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The YF161D1 Mutant of *Synechocystis* 6803 Exhibits an EPR Signal from a Light-Induced Photosystem II Radical†

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ABSTRACT: The currently accepted model for the location of the redox-active tyrosines, D and Z, in photosystem II suggests that they are symmetrically located on the D1 and D2 polypeptides, which are believed to form the heterodimer core of the reaction center. Z, the electron conduit from the manganese catalytic site to the primary chlorophyll donor, has been identified with tyrosine-161 of D1. The YF161D1 mutant of *Synechocystis* 6803 [Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988b) *Biochemistry 27*, 9071-9074; Metz, J. G., Nixon, P. J., Rogner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960-6969], in which this tyrosine has been changed to a phenylalanine, should have no light-induced EPR (electron paramagnetic resonance) signal from a tyrosine radical. This negative result has indeed been obtained in analysis of one of two independently constructed mutants through the use of a non-oxygen-evolving core preparation (Metz et al., 1989). Here, we present an analysis of a YF161D1 mutant through the use of a photosystem II purification procedure that gives oxygen-evolving particles from wild-type *Synechocystis* cultures. In our mutant preparation, a light-induced EPR signal from a photosystem II radical is observed under conditions in which, in a wild-type preparation, we can accumulate an EPR signal from Z+. This EPR signal has a different lineshape from that of the Z+ tyrosine radical, and spin quantitation shows that this radical can be produced in up to 60% of the mutant reaction centers. The EPR lineshape of this radical suggests that photosystem II reaction centers of the YF161D1 mutant contain a redox-active amino acid.

In plants, algae, and cyanobacteria, photosystem II (PSII) catalyzes the light-induced oxidation of water and the reduction of plastoquinone. This photosystem is one of several membrane-associated protein complexes that carry out the initial electron-transfer events in oxygenic photosynthesis. PSII is a chlorophyll-containing complex that is composed of both integral, membrane-spanning polypeptides and extrinsic po-

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lypeptides [for a review, see Babcock (1987), Babcock et al. (1989), and Debus (1992)]. Of the integral membrane proteins, two, called D1 and D2, are known to bind the primary chlorophyll donor, P680, and an intermediate acceptor, which is a pheophytin. The terminal electron acceptors in PSII are two plastoquinones, Q_A and Q_B. A non-heme iron atom and cytochrome b_{559} are also present. A cluster of four manganese is the catalytic site of water oxidation; the 4 oxidizing equivalents that are necessary for the oxidation of water and the release of molecular oxygen are sequentially stored by the water-splitting complex through a chemical mechanism that is not presently understood. The 33-kDa extrinsic protein stabilizes the manganese cluster; its binding site in the complex is not known.

The transfer of electrons from the manganese cluster to P680+ is carried out by the intermediate donor Z (Boska et al., 1983; Gerken et al., 1988). The EPR spectrum of the oxidized form of the species, Z+, is observable as a light-induced transient (Hoganson & Babcock, 1988). Under conditions where the reduction of Z+ is slow because the manganese has been removed from the PSII complex (e.g., by Tris-washing), the EPR spectrum of Z+ can be photoaccumulated in the presence of exogenous acceptors (Babcock & Sauer, 1975a). Also associated with the donor side of PSII is a stable radical, D+, of unknown function and with the same EPR lineshape as the light-induced radical Z+ (Hoganson & Babcock, 1988). The use of isotopic labeling and EPR spectroscopy has shown that D+ is a tyrosine radical (Barry & Babcock, 1987). The similarity of the EPR lineshapes of D+ and Z+ led to the deduction that Z+ must also be a tyrosine radical. Optical spectroscopy has provided experimental evidence to support this proposal (Gerken et al., 1988).

The location of these two redox-active tyrosines in the PSII complex has been explored through the use of site-directed mutagenesis. Since the orientation of the tyrosine phenol ring influences the EPR lineshape, D+ and Z+ are likely to be in conserved protein environments (Barry & Babcock, 1987; Barry et al., 1990). After inspection of the amino acid sequences of D1 and D2, it was proposed (Debus et al., 1988a) that D+ was tyrosine-160 of the D2 polypeptide and Z+ was tyrosine-161 of the D1 polypeptide. Implicit in this model is the idea that D1 and D2 form the heterodimer core of the complex and that there is C_2 structural symmetry on the donor side of PSII (Debus et al., 1988a). Site-specific mutagenesis was used to confirm the assignment of D+ (Debus et al., 1988a; Vermaas et al., 1988). These experiments were performed using the cyanobacterium, *Synechocystis* 6803, which is transformable, undergoes homologous recombination, and is capable of photoheterotrophic growth in the absence of PSII (Williams, 1988). In these experiments, tyrosine-160 of D2 was altered to a phenylalanine residue, which cannot be oxidized. The resulting mutant grew photosynthetically, but lacked the EPR spectrum of the stable tyrosine radical, which can normally be observed in cyanobacterial cells (Debus et al., 1988a; Vermaas et al., 1988).

