of the manganese cluster to be estimated as within 10 Å of tyrosine Z (D1-161).

Electron transfer in photosystem 2 (PS2)[1] follows the absorption of light by the reaction center chlorophyll P680, resulting in the passage of an electron via pheophytin to the primary and secondary plastoquinone acceptors Q_A and Q_B. $P680^+$ is reduced by electrons from the oxygen-evolving com-

† Supported by the U.K. Science and Engineering Research Council (to J.H.A.N. and M.C.W.E.) and by National Institutes of Health (Grant GM 26133 to J.T.W.).
* Address correspondence to this author.
‡ University College London.
§ Rensselaer Polytechnic Institute.

[1] Abbreviations: EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PPBQ, phenyl-1,4-benzoquinone; OEC, oxygen-evolving complex; PS2, photosystem 2; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chl, chlorophyll; H_{pp}, peak to trough line width of EPR spectrum.

0006-2960/92/0431-4562$03.00/0

plex (OEC), which contains a minimum of four Mn atoms [see Babcock et al. (1988) for a review]. The manganese complex is thought to provide the catalytic site for water oxidation and to be the site of charge accumulation, four turnovers of the reaction center being required to produce molecular oxygen. During its cycle, the OEC passes through five different redox states, S_0–S_4 (Kok et al., 1970), four charges being accumulated from S_0 to S_4 and O_2 being evolved at the S_3 to S_0 transition.

Electron transfer between the OEC and P680 is mediated by a tyrosine residue, Y_Z. Site-directed mutagenesis experiments have confirmed that Y_Z is tyrosine-161 of the D1 polypeptide (Debus et al., 1988b; Metz et al., 1989). A second redox-active tyrosine residue, Y_D, is also found on the electron donor side of PS2 and has been identified as tyrosine-160 of the D2 polypeptide (Debus et al., 1988a; Vermaas et al., 1988). Y_D^+ and Y_Z^+ can be observed by EPR as a characteristic EPR line shape termed signal II ($g = 2.0045$, $H_{pp} \approx 1.9$ mT). Signal II has several kinetic forms at physiological temperatures arising from either Y_D^+ or Y_Z^+. Two forms of signal II are from Y_Z^+: signal II very fast (vf) in oxygen-evolving samples and signal II fast (f) in samples where the OEC is blocked [see Babcock et al. (1988)]. Y_Z^+ and Y_D^+ can also be distinguished by their microwave power saturation characteristics which are influenced by interaction with nearby strongly relaxing species (Warden et al., 1976; Yocum & Babcock, 1981).

Both calcium and chloride cofactors have been shown to be required for maximum rates of oxygen evolution [Miyao & Murata, 1984; Ghanotakis et al., 1984; see Coleman (1990) and Yocum (1991) for a review]. The location and number of calcium binding sites, the removal of bound calcium by "calcium depletion" methods, and the effect of these depletion treatments are controversial areas, with a number of conflicting reports in the literature.

In higher plants, each D1/D2 reaction center complex is thought to require a minimum of two calcium atoms in binding sites of different affinities (Ono & Inoue, 1988a; Cammarata & Cheniae, 1987a; Kalosoka et al., 1990). One calcium atom per PS2 is thought to be removed either by NaCl washing in the light, which also results in the loss of the 17- and 23-kDa extrinsic polypeptides (Cammarata & Cheniae, 1987b), or by citrate treatment at low pH in the dark in which the 17- and 23-kDa polypeptides are retained (Ono & Inoue, 1989). It has also been suggested that these treatments do not remove calcium (Shen et al., 1988, 1991). For simplicity, we will refer to the NaCl and citrate treatments as "calcium-depleted".

Thermoluminescence studies on citrate-washed preparations suggested that the S-state cycle was blocked after S_2 formation following calcium depletion (Ono & Inoue, 1989) whereas calcium depletion by NaCl washing in the light appeared to result in a block at the S_3–S_0 transition in EPR studies (Boussac & Rutherford, 1988). This conflict has now been resolved, and the major block appears to be at the S_3–S_0 transition (Boussac et al., 1990b). Calcium depletion has also been shown to slow the kinetics of reduction of photooxidized Y_Z^+ by the Mn cluster (Ghanotakis et al., 1984; Dekker et al., 1984; Ono & Inoue, 1989).

New EPR signals attributed to the OEC were reported by Boussac et al. (1989) in PS2 calcium-depleted by NaCl washing and reconstituted with the 17- and 23-kDa polypeptides. The reconstituted PS2 was shown to have an unusually dark-stable, modified multiline signal ($g = 1.98$, >26 lines spread over 160 mT, $t_{1/2}$ of several hours). The signal was removed by addition of calcium. This dark-stable multiline signal was assigned to a modified form of the normal S_2 state, which gives rise to a multiline signal centered at $g = 2$ attributed to a mixed-valence Mn cluster in an $S = {}^1/_2$ ground state (Dismukes & Siderer, 1980).

Illumination at 273 K and then freezing the calcium-depleted PS2 samples resulted in loss of the modified multiline and generation of a 16.4-mT-wide signal split around the $g = 2$ region, which was assigned to the S_3 state (Boussac et al., 1989). This was supported by experiments showing the appearance of the signal following a single laser flash given to a sample in the S_2 state (Boussac et al., 1990d). It was later shown that the presence of the 17- and 23-kDa extrinsic polypeptides was not necessary for the generation of the modified S_2 or "S3" signals but EDTA, EGTA, or citrate were required in order to observe the dark-stable multiline (Ono & Inoue, 1990b; Boussac et al., 1990b).

Similar EPR signals were observed in citrate-washed samples (Sivaraja et al., 1989; Ono & Inoue, 1990a,b). The citrate-washed preparation was reported to be isolated in an S_1' state, requiring the removal of a single electron to generate the modified S_2 state. Addition of calcium to citrate-washed samples in the dark resulted in the reduction in size of the Y_D^+ EPR signal (Sivaraja et al., 1989; Tso et al., 1991b), an effect not observed with pH 6.5 NaCl-washed samples (Boussac et al., 1989; Lockett et al., 1990) but also observed by Lockett et al. (1990) in pH 8.3 NaCl-washed samples.

It has been suggested that during the S_2–S_3 transition, an oxidation of a histidine residue close to the manganese cluster occurs, the interaction giving rise to the "S3" signal (Boussac et al., 1990d). This might occur (i) as part of the normal S-state transition or (ii) as an auxiliary reaction, resulting from impairment of the normal S_2 to S_3 transition by calcium depletion (Ono & Inoue, 1990b). Boussac et al. (1990d) correlated their EPR data to the oxidation of a histidine residue using transient absorption spectroscopy. Further suggestion of a photooxidizable histidine residue on the donor side of PS2 was provided by the use of the histidine modifier diethyl pyrocarbonate, which resulted in the loss of the thermoluminescence A_T band in Tris-treated PS2 (Ono & Inoue, 1991a,b).

We have further investigated the origin and nature of both the "S3" and modified multiline EPR signals. We have also examined the properties of Y_Z and its relationship to the "S3" signal.

Materials and Methods

Buffers containing MES and sucrose were treated with Chelex prior to the addition of NaCl and EGTA to remove any calcium impurities. All samples were stored at 77 K before use.

PS2 membranes [BBYs; Berthold et al., 1981; P680:Chl ratio 1:(225–250)] were prepared from market spinach by the modified method of Ford and Evans (1983) and were depleted of calcium according to a modified method of Boussac et al. (1989). BBYs were washed in 0.3 M sucrose, 20 mM MES, 10 mM NaCl, and 2 mM EGTA, pH 6.5, and then resuspended to a concentration of 2 mg of Chl/mL in 0.3 M sucrose, 20 mM MES, 2 M NaCl, and 1 mM EGTA; 50 μM PPBQ was added, and the membranes were stirred on ice under room light for 30 min, before being diluted with an equal volume of 0.3 M sucrose, 20 mM MES, 2 M NaCl, and 40 mM EGTA, pH 6.5. The membranes were dialyzed against 0.3 M sucrose, 20 mM MES, and 2 M NaCl, pH 6.5, under room light for 1 h. The calcium-depleted PS2 membranes were then collected by centrifugation or further dialyzed against 0.3 M sucrose/20 mM MES for 3 h in the dark, in order to reconstitute the 17- and 23-kDa extrinsic polypeptides.

The membranes were centrifuged at 40000*g* for 30 min and resuspended in 0.3 M sucrose, 20 mM MES, 10 mM NaCl, and 100 μM EGTA, pH 6.5.

Calcium removal from BBYs by low-pH citrate wash was by the method of Ono and Inoue (1988a). BBYs were washed in 0.4 M sucrose, 0.1 mM MES, and 20 mM NaCl, pH 6.5. After centrifugation at 40000*g* for 30 min, the membranes were resuspended to a concentration of 2 mg of Chl/mL in 0.4 M sucrose, 10 mM citrate, and 20 mM NaCl, pH 3.0. The PS2 membranes were stirring on ice in the dark for 5 min and then diluted 5 times with 0.4 M sucrose, 40 mM MES, and 20 mM NaCl, pH 6.5. The membranes were then collected by centrifugation at 40000*g* for 30 min and resuspended in the same buffer.

The membranes obtained and used as a result of these treatments were depleted of greater than 75% of their oxygen-evolving activity (measured in a Clark-type oxygen electrode at 298 K, using depleted membranes to which 20 mM calcium was added as control).

In experiments designed to examine the effects of chelators, calcium was depleted from PS2 in the presence of 50 μM EGTA by a method similar to that of Boussac et al. (1990b). BBYs were resuspended to 0.5 mg of Chl/mL in 0.3 M sucrose, 25 mM MES, and 2 M NaCl, pH 6.5; 50 μM PPBQ was added, and the membranes were incubated under room light for 30 min; 50 μM EGTA was then added, and the membranes were centrifuged at 40000*g* for 30 min. The membranes were washed in 0.3 M sucrose, 25 mM MES, 30 mM NaCl, and 50 μM EGTA, pH 6.5, and finally resuspended in the same buffer.

