Spectroscopic Evidence from Site-Directed Mutants of *Synechocystis* PCC6803 in Favor of a Close Interaction between Histidine 189 and Redox-Active Tyrosine 160, Both of Polypeptide D2 of the Photosystem II Reaction Center†

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ABSTRACT: The reaction center of photosystem II of oxygenic photosynthesis contains two redox-active tyrosines called Z and D, each of which can act as an electron donor to the oxidized primary electron donor, P_{680}^+. These tyrosines are located in homologous positions on the third transmembrane α-helix of each of the two homologous polypeptides, D1 and D2, that comprise the reaction center. Tyrosine D of polypeptide D2 has been proposed, upon oxidation, to give up its phenolic proton to a nearby basic amino acid residue, forming a neutral radical. Modeling studies have pointed to His190 (spinach numbering) as a likely candidate for this basic residue. As a test of this hypothesis, we have constructed three site-directed mutations in the D2 polypeptide of the cyanobacterium *Synechocystis sp.* PCC6803. His189 (the *Synechocystis* homologue of His190 of spinach) has been replaced by glutamine, aspartate, or leucine. Instead of the normal D· EPR signal (g = 2.0046; line width 16–19 G), PSII core complexes isolated from these three mutants show an altered dark-stable EPR signal with a narrowed line width (11–13 G), and g values of 2.0046, 2.0043, and 2.0042 for the His189Gln, His189Asp, and His189Leu mutants, respectively. Despite the reduced line width, these EPR signals show g values and microwave-power saturation properties similar to the normal D· signal. Furthermore, specific deuteration in one of those mutants at the 3 and 5 positions of the phenol ring of the photosystem II reaction center tyrosines results in a loss of hyperfine structure of the EPR signal, proving that the signal indeed arises from tyrosine. Proton-ENDOR studies of these tyrosine radicals show that one hyperfine coupling component of 3.5–3.6 MHz, observed in the wild-type strain disappears in all three mutants. Upon incubation of wild-type photosystem II core complexes in D_2O, this hyperfine coupling is lost, indicating that it originates from an exchangeable proton, most likely interacting with D· through a hydrogen bond. These results provide strong experimental evidence in favor of a close interaction between His189 and Tyr160 in the D2 polypeptide of photosystem II. This observation provides support for a model in which an imidazole nitrogen of His189 accepts the phenolic proton of Tyr160 upon oxidation of D, forming a back hydrogen bond to the phenolic oxygen of the neutral tyrosyl radical.

The photosystem II reaction center of green plants, algae, and cyanobacteria catalyzes the light-induced oxidation of water and reduction of plastoquinone. The reaction center consists principally of two integral membrane polypeptides (D1 and D2) that show regional homologies to the L and M subunits, respectively, of the purple photosynthetic bacteria reaction center (Trebst, 1986; Nanba & Satoh, 1987; Michel & Deisenhofer, 1988; Tang et al., 1990). The D1 and D2 polypeptides are believed to span the thylakoid membrane five times (Sayre et al., 1986).

Upon photoexcitation of the primary electron donor, P_{680},[1] an electron is transferred from P_{680}* to a pheophytin and subsequently to the primary and secondary plastoquinone acceptors, Q_A and Q_B. P_{680}^+ is reduced by electrons coming from two tyrosyl residues. One is redox-active tyrosine D, identified by isotopic labeling and site-directed mutagenesis experiments as Tyr160 of the D2 polypeptide of the cyanobacterium *Synechocystis* 6803 (Barry & Babcock, 1987; Debus et al., 1988b; Vermaas et al., 1988). Tyr161 is the homolous residue of the *psbD* gene (encoding polypeptide D2) in higher plants. The principal source of electrons for the reduction of P_{680}^+ (Boska et al., 1983; Gerken et al., 1988) is another redox active tyrosine, Z, which has been identified as Tyr161 of the D1 polypeptide (Debus et al., 1988a; Metz et al., 1989). This residue is the same number *psbA* codon in both cyanobacteria and higher plants. Tyr Z· in turn oxidizes a tetranuclear Mn cluster, believed to be the catalytic site of water oxidation [reviewed by Diner (1986), Babcock (1987), and Debus (1992)].

Tyrosyl radicals have also been identified in ribonucleotide reductase (Ehrenberg & Reichard, 1972), prostaglandin H synthase (Smith et al., 1992), galactose oxidase (Whittaker

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[1] Abbreviations: bp, base pairs; Chl, Chlorophyll *a*; cm, chloramphenicol; D, redox-active tyrosine 160 of the D2 polypeptide; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; FM, frequency modulation; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kan, kanamycin; MES, 2-(*N*-morpholino)ethanesulfonic acid; P_{680}, primary electron donor of PSII; P_{700}, primary electron donor of PSI; PSI, photosystem I; PSII, photosystem II; RF, radiofrequency; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; wt, wild type; xxx^R, resistance to antibiotic xxx; Z, redox-active tyrosine 161 of the D1 polypeptide.