In *Synechocystis*, a phenylalanine mutation has also been generated at tyrosine-161 of the D1 polypeptide, which the model predicts to be the intermediate donor, Z+ (Debus et al., 1988b; Metz et al., 1989). In the two independently constructed Tyr-Phe (YF161D1) strains, the mutants could not grow photosynthetically, showed no evidence of oxygen evolution, and were shown to be impaired in electron transfer on the donor side of PSII. Both strains had the EPR signal of the stable radical, D+. There was no further characterization of the mutant described in Debus et al. On the other hand, Metz et al. used a non-oxygen-evolving core PSII preparation to show, using both EPR and optical spectroscopy, that under their sample preparation conditions the mutant exhibited no signal from a light-induced tyrosine radical. This result is consistent with the assignment of Z to tyrosine-161 of the D1 polypeptide.

Recently, an oxygen-evolving PSII preparation from *Synechocystis* 6803 has been developed that has allowed room temperature EPR characterization of PSII particles from wild-type cells and genetically engineered mutants (Noren et al., 1991). We will describe the use of this preparation to further characterize the YF161D1 mutant that was originally described in Debus et al. (1988b). We show that an EPR signal from a light-induced photosystem II radical *can* be observed in photosystem II particles from this mutant. We attribute this signal to an oxidized amino acid residue.

Materials and Methods

Growth of Cells. Mutant and wild-type transformant strains of *Synechocystis* 6803 were a gift of R. J. Debus, and their construction is described in Debus et al. (1988b). The growth of the cells was performed as described in Noren et al. (1991). All media were supplemented with 5 mM glucose, and cells of the YF161D1 mutant were grown for a maximum of 10 days. A total of more than 500 L of YF161D1 cells was used throughout the course of this study; typically, 30 L of mutant culture produced enough purified photosystem II for one EPR sample.

Purification and Characterization of Particles. Purification of photosystem II particles, Tris-washing, oxygen evolution assays, chlorophyll assays, manganese quantitation, and Western blot analysis were performed according to the methods described in Noren et al. (1991) and the references cited therein. Because of the small amount of photosystem II compared to photosystem I in the YF161D1 mutant, fewer fractions (35–37) were pooled after chromatography of the mutant preparation on the final Mono-Q column.

Optical Assays. P700+, the primary donor of photosystem I, and cytochrome b_{559} were quantitated through the use of the following optical difference spectrum: ferricyanide-oxidized minus dithionite-reduced (P700+/cytochrome b_{559}) *or* ferricyanide-oxidized minus ascorbate-reduced (P700+). Dithionite and ascorbate reduction gave the same P700+ content. The spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer. Extinction coeffients of 64 mM^{-1} for P700+ (700–725 nm) (Hiyama & Ke, 1972) and 21.5 mM^{-1} for cytochrome b_{559} (559–577 nm) (Cramer et al., 1986; Cramer & Whitmarsh, 1977) were used in data analysis, unless otherwise noted. In the cytochrome b_{559} quantitation data, the peak of the difference spectrum for both mutant and wild-type samples was 559 nm, but the width at half-maximum amplitude was slightly broader for the wild type: 16 nm (wild type); instead of 12 nm (mutant). The narrow, symmetrical lineshape, bandwidth, and absorption maximum of the mutant difference spectrum reveal that there is little cyt b_6/f contamination in the mutant preparation (Cramer & Whitmarsh, 1977). The slightly broader, asymmetrical bandwidth for the wild-type preparation may be due to a small amount of contamination from cyt f (Ho & Krogmann, 1980).

Room Temperature EPR. Room temperature EPR spectra were recorded at X-band on a Varian E-4 spectrometer equipped with a Varian TE cavity. The spectrometer was interfaced to a Macintosh IIcx computer via a digital voltmeter (Model 195A, Keithley, Cleveland, OH) and a Mac488A bus controller (Iotech, Cleveland, OH). The data acquisition program was a gift from Dr. John Golbeck (University of

Table I: Characterization of Wild-Type and YF161D1 Photosystem II Preparations from *Synechocystis* 6803

	O_2 evolution		chlorophyll/ 4 Mn^{2+} [b]	chlorophyll/ 2 cyt b_{559} [c]	chlorophyll/ D+ [d]	chlorophyll/ P700+ [e]
	thylakoids [a]	particles [a]				
wild type	500	2300	60	60	60	880
YF161D1	0 [f]	0 [f]	≥1600	100	100	280

[a] Micromoles of O_2 per milligram of chlorophyll per hour (±5%). [b] Moles of chlorophyll per 4 moles of Mn^{2+} (±5%). [c] Moles of chlorophyll per 2 moles of cytochrome b_{559} (±10%). [d] Moles of chlorophyll per mole of D+ (±10%). [e] Moles of chlorophyll per mole of P700+ determined optically (±10%). [f] <25 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}.

Nebraska). Data analysis and subtractions were performed on a Macintosh IIcx computer using the graphics software program "Igor" (WaveMetrics, Lake Oswego, OR).