In order to remove calcium bound with high affinity, calcium depletion was performed according to the method of Kalosaka et al. (1990) using the ionophore A23187. This involves extraction at pH 5.0 followed by repeated washings with the ionophore. These samples were finally resuspended in 0.4 M sucrose, 10 mM MES, and 15 mM NaCl, pH 6.0.

To examine the effects of increased pH on the modified multiline and "S3" EPR signals, pH 6.5 NaCl-washed PS2 was centrifuged at 40000*g* for 30 min and then resuspended in 0.3 M sucrose, 20 mM HEPES, 10 mM NaCl, and 100 μM EGTA, pH 7.5. The membranes were again centrifuged, and the pellet obtained was resuspended in the same buffer.

Ammonia-treated samples were prepared under conditions which are intended to minimize effects on the chloride-dependent site. This includes the presence of high chloride concentrations and the absence of sucrose (Boussac et al., 1990a). BBYs were washed in 40 mM HEPES, 20 mM NaCl, and 1 mM EDTA, pH 7.5. The membranes were centrifuged at 40000*g* for 30 min and resuspended in 40 mM HEPES, 15 mM NaCl, 5 mM $MgCl_2$, and 20% (v/v) glycerol, pH 7.5. For EPR, samples contained 1 mM PPBQ and 100 mM NH_4Cl to give a free base NH_3 concentration of 2 mM (Britt et al., 1989). To increase the number of reaction centers to which the slow binding ammonia is bound, dark-adapted samples were frozen and then illuminated at 200 K for 10 min in an ethanol/dry ice bath. The samples were then placed in a water bath at 296 K for 30 s in the dark, followed by rapid freezing at 200 K ["annealing"; see Britt et al. (1989)]. This process forms S_2 at 200 K, the subsequent warming of the sample allowing a more efficient binding of ammonia than is found during transient formation of S_2 during S-state turnover on illumination at 277 K.

EPR Spectrometry. For EPR at cryogenic temperatures, 0.3-mL samples (at approximately 5 mg of Chl/mL) were placed in 3-mm-diameter EPR tubes. Samples were illuminated with a 650-W light source through a 2.5-cm water heat filter for 30 s to generate the dark-stable S_2 state in calcium-depleted samples. Samples were treated as described in the figure legends and then dark-adapted for 30 min before being frozen to 77 K. Additions of DCMU and PPBQ were made from freshly made concentrated stock solutions in dimethyl sulfoxide. To obtain the "S3" signal, samples were illuminated at 277 K for 45 s using an unfiltered 650-W light source (1000 μE m^{-2} s^{-1}) and then frozen to 77 K under illumination. During dark-adaptation, samples were kept on ice at 273 K.

Identical sets of samples in calibrated EPR tubes were made for each experiment using the same preparation and chlorophyll concentration. This enabled control samples to be stored. Using the signals present in these control samples (cytochrome *b*-559 etc.), the EPR spectrometer and measurement conditions were calibrated for experiments which involved EPR data collection on different days.

EPR spectrometry was performed using a Jeol RE1X spectrometer fitted with an Oxford Instruments liquid helium cryostat. Illumination in the EPR cavity was done using a 150-W light source connected to the front of the cavity by a flexible light guide. EPR conditions are given in the figure legends.

For room temperature EPR, 15-μL samples in glass capillary tubes were placed inside EPR tubes. These samples were run as above using the Oxford Instruments cryostat. Spectra were recorded and manipulated using a Dell microcomputer running Asyst software. Y_Z^+ and Y_D^+ were quantified by comparing samples illuminated before and after the addition of 20 mM calcium and by comparing spectra taken during illumination with spectra taken following a 5-min dark period after illumination.

EPR studies at room temperature were also performed with a Varian E-9 spectrometer interfaced to a PDP-11/23A minicomputer operating under Berkeley Software Distribution Unix (TM) (BSD 2.9) compiled with real-time process extensions. Spectra (512 points) were acquired at 0.25-mT magnetic field modulation. Typical acquisition dwell times were 0.5 s per point with a corresponding instrument time constant of 1 s. Actinic illumination of the sample (400–700 nm at 6000 W m^{-2}) was provided by a 1000-W tungsten–halogen source. Two hundred microliter samples were analyzed using a standard Varian flat cell. To permit quantitation of both dark-stable and light-inducible resonances, paired EPR spectra (dark control and illuminated) were acquired as a function of microwave power over the range of 1–144 mW. The protocol for data collection for the dark control consisted of a 20-s preillumination followed by a 30-s dark interval prior to the magnetic field scan. The spectrum for the illuminated sample was obtained subsequent to the dark control scan.

$P_{1/2}$ (the microwave power for half-saturation) was determined graphically as in Styring and Rutherford (1988) from a plot of log (I/P) vs log P, where P is the microwave power and I is the signal amplitude measured as the base line to the lowest field peak of the tyrosine radical [$I = kP_{1/2}/(1 + P/P_{1/2})^{0.5b}$]. $P_{1/2}$ 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.

The microwave power saturation studies at cryogenic temperatures were performed using 25-kHz modulation on the Jeol RE1X spectrometer. This was the lowest available modulation frequency on our spectrometer and was used in order to minimize rapid-passage effects. Rapid-passage conditions are an EPR phenomenon that occurs when the spin–lattice relaxation, T_1^{-1}, is slow compared to the modulation frequency

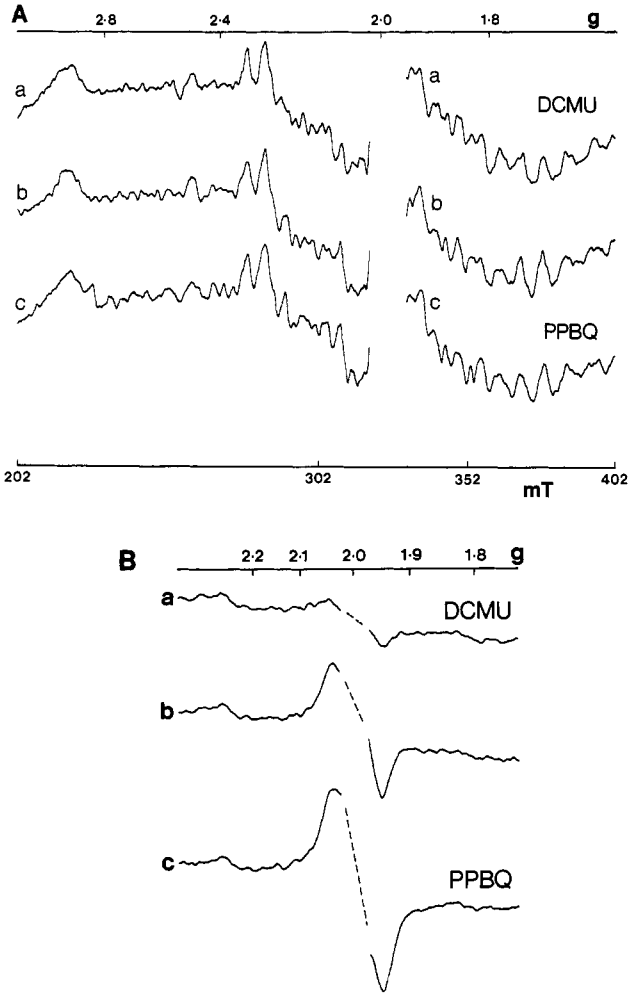

FIGURE 1: EPR spectra showing the effects of DCMU on the formation of the dark-stable multiline S_2 state and the "S3"-split signal. (A) Dark-stable multiline in samples of NaCl-washed, calcium-depleted PS2, illuminated at 277 K and then dark-adapted for 30 min. Samples contain 5 mM EGTA: (a) containing 0.5 mM DCMU; (b) no additions; (c) 0.5 mM PPBQ. Conditions: Microwave power, 10 mW; modulation amplitude, 1.25 mT; temperature, 10 K. (B) "S3"-split signal in samples from panel A, scans a–c, thawed and frozen to 77 K under illumination. EPR conditions as (A) except microwave power 5 mW and gain in (A) 2.5 × gain in (B). The large signal at $g = 2$ from tyrosine radicals has been removed; the dashed line shows which spectra are joined and is not intended to suggest the $g = 2$ line shape.

used (normally 100 kHz). This can lead to errors in the measurement of the $P_{1/2}$ value. Rapid-passage effects on the Y_D^+ signal were observed at cryogenic temperatures by Styring and Rutherford (1988) using 100-kHz modulation, but they were diminished by use of lower modulation frequencies.

The area of the signal from Y_D^+ obtained by double integration was assumed to represent one spin per photosystem 2 center (Miller & Brudvig, 1991) in quantitations of other free radical signals except for Y_Z^+, the method for which is described under Results.

Results

As has previously been demonstrated (Boussac et al., 1989, 1990c), NaCl washing of PS2 at pH 6.5 in the light results in the formation of a dark-stable multiline EPR signal (Figure 1A). The presence of a chelator, either EGTA or citrate, was required in order to observe this signal (Ono & Inoue, 1990b; Boussac et al., 1990b). We routinely illuminated calcium-depleted samples at 277 K before further treatment in order to ensure conversion to the dark-stable multiline S_2 state. Further illumination of the sample at 277 K and then freezing under illumination to 77 K cause the almost complete loss of the dark-stable multiline signal and the appearance of the signal split around $g = 2$ attributed to "S3" (Figure 1B) (Boussac et al., 1989). EPR signals from other PS2 components were also observed. Peaks near $g = 3$ and $g = 2.2$ (Figure 1A) are the g_z and g_y resonances of cytochrome *b*-559. The $g = 1.8$ form of Q_A semiquinone was found in all types of calcium-depleted preparations, indicating that a loss of bicarbonate binding near the non-heme iron occurred during these treatments (not shown). The bicarbonate-bound form of Q_A semiquinone in untreated PS2 membranes is found near $g = 1.9$ (Hallahan et al., 1991).