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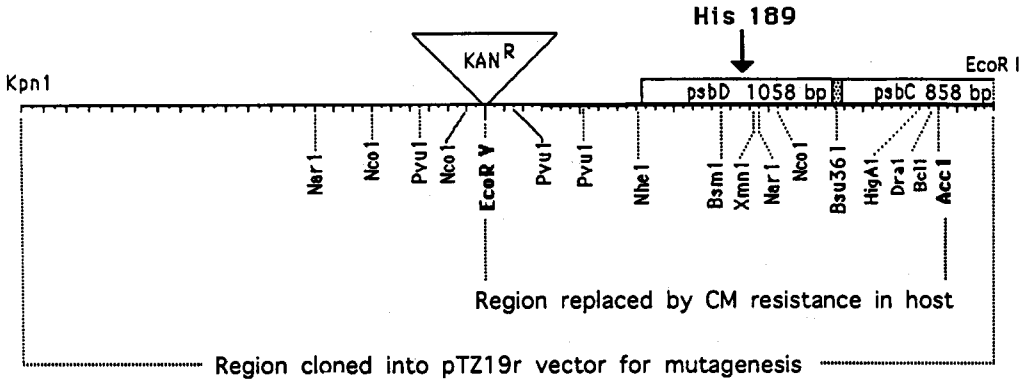

FIGURE 1: Composite map of the *psbD*I gene and flanking regions. The distance between major ticks is 100 bp. Only 858 base pairs of the 1382 bp *psbC* gene were cloned. The hatched area indicates the 17 base pair overlap between the *psbD* and *psbC* genes. The 4.9 kb *Kpn*I–*Eco*RI region was cloned into pTZ19r to enable alterations to be made in the His189 codon of *psbD* gene as indicated by the arrow. A *kan*^{R} gene was inserted into the *Eco*RV site of the cloned DNA. Mutant constructs containing the *Kpn*I–*Eco*RI region, including the *kan*^{R} gene, were used to transform a modified *Synechocystis* host strain in which the *Eco*RV to *Acc*I region had been replaced by a *cm*^{R} gene. Homologous recombination was directed by the *Kpn*I to *Eco*RV region on one end and the *Acc*I to *Eco*RV region on the other end, resulting in the reconstitution of the *psbD* and *psbC* genes. Selection for kanamycin resistance allowed recovery of the desired genotype.

& Whittaker, 1990), and amine oxidase (Jones et al., 1990). Consequently, the elucidation of the role of the protein environment in the function of the Tyr D· radical in the D2 polypeptide of PSII will contribute to our understanding of structure–function relationships in PSII as well as in other enzyme systems in which tyrosyl radicals have been identified.

The physiological function of Tyr D in PSII is as yet unclear, although it has been shown to participate in slow redox reactions with the Mn cluster under special circumstances (Styring & Rutherford, 1987). Both D· and Z· show similar EPR spectra (g = 2.0045, ΔH_{pp} = 19–20 G) at room temperature but arise and decay with very different kinetics (Babcock & Sauer, 1973; Blankenship et al., 1975; Hoganson & Babcock, 1988). The Tyr Z· radical decays on the microsecond to millisecond time scale, depending on the oxidation state of the Mn cluster. The Tyr D· radical decays on the seconds to hours time scale showing multiphasic kinetic components (Styring & Rutherford, 1987; Vermaas et al., 1984).

On the basis of the EPR g value and proton-exchange data from ENDOR and ESEEM, D· has been suggested to be a neutral hydrogen-bonded radical (Rodriguez et al., 1987; Barry & Babcock, 1988; Evelo et al., 1989). That the tyrosyl radical has undergone deprotonation also makes good thermodynamic sense in that the midpoint potential of 0.71–0.76 estimated by Boussac and Etienne (1984) and Vass and Styring (1991) for this species is well below the E_m of the cationic radical (>1.3 V; Harriman, 1987). Babcock et al. (1989) have proposed a model in which the phenolic proton of D is postulated to be hydrogen bonded to a neighboring basic amino acid residue. Upon oxidation, the tyrosine loses the phenolic proton to the base, resulting in the formation of a neutral free radical species. The protonated base now back hydrogen bonds to the phenolic oxygen of the tyrosine neutral radical.

Svensson et al. (1990) have modeled the three-dimensional structure surrounding the tyrosine radicals in PSII. His190 of the D2 polypeptide (His189 in *Synechocystis* 6803), conserved in all primary sequences, was suggested to point into the cavity occupied by D, with one of the nitrogens of the imidazole side chain turned toward the phenolic oxygen of D·. The proposed 2–3-Å distance between the O- and N- is close enough to allow hydrogen bond formation between His190 and D·. Such an interaction was first proposed by Debus et al. (1988b). A similar structure was suggested by Svensson et al. (1990) for Z in the D1 polypeptide, with a hydrogen bond between D1-His190 and Z·. More recently, a similar computer-modeled structure surrounding D has also been proposed by Ruffle et al. (1992), though the Z–His190 distance in D1 is proposed to be greater than the homologous pair in D2.