Illumination of samples in the EPR cavity was performed using a fiber-optic light source (Dolan-Jenner, Model 180). Spectral conditions are given in the figure legends. P700+, in purified cyanobacterial photosystem I (Noren et al., 1991), was used as a g value standard [g = 2.0025 (Hoff, 1987)]. Fremy's salt, $K_2(SO_3)_2NO$, was used as a standard for spin quantitations of PSI and PSII radicals (Babcock et al., 1983; Wertz & Bolton, 1986).

In experiments designed to reduce photooxidized PSII radicals, benzidine was added to the EPR sample from a 10 mM stock solution in ethanol (Babcock & Sauer, 1975b; Yerkes & Babcock, 1980). In the mutant data shown in Figure 3, 800 μM benzidine completely reduced the light-induced photosystem II radical in the YF161D1 mutant. In another experiment, 1000 μM benzidine was required to completely eliminate the mutant signal. Ethanol alone caused a small decrease in the amplitude of the light-induced mutant signal. At 800 μM benzidine, wild-type D+/Z+ was not fully reduced, although there was some variability in the amount of reduction from preparation to preparation.

Results

Table I shows a characterization of thylakoid membranes and photosystem II particles from wild-type and YF161D1 mutant cells. In agreement with previous work (Noren et al., 1991), the wild-type PSII particles are highly active, with rates of 2300 μmol of O_2 (mg of chlorophyll)$^{-1}$ h^{-1}, and have little photosystem I contamination (1 P700+ per 880 chlorophylls). The increase in specific activity over the thylakoid membranes is a factor of 4–5-fold. The wild-type particles have 4 manganese and 2 dithionite-reducible cytochromes b_{559} per 60 chlorophylls.

Cells and thylakoid membranes from the YF161D1 mutant evolve no oxygen (Table I). Because of the low ratio of PSII to PSI in these cells, purification of PSII particles from the mutant by the method of Noren et al. (1991) yields particles with more photosystem I contamination than is found in wild-type particles. Optical quantitation of P700+ content shows 3 times as much P700+ content in the YF161D1 mutant as in wild type (Table I). We find 2 b_{559} per 100 chlorophylls in the mutant. If we estimate the *Synechocystis* PSII reaction center size as 60 chlorophylls (Table I) and the *Synechocystis* PSI reaction center size as 85 chlorophylls (see below, Table II), we find a ratio of approximately 2 PSII/1 PSI in the mutant preparation. In contrast to wild-type particles, there is only a small amount of bound manganese in the mutant particles (4 manganese per approximately 1600 chlorophylls).

Figure 1 shows a Western blot developed with a polyclonal antibody against the purified spinach 33-kDa protein (Noren et al., 1991). The reaction of this antibody against a single polypeptide in photosystem II membranes (Berthold et al., 1981) from spinach (lane 5) and in wild-type cyanobacterial photosystem II particles (lane 1) is shown. In lane 2, as

FIGURE 1: Western blot developed with an antibody against the spinach 33-kDa extrinsic protein. The lanes are (1) untreated *Synechocystis* wild-type PSII particles, (2) Tris-washed wild-type particles, (3) untreated YF161D1 particles, (4) Tris-washed YF161D1 particles, and (5) spinach BBY particles. For the *Synechocystis* particles, 4 μg of chlorophyll was loaded per lane, and 2 μg of chlorophyll was loaded for the spinach BBY particles.

expected, Tris-washing removes the extrinsic 33-kDa protein from the wild-type cyanobacterial particles (Noren et al., 1991). Neither untreated (lane 3) nor Tris-washed (lane 4) mutant samples show any cross-reaction with this antibody. A Coomassie-stained denaturing SDS gel shows that, in the mutant, there is no protein band comigrating with the 33-kDa wild-type polypeptide (data not shown). Western blot analysis with YF161D1 *thylakoid membranes*, however, shows a band that cross-reacts with antibodies against the extrinsic 33-kDa protein (data not shown). We conclude that the extrinsic 33-kDa polypeptide is not present in the YF161D1 mutant particles.

In Figure 2 we show room temperature EPR spectra of Tris-washed wild-type (A) and mutant (B) PSII particles. The EPR spectrum of P700+ in purified cyanobacterial PSI is included for comparison (C). Tris-washing is known to remove manganese and the 33-kDa extrinsic protein from PSII reaction centers (Cheniae & Martin, 1970; Yamamoto et al., 1981). In (A), we show *wild-type* dark-stable D+ (dashed line) and light-induced Z+ (solid line) EPR spectra. Spin quantitation of the dark-stable D+ radical content gives 1 D+ per 60 chlorophyll(s) (chl) (Table I). The light-induced increase in the EPR signal in (A) results from accumulation of Z+ in centers where reduction of the radical has been slowed by removal of manganese. Spin quantitation and comparison to D+ content show that Z+ is produced in 90% of the centers. As has been previously observed, in these wild-type *Synechocystis* particles, D+ and Z+ have identical lineshapes (Noren et al., 1991).