Effect of DCMU on the "S3" Signal and Dark-Stable Multiline. The "S3" signal can be generated as the result of a one-electron oxidation of a sample showing the dark-stable multiline S_2 state (Boussac et al., 1990d). Previous studies have also shown the requirement for an exogenous electron acceptor, PPBQ or dimethylbenzoquinone, acting at the Q_B site, in order to obtain a high yield of "S3" (Boussac et al., 1989; Tso et al., 1991b). The Q_B site inhibitor DCMU has, however, been used in EPR, thermoluminescence, and optical studies on calcium-depleted samples which searched for changes occurring in parallel to the appearance of the "S3" signal (Ono & Inoue, 1990a,b; Boussac et al., 1990d). These studies assumed there was no effect of DCMU on "S3" formation. We have now characterized the effect of DCMU on the formation of the "S3" signal.

Figure 1B shows that the "S3" signal was either greatly reduced in size (Figure 1B, trace a) or absent in NaCl-washed PS2 that had been treated with 0.5 mM DCMU prior to freezing to 77 K under illumination. The size of the dark-stable multiline remained unchanged by the treatment (Figure 1A, traces a–c) as observed previously (Ono & Inoue, 1990a,b). The turnover of the PS2 reaction center and the block at the Q_A to Q_B transfer were confirmed by the increase in the yield of the Q_A iron–semiquinone EPR signal in DCMU-treated samples (not shown).

Figure 1B, trace b, shows that about 50% of the maximum yield of the "S3" signal was obtained without addition of exogenous acceptor. The addition of exogenous acceptor was required to achieve maximum signal size (Figure 1B, trace c). In a recent study, we have shown that Q_B is present in a high percentage (up to 50%) of the reaction centers in our PS2 preparations (Hallahan et al., 1991). Variability in the levels of Q_B between different PS2 preparations may account for the greater yield of "S3" signal in our untreated samples than the 10% found by Tso in the absence of exogenous acceptor (Tso et al., 1991b). Q_B semiquinone is generated by our pretreatment illumination step (Hallahan et al., 1991), and this may account for the relatively high level of DCMU required to abolish the "S3" signal, as the semiquinone species binds more tightly to the Q_B site (Crofts & Wraight, 1983). Substantial reduction in "S3" yield was achieved by addition of DCMU at lower levels or to samples containing PPBQ (not shown).

Decay of the "S3" Signal at Cryogenic Temperatures. When calcium-depleted PS2 was frozen under illumination to trap the "S3" signal and the sample then stored at 77 K in the dark, the "S3" signal decayed. Figure 2b shows that after 7-days storage at 77 K, the signal had decayed by more than 50%. The extent of decay of the "S3" signal varied between calcium-depleted preparations. Both citrate-washed and NaCl-washed preparations exhibited this decay. Presumably this occurs by reduction of the oxidized organic

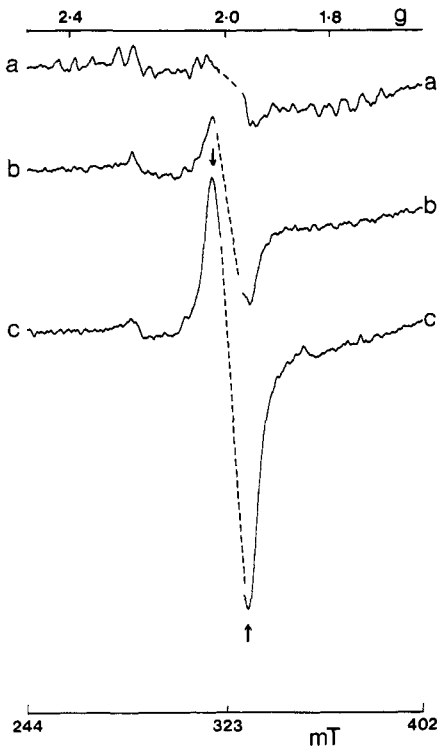

FIGURE 2: EPR spectra showing the decay of the "S3" signal on storage at 77 K in the dark. Samples were NaCl-washed, calcium-depleted PS2 containing 0.5 mM PPBQ and 5 mM EGTA. (a) Illuminated at 277 K and then dark-adapted for 30 min. (b) Frozen under illumination and stored at 77 K for 7 days in the dark. (c) Frozen under illumination and spectrum recorded prior to storage. EPR conditions: microwave power, 5 mW; temperature, 10 K; modulation width, 1.25 mT. The arrows mark the *g*-value positions used for amplitude measurements.

radical or manganese cluster at 77 K by either PS2 electron acceptors or another redox component such as cytochrome *b*-559.

When samples containing DCMU were frozen under illumination, the dark-stable multiline S_2 signal was retained, and increased levels of Q_A semiquinone were generated (not shown). No decay of the dark-stable multiline was observed on storage at 77 K in these samples, Q_A semiquinone decayed slowly with parallel reduction of Y_D^+. This indicates that in calcium-depleted samples with no DCMU added, the decay of the "S3" signal on storage at 77 K was unlikely to be by reduction of the S_2 state of the manganese cluster by Q_A.

The decrease in the size of the "S3" signal on 77 K storage in the dark was not accompanied by a parallel increase in the size of the dark-stable multiline (Figure 2b). Figure 2 shows an example where more than 50% decay of the "S3" signal (Figure 2b) produces only a slight increase in the dark-stable multiline. Although this relationship was difficult to quantify, similar results in several samples confirmed this observation. Therefore, the loss of the dark-stable multiline was not simply caused by the magnetic interaction of S_2 with the oxidized organic radical as indicated previously (Boussac et al., 1989). This result also suggests that an S state higher than S_2 may be formed by room temperature illumination. However, experiments using single-turnover flashes indicate that only the one-electron-transfer step is required to generate "S3" in samples containing the dark-stable multiline (Boussac et al., 1990d). Taken together, the results suggest that conformational changes to the manganese cluster may occur on freezing under illumination and are trapped at 77 K.

Formation of the "S3" Signal at Cryogenic Temperatures. Illumination at 77 K produces one turnover of the PS2 reaction

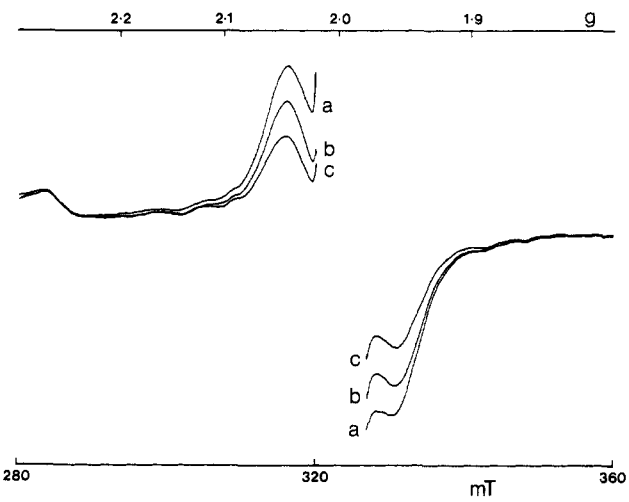

FIGURE 3: EPR spectra showing the properties of the "S3" signal at cryogenic temperatures. A NaCl-washed, calcium-depleted sample containing 5 mM EGTA and 0.5 mM PPBQ was frozen under illumination and stored for 1 week at 77 K. Spectra recorded (a) following 1-min illumination at 10 K, (b) following 7 min in the dark at 10 K following illumination at 10 K, and (c) before illumination at 10 K. EPR conditions as in Figure 2.

center. In both citrate-washed and NaCl-washed PS2 samples, illumination at 8–77 K of a 77 K stored, "S3"-decayed sample resulted in the complete restoration within 15 s of the "S3" signal (Figure 3) with parallel photoreduction of Q_A semiquinone (not shown). The "S3" signal is not formed by 8–200-K illumination of a 273-K dark-adapted sample [not shown, but see Boussac et al. (1989)]. The data suggest that another process accompanies the original oxidation of the organic radical, a process which requires an illumination temperature of greater than 200 K. This change is "frozen in" at 77 K or below.

When a 77-K "S3"-decayed sample was transferred to 200 K for 15 min and returned to 77 K, the ability to regenerate the "S3" signal was lost, and the yield of the dark-stable multiline was increased (not shown). Such temperature sensitivity suggests that the additional event may be a structural change, such as loss or gain of a proton or other ligand.

Following the restoration of the "S3" by illumination below 77 K, a more rapid partial decay of the signal was observed (Figure 3b). The decay, which was accompanied by a parallel loss of the Q_A semiquinone, had a $t_{1/2}$ of approximately 10 min at 10 K. This is more rapid than the reduction of Y_D^+ by Q_A semiquinone which has been estimated at $t_{1/2}$ 50–85 h at 77 K (Nugent et al., 1982, 1987; Kawamori et al., 1987; Inui et al., 1989).

Organic Radicals Observed at Room Temperature. Possible models for the "S3" signal are provided by interactions between organic radicals and transition metals. In PS2, examples of these are the interaction between the Q_A semiquinone and the non-heme iron Fe^{2+} (Butler et al., 1984; Dismukes et al., 1984; Nugent et al., 1992) and the interaction between the Q_A iron–semiquinone and reduced pheophytin (Klimov et al., 1980). We therefore searched for an EPR signal that could be assigned to an organic radical formed on the electron donor side of PS2 and which interacts with the manganese cluster to produce the "S3" signal.