We present here experimental evidence, using magnetic resonance spectroscopy, consistent with hydrogen bond formation between His189 and Tyr160 in the D2 polypeptide of PSII. To test for the presence of this hydrogen bond, we have constructed three site-directed mutants in *Synechocystis* 6803, one of the most widely used organisms for the genetic manipulation of photosynthetic systems (Williams, 1988). The His codon 189 of *psbD* was replaced by codons for Asp, Gln, and Leu. PSII core complexes show altered EPR signals in the dark which are confirmed to arise from the Tyr D· radical by specific isotopic labeling and by EPR analysis. ENDOR studies on the altered D· signal from the mutants and the normal D· signal from wild type show that one proton hyperfine coupling component (3.5 MHz), observed in wild type, is missing in the His189 mutants. This component is clearly D_2O-exchangeable, indicating that this coupling originates from an exchangeable proton of D2-His189 that is likely involved in a hydrogen bond to Tyr D·.

MATERIALS AND METHODS

Strains and Construction of Mutants. All genetic manipulations were performed on a glucose-tolerant (Williams, 1988) and phycocyanin-deficient "olive" strain of *Synechocystis* 6803 (Rögner et al., 1990). The host strain, Tol145, is a double deletion mutant in which both copies of *psbD* have been rendered nonfunctional. Almost all of the *psbD*II gene has been replaced by a spectinomycin resistance cassette (Prentki & Krisch, 1984), leaving only the first 147 and last 13 bases of the DII gene. The *psbD*I gene and part of the *psbC* gene were replaced by a chloramphenicol resistance cassette (Chang & Cohen, 1978) as shown in Figure 1.

The *psbD*I gene and flanking sequences, including 60% of *psbC*, were cloned into plasmid pTZ19r, and site-directed mutations were introduced using the method of Kunkel et al. (1987). Mutant constructs were verified by sequencing. To provide selection for new mutants, a kanamycin resistance cassette with *Hinc*II ends (Taylor & Rose, 1988) was blunt ligated into an *Eco*RV site upstream of *psbD*I.

Plasmid pTZ19r DNA, containing site-directed mutations, was used to transform the *Synechocystis* 6803 host strain in the manner of Williams (1988). *Synechocystis* transformants

were grown with no selection for 24–36 h on filters placed on BG11 + 5 mM glucose (BG) plates. Transformants were selected on BG plates + 15 μg/mL kanamycin until colonies appeared (after 10 days), at which point they were picked to BG plates + 50 μg/mL kanamycin. These colonies were streaked again on BG + kanamycin (50 μg/mL) plates to provide cells for frozen storage and DNA isolation.

The presence and integrity of the mutated *psbD*I gene was verified by Southern hybridization and by directly sequencing PCR amplified DNA in the region of the mutations.

Growth of Cells and Preparation of PSII Core Complexes. Cells of *Synechocystis* were grown photoheterophically at 30 °C for 3–4 days in 18-L carboys containing BG-11 medium (Rippka et al., 1979) plus 5 mM glucose bubbled with 5% CO_2 in air. To specifically deuterate tyrosine, D2-His189Gln mutant cells were grown photoautotrophically in BG-11 medium containing 0.5 mM phenylalanine, 0.25 mM tryptophan, and 0.25 mM 3,5-deuterated tyrosine for 6–7 days following the method of Barry and Babcock (1987). PSII core complexes were purified according to the procedure described earlier (Rögner et al., 1990). The purified protein complex from each cell type was concentrated and transferred to 20 mM MES–NaOH buffer (pH 6.5) containing 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 M mannitol, and 0.03% dodecylmaltoside. For the core complex from the D2-His189Leu mutant sample, 0.3 mM ferricyanide was added to the final buffer, and the sample was illuminated with saturating light for 1 min at 0 °C, followed by 10 min of dark adaptation. All samples were loaded into EPR tubes and frozen in liquid nitrogen in the dark.

D_2O Exchange. The PSII core complex from wild-type cells was transferred to a buffer containing 20 mM HEPES–NaOH (pD 7.5) and 10 mM NaCl prepared with D_2O, followed by 10–12 h of incubation at 4 °C in the dark. The control sample was treated in the same way except using H_2O.

EPR and ENDOR Measurements. EPR spectra were recorded on a Bruker ESP-200 spectrometer equipped with an upgraded computer software system (ESP-300 model) at 150 K using an Oxford ESR-910 helium flow cryostat. The g values were determined from the zero crossing point of the EPR spectrum using wild-type tyrosine D· as a reference (g = 2.0046). The microwave power at half saturation of the EPR signal, $P_{1/2}$, was estimated from the intersection of the two tangents of the power saturation curve, obtained by plotting $\log P$ against $\log(I/P^{1/2})$. ENDOR spectra were recorded at 9.98 GHz using a Bruker ESP300/ER250A EPR/ENDOR spectrometer operating with a TM 110 microwave cavity fitted with a 1–30 MHz coil mounted on a quartz Dewar flask for liquid nitrogen (Bruker). All ENDOR measurements were performed at 100–120 K using a liquid nitrogen flow system (Bruker, variable temperature unit, ER4111VT).