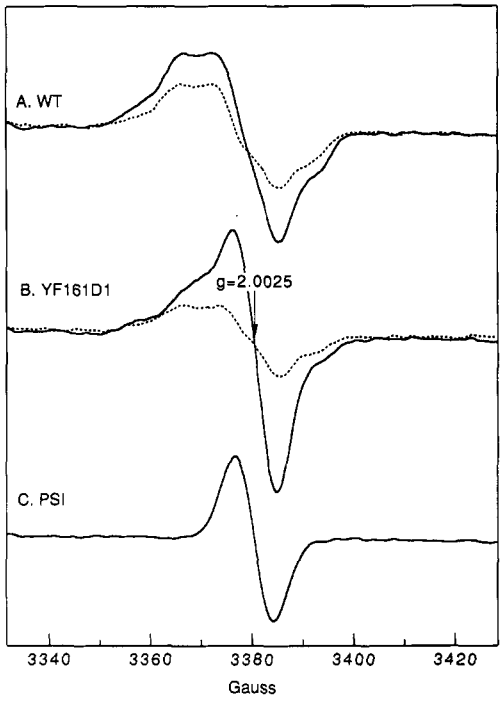

FIGURE 2: Room temperature EPR spectra of Tris-washed photosystem II particles from (A) wild-type and (B) YF161D1 *Synechocystis* 6803. Spectrum C is that of purified PSI particles from *Synechocystis* 6803. In each case, the dashed line was recorded in the dark; the solid line was recorded in the light. Conditions were as follows: modulation frequency, 100 kHz; microwave frequency, 9.49 GHz; microwave power, 3.5 mW; field modulation, 3.2 G; time constant, 2 s; scan time, 4 min. The gain for (A) and (B) was 2.5×10^4, and for (C), it was 1.6×10^4. All spectra were recorded in the presence of 1 mM $K_3Fe(CN)_6$. The chlorophyll concentration of the samples was 0.69 mg of chl/mL for wild type, 0.80 mg of chl/mL for YF161D1, and 0.52 mg of chl/mL for purified PSI. For plotting, the amplitudes of the spectra were scaled by a factor of 0.76.

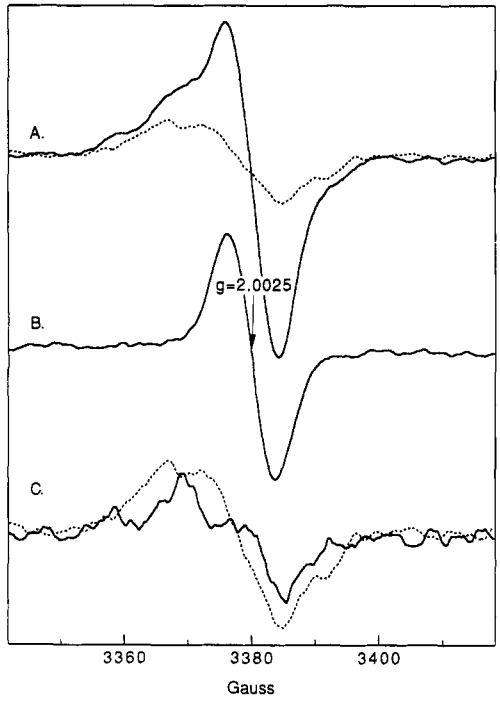

FIGURE 3: Room temperature EPR spectra of the *Synechocystis* 6803 YF161D1 PSII preparation in the presence and absence of benzidine. (A) Spectra of the YF161D1 preparation without added benzidine; the dashed line was recorded in the dark, and the solid line was recorded in the light. (B) Spectrum of the YF161D1 preparation in the presence of 800 μM benzidine. This spectrum was recorded in the light. (C) Spectrum of the light-induced radical in the YF161D1 mutant (solid line) compared to the dark-stable D+ spectrum (dashed line). The chlorophyll concentration of the preparation was 0.5 mg of chl/mL, the gain was 3.2×10^4, and 1 mM $K_3Fe(CN)_6$ was present. For plotting, the amplitude of (C) was increased by a factor of 2 over (A) and (B). Other spectral conditions were as described in Figure 2.

Spectrum 2B shows the EPR spectra recorded on YF161D1 mutant photosystem II particles in the light (solid line) or in the dark after illumination (dashed line). The dark-stable D+ radical has an identical lineshape to the wild-type D+ signal, and the stability of D+ in the two samples is similar. We find 1 D+ per 100 chlorophylls by spin quantitation (Table I). Figure 2B shows that there is a light-induced change in this EPR signal. Since optical quantitation indicates that the P700+ content of the mutant preparation is higher than wild type (Table I), we expect the light-induced EPR spectrum to contain a contribution from P700+. Comparison of Figure 2B and Figure 2C shows that the large 7–8-G signal in the center of the spectrum is due to contaminating PSI. However, the rest of the light spectrum cannot be explained by PSI contamination. The partially resolved hyperfine couplings in the light spectrum (B) are not identical to the EPR lineshape of wild-type Z+. Untreated mutant samples gave a similar light-induced spectrum (for example, see spectrum of Figure 3A).