As shown previously for citrate-washed samples (Ono & Inoue, 1989; Ono et al., 1991), the kinetics of Y_Z^+ reduction are slowed in samples which have been calcium-depleted by NaCl washing. This enables the Y_Z^+ radical to be detected on illumination at room temperature (Figure 4). Initially, 0.5 mM PPBQ was included in these samples as an exogenous

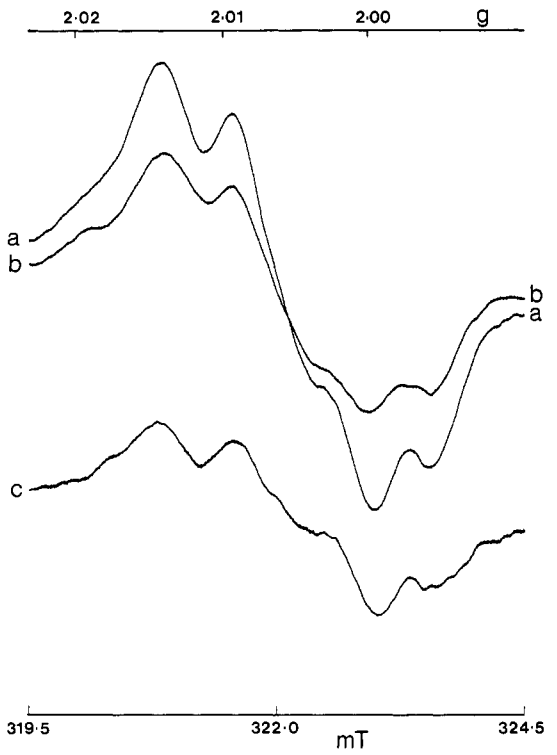

FIGURE 4: EPR spectra of tyrosine radicals at room temperature in NaCl-washed, calcium-depleted PS2. Sample contains 5 mM EGTA, 1 mM ferricyanide, and 1 mM ferrocyanide as electron acceptor. (a) Sample during illumination at 295 K and (b) sample after dark-adaptation for 10 min following illumination showing Y_D^+. (c) Difference spectrum (a − b) showing light-induced Y_Z^+. EPR conditions: microwave power, 20 mW; modulation width, 0.25 mT.

electron acceptor, but under constant illumination, a large PPBQ semiquinone signal appeared in the spectrum at $g = 2$ (not shown). When calcium was added back to the samples, allowing multiple turnovers, the semiquinone signal and Y_Z^+ were not observed on illumination.

To overcome the problems when using PPBQ, 1 mM ferricyanide/1 mM ferrocyanide was used as the exogenous electron acceptor (Hoganson & Babcock, 1988). Figure 4b shows the $g = 2$ spectrum at 295 K in the dark, 10 min after illumination. This is the spectrum of Y_D^+, the stable tyrosine radical. It has a line width of 1.95 ± 0.01 mT and has a $P_{1/2}$ of 27.5 mW. On illumination, Y_Z^+ was formed within the time constant of the spectrometer, superimposed on the spectrum of Y_D^+ (Figure 4a). Y_Z^+ (Figure 4c) had a line width in the calcium-depleted samples of 2.03 ± 0.01 mT, slightly broader than Y_D^+, and had a $P_{1/2}$ of >169 mW. This increase in $P_{1/2}$ shows that Y_Z^+ is undergoing spin relaxation by a neighboring paramagnet, a situation previously observed for Y_Z^+ (Warden et al., 1976; Yocum & Babcock, 1981). Y_Z^+ exhibited a biphasic decay following illumination, 80% of the signal decaying with a $t_{1/2}$ of approximately 1.5 s and 20% with a $t_{1/2}$ of approximately 60 s. Addition of 0.5 mM DCMU or 20 mM calcium was found to suppress the formation of Y_Z^+.

The slow phase of Y_Z^+ reduction required that a short dark-adaptation be performed before Y_D^+ could be measured. The major cause of slow changes to Y_D^+ in the dark in untreated PS2 samples is the oxidation of S_0 to S_1 by Y_D^+ (Vermass et al., 1984). In order to investigate possible changes in Y_D^+ during the short dark period, illuminated and dark-adapted spectra were recorded after addition of 20 mM calcium (which eliminates Y_Z^+) and compared to spectra taken of the same sample before addition of calcium. We conclude that no major changes in Y_D^+ occurred during the short dark period, probably because the manganese cluster was stabilized in S_2.

Quantitation of the amount of Y_Z^+ formed relative to dark-stable Y_D^+ was performed according to the analysis of Randolph (1972). This determination, which compensates for the variance in microwave power dependencies for the two radicals, indicates that Y_Z^+ is produced in a (0.8 ± 0.1):1 ratio with Y_D^+. This shows that calcium-depleted samples can undergo a single electron transfer from Y_Z^+ to the electron acceptors of PS2. Therefore, the electron-transfer chain between P680 and Y_Z remains competent after calcium depletion, and the point of inhibition lies between Y_Z and the OEC. The inability to detect Y_Z^+ in samples supplemented with calcium suggests that calcium restores Y_Z^+ reduction by the OEC.

In addition to the observation of high yields of Y_Z^+, an important finding from this experiment was that no radical other than Y_D^+ and Y_Z^+ could be detected.

Organic Radicals Observed at Cryogenic Temperature. The presence of Y_Z^+ under the conditions used to trap the "S3" signal suggested that Y_Z^+ would also be trapped by illumination and freezing to 77 K. At 50 K in a 10-min dark-adapted sample, reaction centers contain Y_D^+ (Figure 5A). An increased yield of tyrosine radical (measured at 50 K) was obtained by freezing under illumination (Figure 5A). This increase in total yield of tyrosine radical was abolished by addition of 20 mM calcium or greatly reduced by addition of 0.5 mM DCMU to the sample prior to illumination at 277 K, indicating that it was not due to Y_D^+.

It has been shown that the microwave power saturation of Y_D^+ is influenced by the S state of the OEC (Styring & Rutherford, 1988). Boussac et al. (1989) have measured the relaxation properties of Y_D^+ in NaCl-washed preparations at 15 K and showed a $P_{1/2}$ (100 kHz) of 0.25 mW in the presence of the dark-stable multiline, 0.25 mW in the presence of the "S3" signal, and 0.125 mW in the S_1 state formed following calcium addition. The values for the dark-stable S_2 and "S3" states were similar to those found in untreated PS2 (Styring & Rutherford, 1988). Since Y_Z^+ is closer to the manganese cluster, it is expected to have higher $P_{1/2}$ values than Y_D^+. To distinguish Y_Z^+ from Y_D^+ at 50 K in our samples, we performed microwave power saturation studies.

Figure 5B shows the microwave power saturation properties of both the signal present in the dark and the additional radical obtained on freezing under illumination. This reveals that the signal generated by freezing under illumination had a significantly greater $P_{1/2}$ value at 50 K (3 mW at 100 kHz, 3.9 mW at 25 kHz) than the dark-adapted sample containing Y_D^+ (0.3 mW at 100 kHz, 0.6 mW at 25 kHz). This is consistent with the identification of the signal as being Y_Z^+ as it has both the expected line shape and microwave power characteristics. The radical formed in citrate and NaCl-washed samples had very similar properties. This observation represents the first indication that Y_Z^+ may be trapped at cryogenic temperatures.

Quantification of the amount of the trapped Y_Z^+ gave an estimate of (0.25 ± 0.05):1 Y_D^+.

Figure 5A also demonstrates the effect of the power saturation differences of Y_D^+ and Y_Z^+ on the line shape of the two signals, the spectrum in Figure 5A trace b being the line shape of Y_Z^+ at 1 mW and Figure 5A trace c the line shape of Y_D^+ at 0.005 mW. At 1 mW, the line shape of Y_D^+ is distorted by power saturation (Figure 5A, trace a, inner line).

The radical assigned to Y_Z^+ could also be observed at 10 K which will be discussed below. No other organic radical was detected except for a small and variable yield (<10% of reaction centers) of a radical previously assigned to oxidized

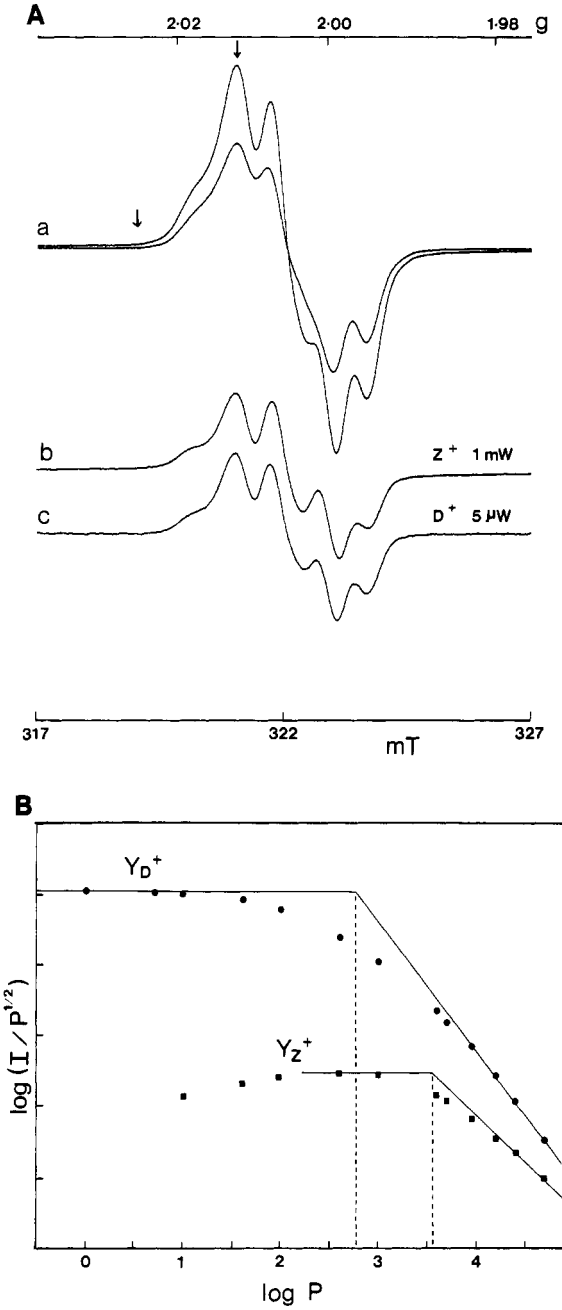

FIGURE 5: Characteristics of tyrosine radicals at 50 K. (A) Spectrum a: the inner line shows the EPR spectrum of Y_D^+ in a calcium-depleted sample dark-adapted for 10 min following illumination. The sample contains 0.5 mM PPBQ and 5 mM EGTA. The outer line shows the spectrum of the same sample after freezing under illumination. The light-induced difference spectrum attributed to Y_Z^+ is shown in trace b. EPR conditions: microwave power, 1 mW; temperature, 50 K; modulation amplitude, 0.25 mT. Trace c shows the spectrum of Y_D^+ with the same EPR conditions except a microwave power of 5 μW. The arrows indicate the g-values used in signal amplitude measurements. (B) Microwave power saturation of tyrosine radicals at 50 K. The graph shows the microwave power saturations of Y_Z^+ and Y_D^+ at 25-kHz modulation frequency. The curves have been displaced vertically for clarity and are not intended to show relative amounts of the two radicals. The dashed lines show the $P_{1/2}$ values (Y_D^+ = 0.6 mW, Y_Z^+ = 3.9 mW).