Assay of Oxygen Evolution. Oxygen evolution under continuous illumination was measured at 25 °C with a microsystem Clark-type oxygen electrode (model 5300, Yellow Spring Instruments). Saturating white light was passed through a heat-reflecting and a high band-pass (>530 nm) filter. Whole cells were suspended in BG11 medium plus 5 mM glucose in the presence of 2 mM DCBQ as an artificial electron acceptor.

Flash-induced oxygen evolution was measured at 25 °C with a Joliot-type oxygen electrode, adapted to fit into a swinging bucket centrifuge rotor, as described by Nixon and Diner (1992). Sample cells, suspended in BG11 plus 5 mM glucose and 100 mM KCl, were spun down to form a homogenous layer on the electrode surface. After 5–10 min of dark adaptation, the sample was illuminated by a series of 15 saturating single-turnover flashes given 533 ms apart. Saturating flashes were supplied by two Stroboslave flash lamps (type 1539-A, GenRad) fired simultaneously. The steady-state oxygen flash yield was measured by taking the average of the oxygen produced by the 12th through the 15th flashes.

Table I: Relative Oxygen Evolving Activity and PSII Reaction Center Concentration in Cells of Olive Wild Type and Mutants[a]

cell type	oxygen rate in continuous light (%)	relative oxygen yield per flash[b] (%)	PSII RC concentration[b] (%)
"olive" wt	100[c]	100	100
His189Gln	30–40	20–30	73
His189Leu	35–41	25–35	46
His189Asp	17–29	13–20	47
Tyr160Met	28–35	15–25	59
Tyr160Phe	36–44	20–30	80

[a] For a detailed description of the measurements, see Materials and Methods. [b] On a per cell basis. [c] The absolute rate of oxygen evolution of "olive" wild-type cells under continuous illumination is 320–360 μmol of O_2 per mg of chlorophyll per hour.

Assay of Chlorophyll a. The chlorophyll *a* content was determined by extraction into methanol. The extinction coefficient at 665.5 nm [79.24 mL/(mg of chl·cm)] was adopted from Lichtenthaler (1987).

Determination of the Relative Concentration of PSII Reaction Centers. The PSII reaction center concentration per cell, relative to wild type, was estimated by measuring the variable chlorophyll fluorescence in the presence of NH_2OH plus DCMU following pretreatment of the cells with benzoquinone and ferricyanide using a flash detection spectrophotometer as described in Nixon and Diner (1992). The cells of the "olive" wild type and mutants were suspended in BG-11 medium at an $OD_{730\,nm}$ of 0.9.

RESULTS

Photoautotrophy and Oxygen Evolution of D2-His198 Mutants. The *psbD*1 gene of *Synechocystis* 6803 was engineered as described under Materials and Methods such that D2-His189 in the gene product was replaced by any one of three different residues, glutamine, leucine, and aspartate. The first two of these were able to grow photoautotrophically at reduced rates on petri plates containing BG-11. While the leucine mutant grew only marginally, the aspartate mutant could not grow at all photoautotrophically.

Table I summarizes the results of oxygen evolution measurements for cells of "olive" wild type and D2-His189 mutants under continuous illumination and flashing light. Also indicated for comparison are two mutants in which D2-Tyr160 was replaced by methionine or phenylalanine. Both of these are capable of photoautotrophic growth, but at reduced rates compared to wild type. The rates of oxygen evolution in continuous light are an indicator of the ability of the strain to grow photoautotrophically, with those mutants showing less than 30% of wild-type rates unable to survive in the absence of glucose. This observation is consistent with what was observed for a series of mutations at D1-Asp170 (Nixon & Diner, 1992).

EPR Spectra. Figure 2 displays the low temperature EPR spectra of PSII core complexes purified from wild-type cells (A) and His189 mutant cells (B–D). A typical dark-stable tyrosine D· EPR spectrum, with a line width of about 19–20 G and a g value of 2.0046, was observed in the wild-type sample. However, the PSII core complexes from all three

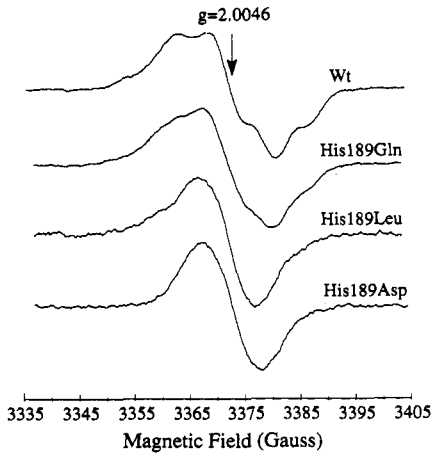

FIGURE 2: Dark-stable EPR spectra of PSII core complexes isolated from wild-type *Synechocystis* 6803 and His189 mutants at ~1 mg of chl/mL. Instrument settings: modulation frequency, 100 kHz; microwave frequency, 9.46 GHz; modulation amplitude, 4 G; microwave power, 0.2 mW; temperature, 150 K.