In order to obtain another measure of the PSI contamination present in the mutant preparation, benzidine was added to the EPR sample. In Figure 3, we show the EPR spectra of the YF161D1 mutant before (A) and after (B) benzidine addition. Benzidine is known to be an electron donor to PSII (Babcock & Sauer, 1975b; Yerkes & Babcock, 1980). In this mutant sample, 800 μM benzidine fully reduced the PSII radicals in the light, leaving only a 7–8-G EPR signal, which is due to P700+. Control experiments using purified PSI showed that 800 μM benzidine had no effect on the amplitude of the light-induced P700+ signal in the presence of 1 mM ferricyanide (Table II). Therefore, we expect to observe the full P700+ EPR amplitude after benzidine addition to the YF161D1 sample in the light. Spin quantitation verifies this expectation (Table II). We compare this EPR spin quantitation to an optical quantitation of P700+ in this sample. For *both* purified PSI and YF161D1 preparations, EPR spin quantitation of P700+ gave a higher concentration of P700+ than did the optical difference measurements using the spinach P700+ extinction coefficient. However, the discrepancy between the optical and EPR measurements was consistent in both samples and could result from a difference in the extinction coefficient of spinach and *Synechocystis* 6803 P700+ (Hiyama & Ke, 1972). We are able to calculate a corrected *Synechocystis* P700+ extinction coefficient of approximately 46 mM^{-1} based on these EPR measurements.

Table II: Optical and EPR Quantitation of P700+ Content

	PSI, no additions	PSI, +800 μM benzidine	YF161D1, +800 μM benzidine
EPR[a]	86	86	180
optical[b]	120	120	280

[a] Moles of chlorophyll per mole of P700+ determined by EPR spin quantitation of the P700+ signal (±5%). [b] Moles of chlorophyll per mole of P700+ determined optically (±10%).

The results described above show that benzidine reduction in the YF161D1 preparation leaves us with the EPR spectrum of the contaminating P700+ alone. In Figure 3C, we use this information about PSI content to obtain the lineshape of the new light-induced radical. The contaminating PSI signal (3B)

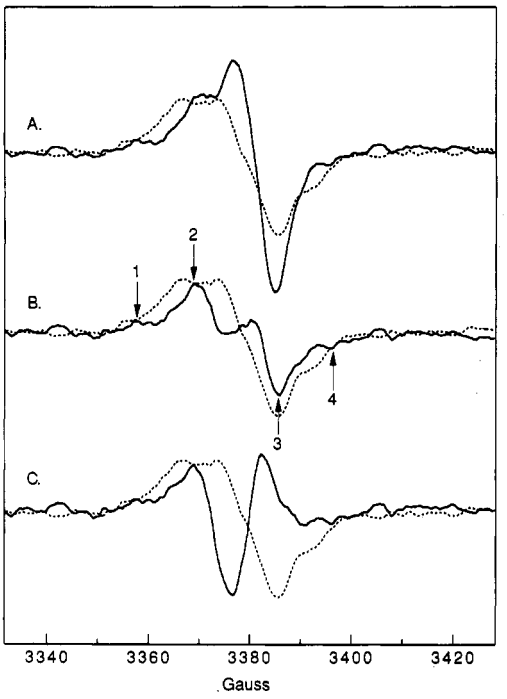

FIGURE 4: Room temperature difference EPR spectra of a Tris-washed *Synechocystis* 6803 YF161D1 PSII sample. In each panel, the dashed line shows the spectrum of the dark-stable D+. (B) Spectrum of the light-induced radical in the YF161D1 mutant that was obtained by the subtraction of the dark spectrum (D+) and P700+. The concentration of P700+ was determined optically. (A) Spectrum obtained by undersubtraction (by a factor of 50%) of the P700+ signal. (C) Spectrum obtained by oversubtraction (by a factor of 50%) of the P700+ signal. The chlorophyll concentration of the sample was 0.8 mg of chl/mL, the gain was 2.5×10^4, and the sample contained 1 mM $K_3Fe(CN)_6$. For plotting, the amplitudes were increased by a factor of 1.5. All other conditions were as described in Figure 2.

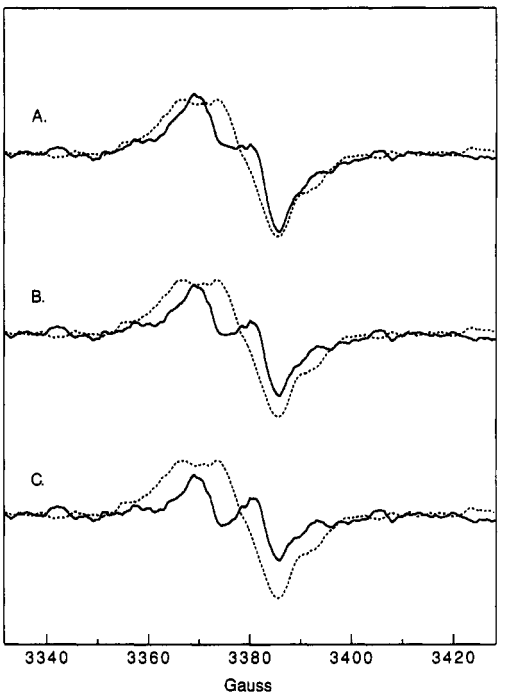

FIGURE 5: Room temperature difference EPR spectra of a Tris-washed *Synechocystis* 6803 YF161D1 PSII sample (same as shown in Figure 4). In each panel, the dashed line shows the spectrum of the dark-stable D+. (B) Same as panel 4B. (A) Spectrum obtained by undersubtraction (by a factor of 20%) of the D+ spectrum. (C) Spectrum obtained by oversubtraction (by a factor of 20%) of the D+ spectrum.