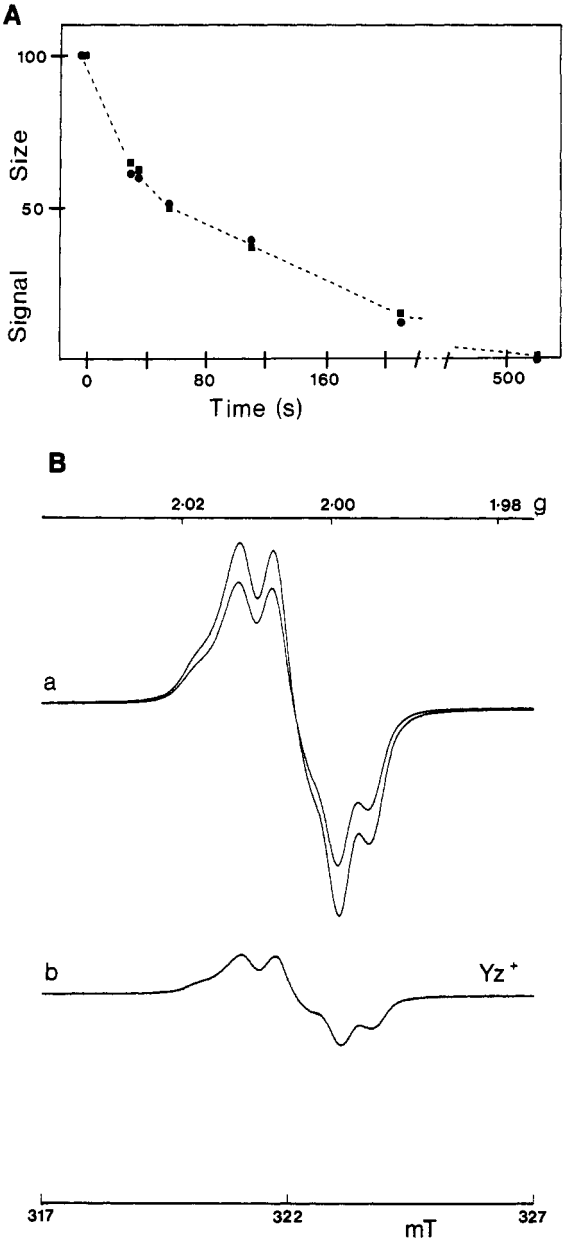

FIGURE 6: (A) Decay of "S3" and Y_Z^+ at 277 K following illumination of a NaCl-washed, calcium-depleted sample. The sample contained 5 mM EGTA and 0.5 mM PPBQ. The sample was illuminated and frozen to 77 K after the times shown, and the two EPR signals were measured. "S3" signals (circles) were measured at 10 K as in Figure 2 and Y_Z^+ (squares) at 50 K as in Figure 5A. (B) EPR spectra showing the decay of Y_Z^+ following illumination at 50 K. A NaCl-washed sample containing 0.5 mM PPBQ and 5 mM EGTA was frozen under illumination and stored for 1 week at 77 K in the dark. Spectrum a: the outer line shows the tyrosine radicals at g = 2 following illumination of the sample at 10 K for 1 min. The inner line shows the spectrum 20 min after illumination. Spectrum b is the difference spectrum showing the line shape of the decayed signal Y_Z^+. EPR conditions as in Figure 5A.

chlorophyll (Nugent et al., 1982).

Relationship between Y_Z^+ and the "S3" Signal. The detection of Y_Z^+ in samples which also contain an "S3" signal suggested that Y_Z^+ could be the organic radical interacting with the manganese cluster. Figure 6A shows the rates of decay following 277-K illumination to form the "S3" signal (measured at 10 K) and the fast-relaxing Y_Z^+ species (measured at 50 K), using a single sample. The signals were trapped by freezing to 77 K at the times shown after illumination, with a series of illuminations being done on each sample. This decay pattern was confirmed by measurements on several samples. The rates of decay of the two EPR signals are essentially identical. The reduction in maximum yield of the "S3" signal from an accumulated illumination of 7 min per sample was <5%, showing that photoinhibition did not affect the experiment. Analysis of several NaCl and ci-

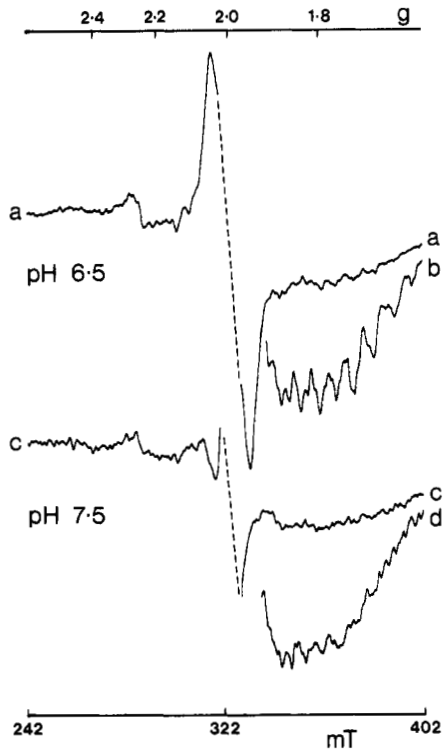

FIGURE 7: EPR spectra of PS2, calcium-depleted by NaCl washing. (a, b) At pH 6.5; (c, d) at pH 7.5. Spectra a and c show the yield of "S3" signal in samples frozen under illumination; spectra b and d illustrate the amount of dark-stable multiline S_2 in 30-min dark-adapted samples. EPR and sample conditions for the "S3" and S_2 signals are as given in Figure 1.

trate-washed preparations having different yields of the "S3" signal indicated a 1:1 stoichiometry between the amount of the two signals trapped by illumination and freezing to 77 K.

Figure 6B shows the spectrum at $g = 2$ of a sample in which the "S3" signal was generated by freezing under illumination and then allowed to decay for 7 days at 77 K. On illumination at 10 K, which restores the "S3" signal (not shown, but see Figure 3), Y_Z^+ (identified by its microwave power saturation properties) was generated and superimposed on the Y_D^+ radical (Figure 6B, trace a, outer line). The subsequent rapid decay of the "S3" signal following illumination at 10 K (see Figure 3) was also accompanied by a decay of a similar percentage of the trapped Y_Z^+ radical (Figure 6B, trace a, inner line, and trace b) and photoreduced Q_A^- (not shown). As discussed above, this recombination of Y_Z^+ with Q_A^- is significantly faster than the decay of Y_D^+/Q_A^- (Nugent et al., 1982, 1987; Kawamori et al., 1987; Inui et al., 1989), supporting the assignment.

The radical assigned to Y_Z^+ therefore has the characteristics expected of a radical interacting with the manganese cluster of the OEC to produce the "S3" signal.

Effect of pH on NaCl-Washed, Calcium-Depleted PS2. Incubations of NaCl-washed PS2 at various pH values revealed that the formation of the dark-stable multiline and the "S3" signals was sensitive to pH. The signals were reduced in size at alkaline pH, and the formation of both signals was greatly decreased by centrifugation and resuspension of the sample at pH 7.5 (Figure 7). Further experiments suggested that the length of exposure of the sample to alkaline pH was a critical factor. Some restoration of the signals could be achieved by titration of the sample to pH 7.5 and then returning to pH 6.5. However, 60-min exposure to pH 7.5 resulted in loss of signals even on return to pH 6.5. The dark-stable tyrosine radical Y_D^+ concentration was also lowered by increasing the pH to 7.5, but Y_Z^+ was still formed on illumination at 295 K (not shown). These results indicate that a loss of integrity of the OEC occurred at pH >7, a much lower pH than found in untreated PS2 membranes, which at pH 7.5 gave a normal multiline EPR spectrum (Beck et al., 1986; Boussac et al., 1990b).

Effect of Chelators on the EPR Characteristics of Calcium-Depleted Preparations. As reported by Boussac et al. (1990b), we confirmed that the presence of the extrinsic 17- and 23-kDa polypeptides was not necessary to generate either the dark-stable multiline or the "S3" signal. The absence of the polypeptides resulted in a narrower splitting of the "S3" signal (Boussac et al., 1990b). When compared directly, the "S3" signal from citrate-washed PS2 had a broader line shape and splitting (18 vs 16 mT) than that seen in NaCl-washed PS2 reconstituted with the extrinsic 17- and 23-kDa polypeptides. The assignment of the "S3" signal to an organic radical magnetically interacting (exchange and/or dipolar coupled) with the manganese cluster (Boussac et al., 1989; Sivaraja et al., 1989) suggests that these changes in splitting will perhaps involve slight structural differences between the interacting species.

In agreement with a previous report (Boussac et al., 1990b), the "S3" signal could still be generated in salt-washed samples prepared in the presence of only 50 μM EGTA, but we observed the signal only in a small percentage of reaction centers. We found that additional EGTA was required in order to observe the maximum yield of this signal (Figure 8). The ability of EGTA to greatly increase the yield of "S3" signal was saturated at approximately 1 mM EGTA. The yield of dark-stable multiline was an indicator of the yield of "S3" signal obtained following illumination at 277 K (Figure 8). The samples prepared with 50 μM EGTA had a lower level of calcium depletion as indicated by retention of 30–40% of the oxygen-evolving activity of calcium-treated samples.