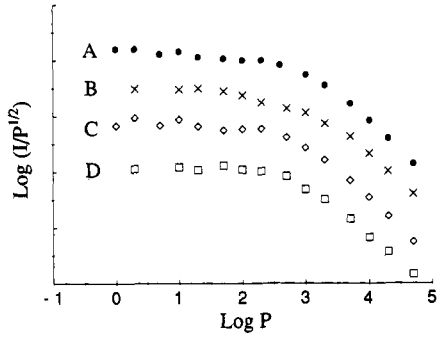

FIGURE 3: Microwave power saturation curves of EPR signals shown in Figure 2. (A) Wild type; (B) His189Gln mutant; (C) His189Leu mutant; (D) His189Asp mutant. Experimental conditions are as in Figure 2 except for the microwave power. The curves have been displaced vertically for clarity and are not intended to show the relative amounts of the signals.

His189 mutants showed altered dark-stable EPR signals, present at a spin concentration about half of that of wild type on a per chlorophyll basis. These signals showed g values of 2.0046, 2.0043, and 2.0042 for the His189Gln, His189Leu, and His189Asp mutants, respectively, and narrowed line widths (ΔH_{pp} = 11–13 G). Their g values and their asymmetric line shapes due to partially resolved hyperfine splittings suggested that these signals originated from tyrosine D· radicals. Similar features were observed for the room-temperature EPR spectra (data not shown).

Power Saturation and Isotopic Labeling. Two types of experiments were carried out to further verify that these signals indeed arise from tyrosine D·, rather than from chlorophyll radicals or from some new paramagnetic species. In the first of these, the microwave power-saturation properties of these signals were investigated. Figure 3 shows the microwave power-saturation curves of EPR signals from the wild type and D2-His189 mutants. All three mutants show saturation curves similar to that of wild type with $P_{1/2}$ = 0.75–0.85 mW at 150 K, suggesting that the EPR signals observed in the mutants and wild type originated from the same paramagnetic species.

A more definitive proof of this point was obtained by deuterating the 3- and 5-positions of the phenolic ring of the reaction center tyrosines in one of the His189 mutants (His189Gln) by growing the cells under conditions of functional aromatic amino acid auxotrophy [see Materials and Methods and Barry and Babcock (1987)]. The EPR spectrum, recorded in the dark, of the PSII core complex isolated from these cells shows the loss of the hyperfine structure of D·, compared to the same mutant grown under normal growth conditions with fully protonated tyrosine (Figure 4). The EPR spectrum observed here in the 3,5-deuterated sample is similar to those of 3,5-deuterated model tyrosine and D· radicals observed in 3,5-deuterated wild-type *Synechocystis* cells (Barry et al., 1990). This result clearly demonstrates that the EPR signal observed in the His189Gln mutant indeed arises from tyrosine. This observation, the partially resolved hyperfine structure of the radicals observed for the other two mutants (Figure 2), the elevated g values at the zero crossing points observed for the EPR spectra in all the mutants, the similar microwave power saturation dependence (Figure 3), and the fact that the signals are obtained in purified PSII core complexes, all argue that in all three mutants the EPR spectra arise from tyrosine D·. The deuteration experiment (Figure 4) also argues against the possibility that these narrow signals arise from a mixture of tyrosine radical and some 10 G narrow radical (e.g., chl^+ radical), frequently observed in illuminated PSII core complexes. Were this the case, a 10 G signal should have become discernible in the 3,5-deuterated sample (Figure 4, dashed line) owing to differences in the g values of chlorophyll cation radicals (2.0025) and neutral tyrosyl radicals (2.0045). No such chlorophyll radical is observed. We conclude from these experimental results that the replacement of His189 by Asp, Leu, or Gln in the D2 polypeptide causes a change in the hyperfine structure and line width of the tyrosine D· EPR signal. These observations indicate that His189 and Tyr160 are close enough to interact and that the loss of this interaction causes either a change(s) in radical orientation and/or a change in the spin-density distribution of Tyr D·.

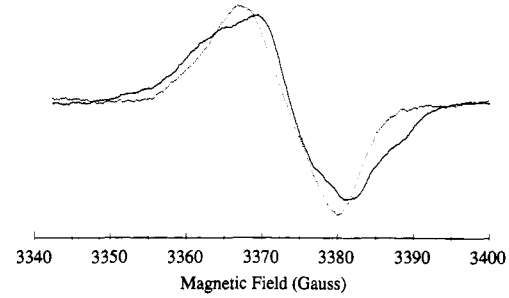

FIGURE 4: Dark-stable EPR spectra of PSII core complexes purified from His189Gln mutant cells grown on normal BG-11 medium (solid line) or in the presence of 3,5-deuterated tyrosine (dashed line, see Materials and Methods). Instrument settings are as in Figure 2.