will be subtracted from the light spectrum (3A, solid line). In addition, since the dark-stable D+ signal (3A, dashed line) makes a constant contribution to the spectrum, it must also be subtracted. The lineshape of the light-induced radical in the YF161D1 mutant, obtained in this fashion, is shown in Figure 3C. The dark-stable D+ signal (3A) is repeated in 3C (dashed line) for comparison. The lineshape of the new radical is different from D+. Spin quantitation and comparison of D+ content indicate that this light-induced radical is present in up to 60% of the centers immediately after a 10-min dark-adaptation in the EPR cavity. This radical is more labile than wild-type Z+. After 60 min of repeated illumination at room temperature, the amplitude of the Z+ signal decreased by 40% in the wild-type sample, while the amplitude of the light-induced radical decreased by 60% in the YF161D1 mutant sample.

To show that the new lineshape of the YF161D1 radical is not due to error in the subtraction of P700+, we present the EPR spectra in Figure 4, which were recorded on a Tris-washed sample. The dark spectrum of D+ is repeated in each panel for comparison (dashed line). The solid spectrum in 4B was generated by subtracting both D+ and the EPR signal of P700+. In this case, we employed optical quantitation, using our corrected *Synechocystis* extinction coefficient, to decide on the amplitude of the P700+ signal to be subtracted. The lineshape of the radical in 4B agrees with the lineshape generated by benzidine reduction/subtraction (Figure 3C). Undersubtraction (4A) and oversubtraction (4C) of P700+ are also shown. Under- and oversubtraction does not affect the spectrum at field positions 1, 2, and 4, which distinguish the lineshape of this radical from D+/Z+. Similar experiments using wild-type photosystem II particles, purposely chosen to have PSI contamination, demonstrate that, in wild type, we can recover the lineshape of Z+ by this subtraction method and that the lineshape of the light-induced YF161D1 radical cannot be produced in this sample by under- or oversubtraction of P700+ (data not shown).

Spin quantitation of D+ in the mutant photosystem II particles shows that there is 1.0 D+ per reaction center in the dark following illumination. Therefore, we do not expect D to be further oxidized in the light. However, in order to demonstrate that small errors in the estimate of the D+ content in the light do not seriously affect the lineshape of the new light-induced radical, we present the data in Figure 5. The spectrum of D+ is repeated in each panel in the dashed line. Spectrum 5B shows the same sample and the same subtraction parameters that were used in Figure 4B. Generation of spectrum B involves a straightforward 1:1 subtraction of the D+ dark signal. In 5A, we show the effects of undersubtraction of D+ by 20%, and in 5C, we show the effects of oversubtraction of D+ by 20%. The lineshape of the new radical can be clearly distinguished from D+ even with over- and undersubtraction of the dark-stable signal.

DISCUSSION

Cells of the YF161D1 mutant cannot grow photosynthetically and are completely inactive in oxygen evolution. Purified PSII particles from the YF161D1 mutant are, accordingly, inactive. We find that the mutant preparation has a very low manganese content with only 0.2–0.3 manganese per 2 cytochromes b_{559}. This is in contrast to the active particles prepared from wild-type cells, which show 4 manganese per 2 b_{559}. In the dark, we observe a normal D+ EPR lineshape in photosystem II particles from YF161D1, in agreement with earlier reports (Debus et al., 1988b; Metz et al., 1989). Photosystem II particles from the YF161D1 mutant show 1.0 D+ per 2 cytochromes b_{559}, as do wild-type particles. In our mutant

preparation, D+ has stability properties that are similar to those of wild type.

When compared to wild-type preparations, particles from the mutant are deficient in their content of the 33-kDa extrinsic protein. Although this polypeptide is present in thylakoid membranes of the mutant, it may not be tightly bound (Jansson et al., 1987). We propose that the YF161D1 mutation changes the binding affinity of the reaction center for the 33-kDa protein. This alteration may be the result of pleotropic structural alterations in the mutant, or the tyrosine that is altered in the mutant may play a role in the assembly or stabilization of the manganese cluster [for example, see Blubaugh and Cheniae (1990)] and loss of the 33-kDa protein may follow as a consequence of manganese loss (Ghanotakis et al., 1984).

In Tris-washed wild-type preparations, Z+ is produced in 90% of the centers in the light. In the YF161D1 mutant, we see a substantial change in the EPR lineshape in the light. We can obtain the lineshape of the photooxidized photosystem II radical by the benzidine reduction/subtraction technique. This new radical is produced in up to 60% of the mutant centers and has an altered lineshape from that of D+/Z+. The radical has the same spin content and lineshape in Tris-washed and non-Tris-washed mutant preparations; this is an expected result since the mutant does not have a stoichiometric amount of manganese per reaction center. The YF161D1 radical is more labile than wild-type Z+.