We investigated the possibility that these results were caused by a failure to deplete calcium at low EGTA concentrations. Citrate is unable to remove calcium from untreated PS2 membranes at pH 6.5 (Ono & Inoue, 1988a). As shown in Figure 8, the addition of 40 mM citrate to the NaCl-washed sample containing 50 μM EGTA gave rise to an increased S_2 and "S3" signal size, although the signals were smaller than those observed in the presence of 10 mM EGTA.

To ensure a more complete calcium depletion, samples prepared using the ionophore A23187 (Kalosaka et al., 1990) were examined for the presence of dark-stable multiline and the ability to form the "S3" signal. This treatment produced samples with less than 10% of the oxygen rates of calcium-treated samples. Without addition of EGTA to the EPR sample, a low yield of both the dark-stable multiline (in samples frozen in the dark) and "S3" signals (in samples frozen under illumination) was obtained. This may be due to the inclusion of 1 mM EGTA in some stages of sample preparation by this method. In the presence of 5 mM EGTA, the yield of dark-stable multiline was substantially increased, and the yield of "S3" signal and Y_Z^+ was doubled (not shown). The properties of the dark-stable multiline and the "S3" signal were very similar to those seen in pH 6.5 NaCl-washed samples.

These findings show a relationship between the chelators EGTA and citrate and the appearance of the "S3" signal, perhaps indicating a direct effect of chelator on the manganese cluster in calcium-depleted samples.

Detection of a Split "S3"-Type EPR Signal in PS2 Treated with Ammonia. The effects of ammonia as an inhibitory analogue of water have been investigated in several labora-

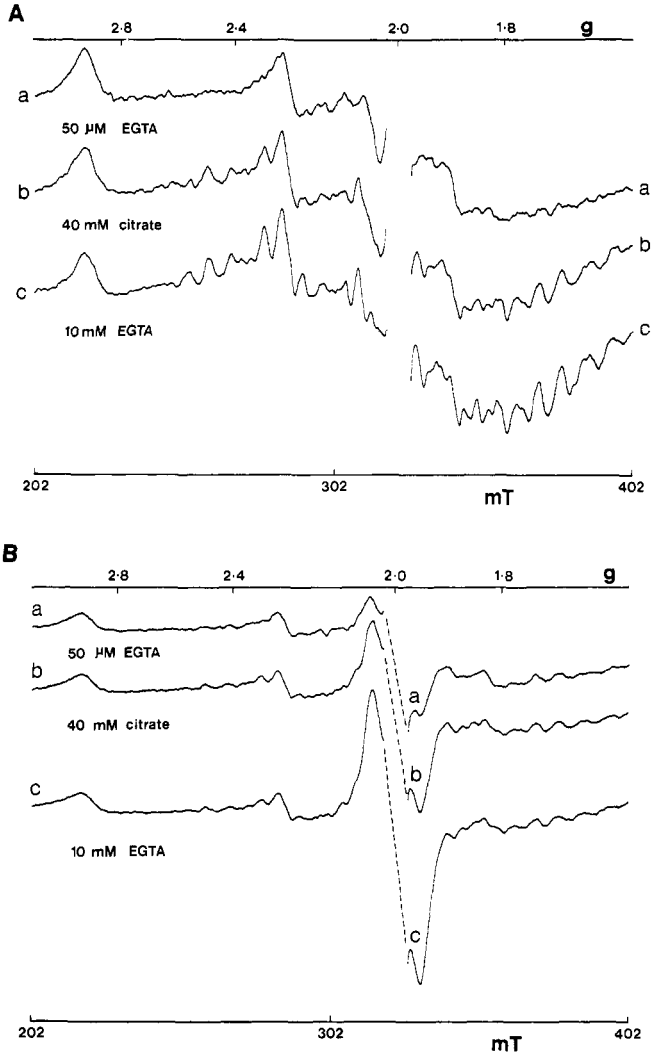

FIGURE 8: EPR spectra showing the effect of chelators on the dark-stable multiline S_2 state and the "S3" signal. (A) Samples illuminated and then dark-adapted for 30 min. (B) Samples frozen under illumination. (a) Containing 50 μM EGTA; (b) containing 50 μM EGTA plus 40 mM citrate; (c) containing 10 mM EGTA. EPR and sample conditions as in Figure 1.

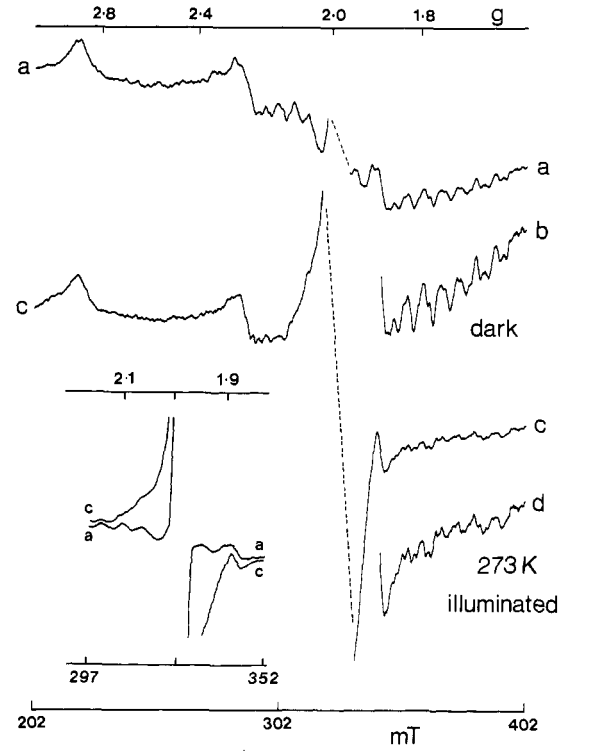

FIGURE 9: EPR spectra showing an "S3"-type signal in PS2 treated with 100 mM ammonium chloride at pH 7.5. (a) 30-min dark-adapted sample; (b) as in (a) but gain increased to show stable multiline S_2; (c) sample frozen under illumination; (d) as in (c) but gain increased as above to show lower yield of dark-stable multiline. The inset shows spectra a and c in the $g = 2$ region and illustrates the broad radical formed by freezing under illumination. EPR conditions as Figure 2.

tories. Ammonia binds to at least two sites in the OEC, one in competition with and one independent of chloride binding (Isawa et al., 1969; Sandusky & Yocum, 1983, 1984, 1986). The binding to the chloride site induces a stable $g = 4.1$ EPR signal from the S_2 state (Beck & Brudvig, 1986). Ammonia binding to the second site modifies the S_2 multiline EPR signal (Beck & Brudvig, 1986; Beck et al., 1986; Andreasson et al., 1988). This second site was shown to involve direct binding to manganese (Britt et al., 1989) perhaps being the site normally occupied by water. Ammonia treatment also causes a slowing of Y_Z^+ reduction without loss of the manganese cluster (Yocum & Babcock, 1981).

Figure 9 shows that in PS2 samples (not calcium-depleted) at pH 7.5, supplemented with 100 mM NH_4Cl and "annealed" by the process described under Materials and Methods, a new signal was detected by freezing the sample under illumination (Figure 9c). The signal was greatly decreased in size if the "annealing" step was omitted, even though ammonia binding to the S_2 state was indicated by an alteration in the splitting of the multiline signal (not shown).

The new signal can be seen as a broad resonance near $g = 2$ which was absent following incubation of the sample for 10 min at 273 K in the dark (Figure 9a). The signal had similar microwave power saturation and temperature dependence characteristics to the "S3" signal discussed above, indicating fast relaxation by a transition metal. The spectrum of the dark-adapted sample contains some S_2 multiline EPR signal, having the increased stability and reduced line width typical of ammonia-treated samples (Figure 9b) (Beck et al., 1986; Beck & Brudvig, 1986; Boussac et al., 1990a). This S_2 signal is decreased by freezing under illumination (Figure 9d).

An EPR signal with almost identical properties (broad unresolved line width, g-value, microwave power, and temperature dependence) was reported by Baumgarten et al. (1990) from chloride-depleted and fluoride-treated preparations. This was assigned to an oxidized organic radical interacting with the manganese cluster, and the similarities to the interaction seen in calcium-depleted samples were discussed (Baumgarten et al., 1990).

The effects shown in Figure 9 may be caused by ammonia binding to the non-chloride binding site. The high chloride concentrations and the absence of sucrose in our samples suppress binding to the chloride site. This was confirmed by the absence of stabilization of the $g = 4.1$ form of S_2 [not shown, but see Boussac et al. (1990a)]. However, we cannot completely rule out the possibility that ammonia binds to the chloride site under these conditions. The requirement for the "annealing" step may be to ensure an increased binding of ammonia to S_2 over that occurring in the cycling of S states under continuous illumination at 277 K. Following the annealing treatment, S-state turnover must be blocked at S_2 or above, at least in some centers, or the bound ammonia would be released by the cycling of S states.

In ammonia-treated samples treated to trap the interaction signal, Y_Z^+ was also observed at 50 K (not shown). This trapping of Y_Z^+ is consistent with the slowing of Y_Z^+ reduction at room temperature found in ammonia-treated samples

(Yocum & Babcock, 1981). The yield of Y_Z^+ was greatly increased in samples which had undergone the "annealing" step in parallel with the increased yield of the "S3"-type signal. We suggest that as in the case of calcium-depleted samples, the interaction is likely to be between Y_Z^+ and the manganese cluster.

Discussion

We have investigated the EPR characteristics of the "S3" EPR signal in "calcium-depleted" PS2 preparations using standard preparative methods from the literature (Boussac et al., 1989; Ono & Inoue, 1988a; Kalosaka et al., 1990). The detection of "S3"-type signals is not specifically due to calcium depletion, a view reinforced by the observation of a similar signal in chloride-depleted preparations (Baumgarten et al., 1990) together with our observation of a similar signal in ammonia-treated PS2. The "S3"-type signals in chloride-depleted and ammonia-treated samples do, however, have some distinct character, such as the poorer resolution of the splitting, which indicates different structural properties between these and calcium-depleted samples. We also suggest that the split signal formed in samples treated with the amine inhibitor hydroxylamine (Beck & Brudvig, 1987; Sivaraja & Dismukes, 1988a,b), but previously attributed to a form of Q_A semiquinone, is from a similar interaction.