Proton ENDOR. Proton ENDOR experiments were carried out to investigate further the nature of this interaction. Figure 5A shows the proton ENDOR spectrum obtained from PSII core complexes incubated in H_2O and isolated from cells grown in H_2O. Also shown (Figure 5B) is a proton ENDOR spectrum obtained from PSII core complexes incubated in D_2O and isolated from cells grown in H_2O. One proton hyperfine coupling component (3.5 MHz) is clearly D_2O-exchangeable. The rate of exchange is extremely slow as, even after 14 h of incubation at 0 °C, there is still a trace of 3.5-MHz signal left in the D_2O sample due to incomplete exchange. Such extremely slow D_2O/H_2O-exchange kinetics for the D· radical have been reported previously (Rodriguez et al., 1987; Evelo et al., 1989). The D_2O exchangeability of the 3.5-MHz coupling indicates that it originates from an exchangeable proton interacting with the tyrosine D· radical. This result agrees with a previous ENDOR study on the D· radical using Tris-washed PSII particles from spinach (Rodriguez et al.,

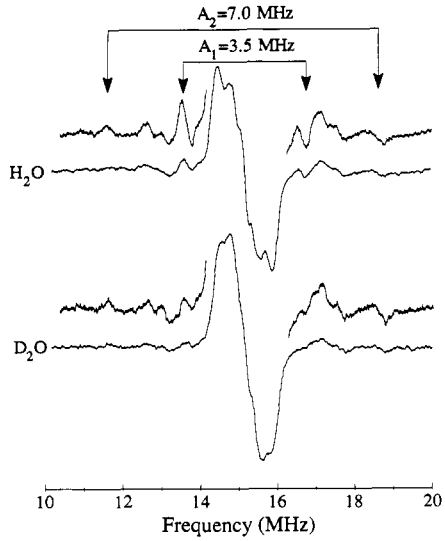

FIGURE 5: Proton-ENDOR spectra of wild type PSII core complex dissolved in H_2O buffer (upper) or exchanged with D_2O as described under Materials and Methods (lower). Instrument conditions: microwave frequency, 9.98 GHz; microwave power, 4 mW; maximum RF power, 150 W; FM amplitude, 140 kHz (for whole region scan) and 70 kHz (for insert narrow scan); magnetic field, 3557 G; temperature, 105 K; number of scans, 20 (for whole region scan) and 50 (for insert narrow scan). The hyperfine coupling constants are given as the peak to peak splitting as shown.

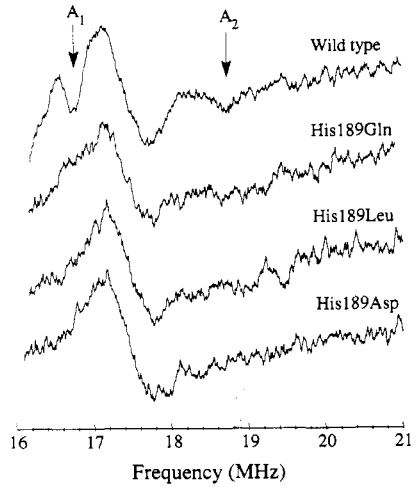

FIGURE 6: Comparison of the proton-ENDOR spectra of PSII core complexes isolated from wild-type cells and His189 mutants. A1 and A2 indicate the two hyperfine coupling components (3.5 and 7.0 MHz) shown in Figure 5. Instrumental conditions: FM amplitude, 140 kHz; number of scan, 20. For details, see Figure 5.

1987). However, the loss of another proton hyperfine coupling at 7.0 MHz, assigned to the same D_2O-exchangeable proton in PSII particles from spinach by Rodriguez et al. (1987), is not observed here. We cannot exclude, however, a subcomponent at 7.0 MHz that is exchangeable. Figure 6 displays the results of proton-ENDOR experiments using PSII core complexes isolated from the His189 mutants. The D_2O-exchangeable 3.5-MHz component completely disappears in all three mutants. These results indicate that residues His189 and Tyr160 of the D2 polypeptide interact magnetically over a short distance and are possibly linked by a hydrogen bond. Given the present resolution, it is unclear whether the hyperfine coupling of 7.0 MHz is reduced or not in the His189 mutants.