The YF161D1 mutant has been characterized previously (Debus et al., 1988b; Metz et al., 1989). The studies described in Debus et al. (1988b) did not include an EPR study of photosystem II particles, since there was no photosystem II preparation available at that time. On the other hand, the characterization described in Metz et al. (1989) did employ EPR and optical studies of mutant photosystem II particles, which were purified through the use of a core preparation (Rogner et al., 1990). While the EPR spectrum of D+ was observed in the mutant, Metz et al. saw no evidence for oxidation of an additional photosystem II amino acid residue. However, we believe that this negative result was caused by the type of biochemical preparation that was employed and by the lability of the new signal. The Rogner core preparation gives non-oxygen-evolving wild-type particles; this purification procedure includes an overnight sucrose gradient step and concentration of the sample through the use of an ultrafiltration device. This purification could lead to an increased lability of the new radical as compared to wild-type Z+. In contrast, our preparation involves no overnight steps, concentration is performed quickly through PEG precipitation, and the samples are maintained in high concentrations of glycerol throughout the purification. High rates of oxygen evolution in the wild type attest to the speed and relative gentleness of our procedure. Alternatively, the new signal could arise from a PSII protein component that is present in our preparation, but absent in the Rogner preparation.

The identity of the radical that we observe in the mutant preparation is unknown. The spectrum that we obtain has an approximate 1:3:3:1 pattern, a splitting of 8–10 G, and an apparent g value of 2.004, allowing us to clearly distinguish this radical from organic free radicals arising from chlorophyll, pheophytin, and Qa, which all have a narrower, featureless EPR spectrum [see Petersen et al. (1990) and Miller and Brudvig (1991) and references cited therein].[1] A chlorophyll cation radical from photosystem II, which might be accumulated under the oxidizing conditions of this room temperature experiment, for example, shows no partially resolved hyperfine splitting, has a 8–10-G line width, and has a lower g value. The high g value reflects the presence of oxygen or nitrogen in the radical (Wertz & Bolton, 1986). Since quantitation indicates that photosystem II preparations do not contain enough extractable quinone to account for a donor-side redox-active quinone (de Vitry et al., 1986; Takahashi & Satoh, 1987; Tabata et al., 1985), we believe this radical to be an oxidized amino acid residue. It has recently been suggested that histidine is redox-active in photosystem II (Boussac et al., 1990; Ono & Inoue, 1991). Other amino acids, such as tryptophan, are thought to be redox-active in some proteins [for a review, see Stubbe (1989)]. Also, it has been observed that amino acid residues can form covalently linked redox cofactors [for example, see Ito et al. (1991) and Janes et al. (1990)]. Assignment of the identity of the oxidized species awaits isotopic labeling.

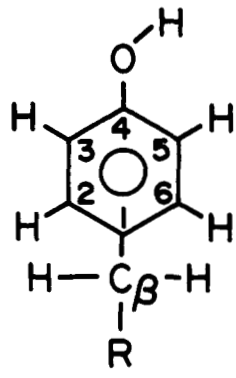

FIGURE 6: Numbering scheme for tyrosine.

Although we have no definitive evidence concerning the identity of the new radical, our observations can be reconciled with the idea that the radical arises from a redox-active tyrosine in a different environment than D+/Z+. One reason to consider tyrosine is the ample evidence that already exists for the oxidation of tyrosine in photosystem II (Barry & Babcock, 1987). The isotropic g value of the radical is similar to the g value of D+/Z+ [see Barry and Babcock (1987) and references cited therein]. Moreover, the lineshape of tyrosine radicals is sensitive to protein environment (Sealy et al., 1985; Bender et al., 1989; Barry et al., 1990; Kulmacz et al., 1990; Fishel et al., 1991). Therefore, while there is no precedent for an approximate 1:3:3:1 lineshape in powder samples of a tyrosine radical,[2] such a lineshape can be rationalized as indicating the presence of three accidentally degenerate protons. For unoriented samples like the ones used here, it may be possible to obtain such a spectrum by an accidental degeneracy of the 3,5-protons and one β-methylene proton (Figure 6), if the anisotropy of the hyperfine tensor for the 3,5-protons is small (Bender et al., 1989).

Changes in spin density distribution or in the geometry at the methylene–C_1 bond are responsible for alterations in the lineshape of tyrosine radicals (Wertz & Bolton, 1986; Sealy et al., 1985; Barry & Babcock, 1988). In D+, the coupling to the 3,5-protons has been reported to be 6.5 G, and the coupling to one of the inequivalent β-methylene protons has been reported to be 10 G (Barry et al., 1990). A small redistribution in unpaired spin density in the phenol ring could

[1] The anion radicals of Qa and pheophytin also interact with the non-heme iron or with $Fe^{2+}Qa^-$, respectively, to give rise to low-temperature EPR signals that are broader than the signal that we observe.