It has been proposed that chelator binding (Sivaraja et al., 1989) and ammonia treatment (Ono & Inoue, 1988b) cause a reduction in the redox potential of the S_2 state. Vass and Styring (1991) have proposed that in untreated samples the E_m of the S_0/S_1 couple is only 210 mV below that of the S_2/S_1 couple. Small shifts in redox potential may therefore cause the changes in stability of the S states and of the manganese complex which we have observed. The shift of S_2 to a lower redox potential is also indicated by the thermoluminescence experiments of Ono and Inoue (1990b) on calcium-depleted samples.

The S_2 EPR multiline is stabilized in ammonia-treated and chelator-treated samples, but no stabilization of this signal was seen in chloride-depleted and fluoride-treated samples (Baumgarten et al., 1990). There is evidence that the S_2 state is stabilized in chloride-depleted samples, but in an EPR-silent form (Ono et al., 1986). Chloride depletion of PS2 induces a block beyond S_2 in the S-state cycle (Theg et al., 1984; Itoh et al., 1984; Ono et al., 1986). Therefore, in all samples which show an "S3"-type signal, the normal S_2 state is perturbed, either favoring other forms of S_2 such as the $g = 4.1$ form or altering the line shape and stability of the multiline form [see Rutherford et al. (1991) for a review]. This is consistent with the current view that calcium and chloride are required for structural rearrangements which occur in the higher S states and which involve binding of the substrate water (Rutherford et al., 1991).

To achieve maximum yields of Y_Z^+ or "S3", it is essential to reoxidize Q_A semiquinone using an exogenous electron acceptor because of the rapid recombination reaction which occurs even at cryogenic temperatures. DCMU treatment prevents reoxidation of Q_A semiquinone, lowering the yield of Y_Z^+ at room temperature or Y_Z^+ and "S3" at cryogenic temperatures due to the increased back-reaction. The paper showing some "S3" formation in the presence of 50 μM DCMU (Ono & Inoue, 1990b) did not show the "S3" yield in the presence of electron acceptors and so cannot be compared with our results. Previous reports (Sivaraja et al., 1989; Tso et al., 1991a; Boussac et al., 1990b) suggested that a one-electron turnover of PS2 in the presence of DCMU gives only the S_1–S_2 transition. In samples not exposed to illumination before DCMU treatment, this accounts for the lack of "S3" signal formation. Figure 1 shows that due to the pretreatment, our samples were already in the dark-stable S_2 state prior to illumination. The absence of the "S3" signal in our DCMU-treated samples was therefore not due to the presence of S_1 but was caused by an increased reduction of the organic radical via Q_A semiquinone. Tso et al. (1991a) observed a similar inhibition of "S3" signal formation by DCMU in a "double turnover" experiment using citrate-washed PS2. They suggested that loss of the signal was due to the formation of an EPR-silent S_3 state, analogous to the normal S_3 state. The presence of the dark-stable S_2 in illuminated DCMU-treated samples suggests that this is not the case.

The ability to generate the dark-stable S_2 and "S3" signals in calcium-depleted samples is sensitive to pH, both being lost on raising the pH to 7.5. The instability at higher pH values appears to be through changes to the manganese complex, as Y_Z could still be oxidized at pH 7.5. The instability at higher pH may also result from effects on the chloride binding site. The affinity for chloride is lower at higher pH (Theg & Homann, 1982; Homann, 1988), probably through deprotonation of a ligand at the binding site.

Possible Role of Y_Z^+ in "S3" Formation. No photooxidation of Y_Z occurs at <77 K in dark-adapted samples, suggesting electron transfer to the OEC is prevented at <77 K by a block at Y_Z. When samples are frozen to 77 K with Y_Z in the oxidized state, it is slowly reduced, and the "oxidized" conformation is retained. As found with Y_D (Nugent et al., 1982, 1987; Inui et al., 1989), Y_Z can then be photooxidized at liquid helium temperatures. Y_Z^+ has been suggested to be stabilized by movement of the ring hydroxyl proton toward a nearby histidine (Babcock et al., 1988; Gerken et al., 1988; Svensson et al., 1990). The oxidation and reduction of Y_Z (and "S3") shows a temperature dependence consistent with a structural change accompanying the redox-state change. Our results also show that the oxidized conformation of the OEC and Y_Z can relax at 200 K. At 200 K, ligand exchange can occur in the manganese cluster as demonstrated by ammonia binding (Boussac et al., 1991a).

We have shown using calcium-depleted and ammonia-treated samples that Y_Z^+ is obtained at room temperature on illumination and that a proportion of this can be trapped by freezing to cryogenic temperatures. The characteristics of formation and decay of Y_Z^+ in calcium-depleted samples match those of the "S3" signal. We therefore propose that the "S3" signal could arise from an interaction between Y_Z^+ and the manganese cluster. However, we cannot completely rule out that the interaction between the manganese cluster and, for example, oxidized histidine may be strong enough to prevent the observation of an EPR signal from the oxidized histidine.

Evidence for Histidine as the Organic Radical in the "S3" State. The "S3" signal is formed by interaction between the manganese cluster (in S_2 or possibly S_3 states) and an organic radical. Of the interactions which occur in PS2, the pheophytin/iron–semiquinone interaction, between an $S = {}^1/_2$ organic radical and an $S = {}^1/_2$ spin system (Klimov et al., 1980), may be the best model. This has similar characteristics to the "S3" system which is proposed to be an interaction between an $S = {}^1/_2$ organic radical and the $S = {}^1/_2$, S_2 state (Boussac et al., 1990d). The line shape and magnitude of the splitting around $g = 2$ (Klimov et al., 1980) of the pheophytin/iron–semiquinone interaction resemble those of the "S3" signal. This analogy has already been used to estimate, using the point dipole relationship, the distance between the

organic radical and the manganese cluster as being 7 Å (Baumgarten et al., 1990). The pheophytin/iron–semiquinone split $g = 2$ signal is observed below 10 K at high microwave powers while at higher temperatures and low microwave powers it is possible to observe the pheophytin radical (Klimov et al., 1980).

The EPR signal from "S3" only indicates that a nonspecific organic radical is involved in the interaction with the manganese complex. A simulation of the "S3" signal used a model system for the S_2 multiline and calculated its interaction with a $g = 2$ radical (Boussac et al., 1990d). This simulation would equally support the involvement of oxidized histidine or Y_Z^+ in the interaction.

The optical spectra presented by Boussac et al. (1990d) to indicate histidine oxidation involved the use of DCMU. However, the spectrum was measured as a kinetic transient, and it is probable that formation of some "S3" (and Y_Z^+) would have occurred. The spectrum obtained was different from the normal S_2 to S_3 transition but was similar to that of Y_Z^+ (Gerken et al., 1988) and other tyrosine radicals. The spectrum shown by Boussac et al. (1990d) is similar, for example, to that of the tyrosine radical of ribonucleotide reductase (Bollinger et al., 1991). The differences from the Y_Z^+ spectrum previously observed may be due to an electrochromic shift occurring in preparations where oxygen evolution is impaired. We therefore argue that the evidence for histidine oxidation is weak and would equally support oxidation of Y_Z^+.

Origin of the "S3" Signal. Illumination at 277 K of calcium-depleted PS2 poised in S_2 could be producing $S_2\ Y_Z^+$, S_3, or $S_3\ Y_Z^+$, assuming a block in S-state turnover. The "S3" signal could then arise from either S_3, $S_3\ Y_Z^+$, or $S_2\ Y_Z^+$. The block in calcium-depleted samples has been assigned to the S_3 state, but, for example, the $S_2\ Y_Z^+$ state would not have been distinguished in those experiments (Boussac & Rutherford, 1988; Boussac et al., 1990b).

There are three observations which are difficult to incorporate into any explanation. (1) The modified multiline signal of the S_2 state is removed or greatly decreased under conditions which generate the "S3" signal. (2) Y_Z^+ is only present in about 25% of reaction centers when the "S3" signal is observed. (3) The Y_Z^+ spectrum can still be observed when the "S3" signal is present.

The relationship between the formation, rates of decay, and yields of "S3" and Y_Z^+ at different temperatures supports the involvement of Y_Z^+ in forming the "S3" signal, i.e., $S_3\ Y_Z^+$ or $S_2\ Y_Z^+$. Evidence from flash studies shows that only one flash above the S_2 state is required to generate the "S3" signal (Boussac et al., 1990d; Baumgarten et al., 1990), suggesting $S_2\ Y_Z^+$ leads to "S3" formation. The loss of the dark-stable multiline S_2 state on freezing under illumination supports the loss of this form of the S_2 state. We have presented evidence which separates "S3" signal formation and decay from dark-stable S_2 changes. This indicates that structural changes may occur to the manganese complex under illumination at 277 K, perhaps involving chelator binding, which can be "frozen in" to account for the loss of the dark-stable S_2.

Therefore, we suggest that if $S_2\ Y_Z^+$ is formed by illumination at room temperature, then as the temperature is lowered during freezing under illumination, first the EPR-silent state of the manganese cluster is frozen in. Then as the temperature is lowered further, Q_A to Q_B electron transfer is inhibited, allowing those centers with Q_A^- to reduce Y_Z^+, lowering the yield of Y_Z^+ trapped at 77 K.

Our analysis implies that the "S3" signal arises from the modified S_2 state interacting with Y_Z^+. The "S3" signal may originate primarily from the manganese cluster. This accounts for the presence of the Y_Z^+ signal, the "S3" signal arising from the interacting S_2 state. The re-addition of calcium to calcium-depleted samples may allow transient formation of some native S_3 before decay via S_2 to S_1. In ammonia-treated samples, the slowing of the S_2 to S_3 step would allow $S_2\ Y_Z^+$ to be trapped, to also give an "S3"-type signal.