DISCUSSION

$D^{\cdot}$ has been proposed to be a hydrogen-bonded neutral (deprotonated) tyrosine free radical in which the phenolic proton is shared between the tyrosine and a nearby amino acid (Rodriguez et al., 1987; Babcock et al., 1989; Evelo et al., 1990). Loss of the phenolic proton of D, resulting in the formation of a neutral free radical, is probably required for tyrosine oxidation, in light of its estimated midpoint potential (Boussac & Etienne, 1984; Vass & Styring, 1991; $E_{m,7}$ = 0.71–0.76 V) much below the E_m of the tyrosyl radical cation (>1.3 V; Harriman, 1987) . Early ENDOR and ESEEM experiments have shown that $D^{\cdot}$ interacts with an exchangeable proton (Rodriguez et al., 1987; Evelo et al., 1989). Two proton hyperfine couplings of 3.5 and 7.0 MHz were assigned to a single exchangeable proton in ENDOR studies of Tris-washed PSII membrane fragments of spinach (Rodriguez et al., 1987). However, in this paper we have only been able to confirm the 3.5-MHz component to be D_2O-exchangeable using PSII core complexes isolated from H_2O-grown *Synechocystis* cells. We do not see exchange at 7.0 MHz, though in this region there may be two or more components (M. Espe and G. T. Babcock, personal communication), one of which might be exchangeable and not observable with the 70-kHz frequency modulation amplitude used in the ENDOR experiment of Figure 5. It is also possible that there are species differences between *Synechocystis* 6803 and spinach. It is likely that the origin of the 3.5-MHz coupling is the former phenolic proton that is now bound to a nearby basic amino acid. This proton remains in close proximity to the tyrosine radical, probably hydrogen bonding to the phenolic oxygen of the neutral tyrosyl radical. The disappearance of the 3.5-MHz component in all three of the D2-His189 mutants argues strongly that His189 in the D2 polypeptide is the bearer of the coupled proton and the probable acceptor of the D phenolic proton.

While likely, we cannot at present be absolutely certain that the exchangeable proton that we attribute to His189, within a short distance of Tyr $D^{\cdot}$, is hydrogen-bonded to the phenolic oxygen. Further ENDOR studies should indicate whether there is an isotropic component to the coupling between the exchangeable proton, detected at 3.5 MHz, and $D^{\bullet}$. Evidence of such a component and its implication of orbital overlap would strengthen the assignment of this exchangeable proton to a hydrogen bond.

The replacement of His189 by Gln, Leu, and Asp in the D2 polypeptide results in an unexpected narrowing of the dark-stable EPR signals from 19–20 G to 11–13 G with a partial loss of hyperfine structure (Figure 2). In work done independently and in parallel with our own, Styring et al. (1993) and Tommos et al. (1993) have recently reported the replacement of D2-His189 in *Synechocystis* 6803 by Leu and Tyr and have observed a nearly structureless and somewhat narrower (9–10 G) radical in thylakoid membranes from these two mutants. A potential criticism of the EPR result in these papers and in the present publication is that an apparent narrowing of the radical signal can be caused by the appearance of some new paramagnetic species other than tyrosine that superimposes itself over the normal tyrosine $D^{\cdot}$ signal (e.g., 9–10 G chl^{+} radical) . The g values, the partially resolved hyperfine structure, and the microwave power saturation properties of the signals presented here (Figure 3) argue that this is not the case in this work. Figure 4 provides definitive proof that this signal arises from tyrosine, as specific deuteration of the 3- and 5-positions of the reaction center tyrosines in one of the mutants (D2-His189Gln) results in a loss of hyperfine structure. This experiment also argues that there is no overlapping 9–10 G narrow radical. The experimentof Figure 4 and the similarity in the ENDOR spectra

(Figure 6) together argue that the radical generated in all three mutants arises from tyrosine D.

The reason for the narrowing of the tyrosine radical in the His189 mutants is as yet unclear. There are, however, several possible explanations. The EPR line shape of tyrosine D· is determined mainly by hyperfine coupling to one proton at the β-methylene position and to the two protons at the 3 and 5 ring positions (Barry et al., 1990; Hoganson & Babcock, 1992). For the 3 and 5 ring positions, the proton hyperfine coupling is determined by the spin density on the ring carbon to which the proton is bonded and a proportionality constant. The hyperfine couplings of the β-methylene protons depend on both the C1 ring carbon spin density and the dihedral angle which is defined by the projection of the Cβ–Hβ bond onto the C1 ring carbon p_z orbital. A change in spin density at the C3, C5, or C1 ring carbons due to the loss of hydrogen-bonding in the His189 mutants could contribute to an EPR signal narrowing. Another possibility is that, in the mutants, the tyrosine radical could have become more flexible through the loss of a hydrogen-bond. The phenolic ring of D2-Tyr160 may have undergone a rotation or may exist in a mix of rotomeric states of the side chain. These would give, respectively, a new dihedral angle or a mix of dihedral angles for the projection of the β-methylene proton onto the C1-p orbital. The new projection or the interference between the EPR spectra of the rotomers could produce line width narrowing. One additional possible explanation for the narrowing is that, upon loss of the hydrogen bond, a change in orientation of tyrosine D enhances orbital overlap with a neighboring aromatic amino acid (e.g., Phe or Trp). A sharing of spin density between D· and such an amino acid could induce a narrowing of the EPR signal, as has been observed for the special bacteriochlorophyll pair of $P870^+$ of the bacterial reaction centers when compared to the bacteriochlorophyll monomeric cation (Norris et al., 1971; Feher et al., 1975). An investigation of the spin-density distribution and of the cause of the narrowing of the EPR signal in these mutants is now under way.