[2] See O'Malley et al. (1984), Brok et al. (1985), and Rutherford (1985) for the D+ lineshape in oriented samples, which does show a 1:3:3:1 pattern when the field is perpendicular to the membrane.

produce equal splittings to the 3,5-protons and one methylene proton, since for both 3,5-protons and β-methylene protons the hyperfine coupling is directly proportional to spin density (Wertz & Bolton, 1986). Recent studies of the indanetrione anion radical have indicated that spin density rearrangements can accompany changes in hydrogen-bonding interactions to the molecule's oxygen atoms (Reiter et al., 1990). It has been proposed that Z is hydrogen-bonded, perhaps to a histidine (Eckert & Renger, 1988; Babcock et al., 1989; Svensson et al., 1990). Thus, the lineshape of the YF161D1 radical could be due to an oxidized tyrosine with a different hydrogen-bonding interaction to the phenol oxygen than tyrosine Z. Of course, other combinations of changes in methylene–C_1 bond geometry and alterations in spin density can also explain the new spectral lineshape, and simulations of the powder EPR spectrum are necessary.

If Z is tyrosine-161 of D1 as the currently accepted structural model suggests, then the YF161 mutation replaces tyrosine Z with a nonoxidizable phenylalanine (Debus et al., 1988a; Vermaas et al., 1988). Such a substitution can account for the dramatic alterations in electron-transfer properties that are observed in this mutant (Debus et al., 1988b; Metz et al., 1989). Since, in the context of this model, tyrosine Z is not present in the YF161D1 mutant, we explain our observations by proposing that another reaction center amino acid is oxidized. This amino acid may be a tyrosine in a different location than tyrosine Z, accounting for the change in EPR lineshape and the increased lability that we observe for this radical. There is precedent for such a result in previous attempts to identify a free radical in cytochrome *c* peroxidase through site-directed mutagenesis (Fishel et al., 1991). It has also been observed that another tyrosine can be oxidized in ribonucleotide reductase in the absence of the normally oxidized Y122 (Bollinger et al., 1991). Interestingly, it has been proposed that this second redox-active tyrosine is active on the normal electron-transfer pathway in this protein.

However, if the mutant radical is a tyrosine, our work does not exclude the unlikely possibility that Z+ is still present in the YF161 mutant and that the YF161D1 mutation has a dramatic effect on the *environment* of Z+, producing the alteration in lineshape and the alteration in electron-transfer properties that are observed in the mutant.

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Registry No. Tyrosine, 60-18-4.

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Potential Ligands to the [2Fe-2S] Rieske Cluster of the Cytochrome bc_1 Complex of *Rhodobacter capsulatus* Probed by Site-Directed Mutagenesis†

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ABSTRACT: The Rieske protein of the ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex or b_6f complex) contains a [2Fe-2S] cluster which is thought to be bound to the protein via two nitrogen and two sulfur ligands [Britt, R. D., Sauer, K., Klein, M. P., Knaff, D. B., Kriauciunas, A., Yu, C.-A., Yu, L., & Malkin, R. (1991) *Biochemistry 30*, 1892-1901; Gurbiel, R. J., Ohnishi, T., Robertson, D. E., Daldal, F., & Hoffman, B. M. (1991) *Biochemistry 30*, 11579-11584]. All available Rieske amino acid sequences have carboxyl termini featuring two conserved regions containing four cysteine (Cys) and two or three histidine (His) residues. Site-directed mutagenesis was applied to the Rieske protein of the photosynthetic bacterium *Rhodobacter capsulatus*, and the mutants obtained were studied biochemically in order to identify which of these conserved residues are the ligands of the [2Fe-2S] cluster. It was found that His159 (in the *R. capsulatus* numbering) is not a ligand and that the presence of the Rieske protein in the intracytoplasmic membrane is greatly decreased by alteration of any of the remaining six His or Cys residues. Among these mutations, only the substitution Cys155 to Ser resulted in the synthesis of Rieske protein (in a small amount) which contained a [2Fe-2S] cluster with altered biophysical properties. This finding suggested that Cys155 is not a ligand to the cluster. A comparison of the conserved regions of the Rieske proteins with bacterial aromatic dioxygenases (which contain a spectrally and electrochemically similar [2Fe-2S] cluster) indicated that Cys133, His135, Cys153, and His156 are conserved in both groups of enzymes, possibly as ligands to their [2Fe-2S] clusters. These findings led to the proposal that Cys138 and Cys155, which are not conserved in bacterial dioxygenases, may form an internal disulfide bond which is important for the structure of the Rieske protein and the conformation of the quinol oxidation (Q_o) site of the bc_1 complex.

The ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex)[1] is a key component of photosynthetic and respiratory electron-transport chains in many organisms, and is a site where energy transfer is coupled to ATP synthesis (Dutton, 1986). The subunit composition of this membrane-bound complex varies with the organism, but irrespective of the source, all have three components with the following functional prosthetic groups: a cytochrome *b* containing two *b* hemes, a cytochrome c_1 containing a *c* heme, and an iron–sulfur protein, commonly referred to as the Rieske iron–sulfur (Fe-S) protein, which

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[1] Abbreviations: bp, base pair(s); bc_1 complex, ubiquinol–cytochrome *c* oxidoreductase; cyt, cytochrome; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; E_m, redox midpoint potential; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; Q_o, quinol oxidation site; Q_i, quinone reduction site; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ps, photosynthetic growth.

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