Conclusion

Our conclusion is that the "S3" signal most probably arises from the S_2–Y_Z^+ interaction. This conclusion is consistent with the results of Hoganson and Babcock, (1988) and Gerken et al. (1988), which showed that Y_Z is the sole electron carrier between P680 and the OEC.

Assuming the interaction is between Y_Z^+ and the manganese complex, we can now suggest a more specific location for the site of water oxidation. Previous estimates of the distance between Y_Z and the OEC have been in escess of 10 Å and up to 20 Å, largely because of the lack of broadening of the Y_Z^+ EPR spectrum (Hoganson & Babcock, 1988). Baumgarten et al. (1990) calculated, using the point dipole relationship, a distance of 7 Å between the interacting species forming the "S3" signal. This measurement is not very accurate, as the interaction is much more complex, but by using this, we would estimate that Y_Z is significantly less than 10 Å from the manganese cluster. This is supported by the microwave power saturation data which show that Y_Z^+ interacts with the manganese cluster even at room temperature, indicating the close proximity of the two species.

Acknowledgments

We thank Dr. Dugald Maclachlan, Dr. Stephen Rigby, and Dr. Peter Heathcote for helpful comments and discussion.

References

Andreasson, L.-E., Hansson, O., & von Schenck, K. (1988) *Biochim. Biophys. Acta 936*, 351–360.

Babcock, G. T., Barry, B. A., Debus, R. J., Hoganson, C. W., Atamian, M., McIntosh, L., Sithole, I., & Yocum, C. F. (1989) *Biochemistry 28*, 9557–9565.

Baumgarten, M., Philo, J. S., & Dismukes, G. C. (1990) *Biochemistry 29*, 10814–10822.

Beck, W. F., & Brudvig, G. W. (1986) *Biochemistry 25*, 6479–6486.

Beck, W. F., & Brudvig, G. W. (1987) *Biochemistry 26*, 8285–8295.

Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1986) *J. Am. Chem. Soc. 108*, 4018–4022.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.

Bollinger, J. M., Edmondson, D. E., Huynh, B. H., Filley, J., Norton, J. R., & Stubbe, J. (1991) *Science 253*, 292–298.

Boussac, A., & Rutherford, A. W. (1988) *Biochemistry 27*, 3476–3483.

Boussac, A., Zimmermann, J. L., & Rutherford, A. W. (1989) *Biochemistry 28*, 8984–8989.

Boussac, A., Rutherford, A. W., & Styring, S. (1990a) *Biochemistry 29*, 24–32.

Boussac, A., Zimmermann, J. L., & Rutherford, A. W. (1990b) *FEBS Lett. 277*, 69–74.

Boussac, A., Zimmermann, J. L., & Rutherford, A. W. (1990c) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 713–716, Kluwer Academic

Publishers, Dordrecht, The Netherlands.
Boussac, A., Zimmermann, J. L., Rutherford, A. W., & Lavergne, J. (1990d) *Nature 347*, 303–306.
Britt, R. D., Zimmermann, J. L., Sauer, K., & Klein, M. P. (1989) *J. Am. Chem. Soc. 111*, 3522–3532.
Butler, W. F., Carlo, R., Fredkin, D. R., Isaacson, R. A., Okamura, M. Y., & Feher, G. (1984) *Biophys. J. 45*, 947–973.
Cammarate, K., & Cheniae, G. (1987a) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 1, pp 617–620, Martinus Nijhoff, Dordrecht, The Netherlands.
Cammarata, K., & Cheniae, G. (1987b) *Plant Physiol. 84*, 587–595.
Coleman, W. J. (1990) *Photosynth. Res. 23*, 1–27.
Crofts, A. R., & Wraight, C. A. (1983) *Biochim. Biophys. Acta 726*, 149–185.
Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988a) *Biochemistry 27*, 9071–9074.
Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.
Dekker, J. P., Ghanotakis, P. F., Plijter, J. J., Van Gorkom, H. J., & Babcock, G. T. (1984) *Biochim. Biophys. Acta 767*, 515–523.
Dismukes, G. C., & Siderer, Y. (1980) *FEBS Lett. 121*, 78–80.
Dismukes, G. C., Frank, H. A., Friesner, R., & Sauer, K. (1984) *Biochim. Biophys. Acta 764*, 253–271.
Ford, R. C., & Evans, M. C. W. (1983) *FEBS Lett. 160*, 159–163.
Gerken, S., Brettel, K., Schlodder, E., & Witt, H. T. (1988) *FEBS Lett. 237*, 69–75.
Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1984) *FEBS Lett. 167*, 127–130.
Hallahan, B. J., Ruffle, S. V., Bowden, S. J., & Nugent, J. H. A. (1991) *Biochim. Biophys. Acta 1059*, 181–188.
Hoganson, C. W., & Babcock, G. T. (1988) *Biochemistry 27*, 5848–5855.
Homann, P. H. (1988) *Biochim. Biophys. Acta 94*, 1–13.
Inui, T., Kawamori, A., Kuroda, G., Ono, T., & Inoue, Y. (1989) *Biochim. Biophys. Acta 973*, 147–152.
Isawa, S., Heath, R. L., & Hind G. (1969) *Biochim. Biophys. Acta 180*, 388–398.
Itoh, S., Yerkes, C. T., Koike, H., Robinson, H. H., & Crofts, A. R. (1984) *Biochim. Biophys. Acta 766*, 612–622.
Kalosaka, K., Beck, W. F., Brudvig, G. W., & Cheniae, G. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 721–724, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Kawamori, A., Satoh, J., Inui, T., & Satoh, K. (1987) *FEBS Lett. 217*, 134–138.
Klimov, V. V., Dolan, E., Shaw, E. R., & Ke, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7227–72313.
Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475.
Lockett, C. J., Demetriou, C., Bowden, S. J., & Nugent, J. H. A. (1990) *Biochim. Biophys. Acta 1016*, 213–218.
Metz, J. G., Nixon, P. J., Rogner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.
Miller, A. F., & Brudvig, G. W. (1991) *Biochim. Biophys. Acta 1056*, 1–18.
Miyoa, M., & Murata, N. (1984) *FEBS Lett. 168*, 118–120.
Nugent, J. H. A., Evans, M. C. W., & Diner, B. A. (1982) *Biochim. Biophys. Acta 682*, 106–114.
Nugent, J. H. A., Demetriou, C., & Lockett, C. J. (1987) *Biochim. Biophys. Acta 894*, 534–542.
Nugent, J. H. A., Doetschman, D. C., & Maclachlan, D. J. (1992) *Biochemistry 31*, 2935–2941.
Ono, T., & Inoue, Y. (1988a) *FEBS Lett. 227*, 147–152.
Ono, T., & Inoue, Y. (1988b) *Arch. Biochem. Biophys. 264*, 82–92.
Ono, T., & Inoue, Y. (1989) *Biochim. Biophys. Acta 973*, 443–449.
Ono, T., & Inoue, Y. (1990a) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 741–744, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Ono, T., & Inoue, Y. (1990b) *Biochim. Biophys. Acta 1020*, 269–277.
Ono, T., & Inoue, Y. (1991a) *FEBS Lett. 278*, 183–186.
Ono, T., & Inoue, Y. (1991b) *Biochemistry 30*, 6183–6188.
Ono, T., Zimmermann, J. L., Inoue, Y., & Rutherford, A. (1986) *Biochim. Biophys. Acta 851*, 193–201.
Ono, T., Kusunoki, M., Matsushita, T., Oyanagi, H., & Inoue, Y. (1991) *Biochemistry 30*, 6836–6841.
Randolph, M. L. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 119–152, Wiley Interscience, New York.
Rutherford, A. W., Boussac, A., & Zimmerman, J. L. (1991) *New J. Chem. 15*, 491–500.
Sandusky, P. O., & Yocum, C. F. (1983) *FEBS Lett. 162*, 339–343.
Sandusky, P. O., & Yocum, C. F. (1984) *Biochim. Biophys. Acta 766*, 603–611.
Sandusky, P. O., & Yocum, C. F. (1986) *Biochim. Biophys. Acta 849*, 85–93.
Shen, J. R., & Katoh, S. (1991) *Plant Cell Physiol. 32*, 439–446.
Shen, J. R., Satoh, K., & Katoh, S. (1988) *Biochim. Biophys. Acta 936*, 386–394.
Sivaraja, M., & Dismukes, G. C. (1988a) *Biochemistry 27*, 3467–3475.
Sivaraja, M., & Dismukes, G. C. (1988b) *Biochemistry 27*, 6297–6306.
Sivaraja, M., Tso, J., & Dismukes, G. C. (1989) *Biochemistry 28*, 9459–9464.
Styring, S., & Rutherford, A. W. (1988) *Biochemistry 27*, 4915–4923.
Svensson, B., Vass, I., Cedergren, E., & Styring, S. (1990) *EMBO J. 9*, 2051–2059.
Theg, S. M., & Homann, P. H. (1982) *Biochim. Biophys. Acta 679*, 221–234.
Theg, S. M., Jursinic, P. A., & Homann, P. H. (1984) *Biochim. Biophys. Acta 766*, 636–646.
Tso, J., Sivaraja, M., & Dismukes, G. C. (1991a) *Biochemistry 30*, 4734–4739.
Tso, J., Sivaraja, M., Philo, J. S., & Dismukes, G. C. (1991b) *Biochemistry 30*, 4740–4747.
Vass, I., & Styring, S. (1991) *Biochemistry 30*, 830–839.
Vermaas, W. F. J., Renger, G., & Dohnt, G. (1984) *Biochim. Biophys. Acta 764*, 194–202.
Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.
Warden, J. T., Blankenship, R. E., & Sauer, K. (1976) *Biochim. Biophys. Acta 423*, 462–478.
Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1–15.
Yocum, C. F., & Babcock, G. T. (1981) *FEBS Lett. 130*, 99–102.