It is known that the midpoint potentials ($E_{m,6}$) of Z·/Z and D·/D are quite different, approximately 1.0 and 0.75 V, respectively. The slow exchange between the solvent phase and the immediate environment of the tyrosyl radical (hours for D·; Rodriguez et al., 1982) implies that, on the time scale of tyrosine D oxidation, the pK_a of the proton acceptor, whether hydrogen-bonded or not, is a critical determinant of the E_m of the tyrosyl radical. Should the proton acceptor be hydrogen-bonded to the phenolic oxygen, the strength of the hydrogen bond, determined by the pK_a of the proton acceptor, is another way in which the protein can modulate the midpoint potential of the tyrosyl radical to suit the needs of the components coupled to its oxidation and reduction. The modeling of Ruffle et al. (1993) has suggested that the distances between the Z and D tyrosines and the homologous histidines, D1-His190 and D2-His189, respectively, are inequivalent and longer in the first case than in the second. This observation may imply that while D2-His189 may be the proton acceptor for D2-Tyr160 and hydrogen bonded to it, the proton acceptor for D1-Tyr161 may not be D1-His190 but a different more acidic residue. Alternatively, D1-His190 may still be a non-hydrogen-bonded proton acceptor, but the presence of local charged groups lowers the pK_a of this residue. That this residue is critical to the function of tyrosine Z has been shown by the introduction at D1-His190 of the site-directed replacements Gln, Asp, Glu, Trp, and Tyr . We have found that in such mutants the electron transfer from Z to P_{680}^+ slows down about 200-fold (Diner et al., 1991). None of these mutants, however, evolve oxygen and are consequently incapable of photoautotrophic growth. Despite these marked kinetic effects, loss of D1-His190 in the Asp, Gln, and Glu mutants appears to have little effect on the EPR spectrum of Z· (Tang, Nixon, and Diner, manuscript in preparation) implying that the interaction between D1-His190 and D1-Tyr161 is different from that between D2-His189 and D2-Tyr160. This difference also argues that the effect of the replacement of D2-His189 on the EPR spectrum of D· is the result of the loss of a specific interaction between these residues and not the consequence of a general conformational change in protein structure associated with histidine replacement.

The role of tyrosine D and of His 189 in its function is still unclear. In all the His189 mutants, the spin concentration of the dark-stable EPR signal is about half of that of wild type on a per chlorophyll basis, indicating that Tyr D· in the mutants is either more difficult to generate or decays faster than in wild type. Tommos et al. (1993) have reported a shorter life time for the dark-stable radicals in the D2-His189Tyr and His189Leu mutants compared with the normal D· radical in wild type, though these lifetimes are still measured on the hours time scale. We have found that the His189Leu and His189Asp mutants can, respectively, barely grow photosynthetically or not at all. Only D2-His189Gln cells can grow photoautotrophically in BG11 medium, but the growth rate is much slower than for wild type cells. These results indicate the importance of His189 to the physiological function of tyrosine D. This observation is reinforced by the slow growth of mutants D2-Tyr160Met and Phe, both of which lack tyrosine D. We find that in all these site-directed mutants the loss of oxygen evolution exceeds that of the decrease in the number of PSII centers (see Table I). This observation contrasts in degree with that of Tommos et al. (1993) for the D2-His189 mutants where the losses in PSII centers and O_2 evolution, measured in continuous light, were reported to be equivalent. The reason for this difference is not immediately clear, though the flash O_2 measurement may be a more accurate indicator of the relative number of active O_2-evolving centers. If so, then any loss in function of tyrosine D may result in an impairment in the ability to assemble or to maintain assembled the Mn cluster required for O_2 evolution. In a recent paper by Kirilovsky et al. (1992), the Mn cluster in a mutant lacking tyrosine D, D2-Tyr160Phe, was more unstable to thylakoid isolation and detergent treatment than was the wild-type strain.

Tommos et al. (1993) used thylakoid membranes rather than PSII core complexes as the experimental material for the study of the D2-His189 mutations, having found an alteration in the line shape of D· in detergent-solubilized complexes of D2-His189Tyr. Contrary to this observation, it has been observed in spinach that the EPR spectra are identical in both types of material (Satoh, 1983). Furthermore, the EPR spectrum we report here for Tyr D· in core complexes of wild-type *Synechocystis* 6803, prepared according to Rögner et al. (1990), is identical in line shape to that reported by Tommos et al. (1993) in thylakoid membranes isolated from the same organism. Core complexes of *Synechocystis* 6803 isolated by different procedures differ in how native they are. We have observed in our own lab that the harshness of the detergent treatment can influence the line shape of the D· radical. The use of purified PSII core complexes, prepared as we have described here, (a) eliminates EPR spectral contamination by P_{700}^+, rising from the 5–10-fold higher concentration of PSI than PSII centers (Rögner et al., 1990) as well as other paramagnetic species external to PSII and (b)

allows the EPR and ENDOR samples to be considerably more concentrated with a resulting gain in signal/noise.

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