Modified EPR Spectra of the Tyrosine$_D$ Radical in Photosystem II in Site-Directed Mutants of *Synechocystis* sp. PCC 6803: Identification of Side Chains in the Immediate Vicinity of Tyrosine$_D$ on the D2 Protein†

Cecilia Tommos,‡ Lars Davidsson,‡ Bengt Svensson,‡ Cathy Madsen,§ Wim Vermaas,‡,§ and Stenbjörn Styring*,‡

Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden, and Department of Botany and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601

Received October 21, 1992; Revised Manuscript Received February 11, 1993

ABSTRACT: The oxidizing side of photosystem II contains two redox-active tyrosyl side chains, Tyr$_Z$ and Tyr$_D$, and a cluster of Mn atoms involved in water oxidation. The structural environment of these components is unknown, and with computer-assisted modeling we have created a three-dimensional model for the structures around Tyr$_Z$ and Tyr$_D$ [Svensson et al. (1990) *EMBO J. 9*, 2051–2059]. Both tyrosines are proposed to form hydrogen bonds to nearby histidine residues (for *Synechocystis* 6803, these are His190 on the D1 and His189 on the D2 proteins). We have tested this proposal by electron paramagnetic resonance (EPR) spectroscopy of Tyr$_D^{ox}$ in mutants of the cyanobacterium *Synechocystis* 6803 carrying site-directed mutations in the D2 protein. In two mutants, where His189 of the D2 protein is changed to either Tyr or Leu, the normal EPR spectrum from Tyr$_D^{ox}$ is replaced by narrow, structureless radical signals with *g*-values similar to that of Tyr$_D^{ox}$ ($g \approx 2.0050$). The new radicals copurify with photosystem II, are dark-stable, destabilized by elevated pH, and light-inducible, and originate from radicals formed by oxidation. These properties are similar to those of normal Tyr$_D^{ox}$, and we assign the new spectra to Tyr$_D^{ox}$ in an altered environment induced by the point mutation in His189. In a third mutant, where Gln164 of the D2 protein was mutated to Leu, we also observed a modified EPR spectrum from Tyr$_D^{ox}$. This is also consistent with the model in which this residue is found in the immediate vicinity of Tyr$_D^{ox}$. Thus the results provide experimental evidence supporting essential aspects of the structural model.

Photosystem II (PSII)[1] (Andersson & Styring, 1991; Vermaas & Ikeuchi, 1991) is in many respects homologous to the structurally solved and well-studied photosynthetic reaction center from purple bacteria (Deisenhofer et al., 1985). Structurally, the topological organization of the PSII reaction center proteins D1 and D2 is similar to that of the L and M subunits of the reaction center from purple bacteria (Trebst, 1986; Michel & Deisenhofer, 1988; Svensson et al., 1990). Functionally, the acceptor side of PSII strongly resembles that in reaction centers of purple bacteria. However, the oxidizing (donor) side of PSII involves a unique set of cofactors and prosthetic groups which have no correspondence in the bacterial reaction centers.

The electron transport pathway on the PSII donor side is rather well understood. After light-induced oxidation of the primary donor P_{680}, P_{680}^+ is reduced by the water-splitting system via a redox-active component. In its oxidized form this component gives rise to a characteristic radical EPR signal called Signal II$_{(v)f}$, where (v)f stands for (very) fast and denotes the time scale of its oxidation and rereduction (Babcock et al., 1976; Hoganson & Babcock, 1988). An EPR signal indistinguishable in shape, but with much slower kinetics, originates from the oxidized form of another accessory donor in PSII; this EPR signal is known as Signal II$_{slow}$ (Babcock & Sauer, 1973). Signal II$_{slow}$ was found to originate from a tyrosyl radical (Barry & Babcock, 1987), and using site-directed mutagenesis in the cyanobacterium *Synechocystis* sp. PCC 6803, this signal was shown to originate from the oxidized form of Tyr160[2] of the D2 protein (Debus et al., 1988a; Vermaas et al., 1988). This Tyr residue is commonly referred to as Tyr$_D$. With similar techniques, the species giving rise to Signal II$_{vf}$ has been identified as the symmetrically located residue, Tyr161 of the D1 protein (Debus et al., 1988b; Metz et al., 1989); this Tyr is known as Tyr$_Z$. Signal II$_{slow}$ has been extensively studied and is, because of its stability in the dark, a very useful intrinsic spectroscopic probe for the structure and function of PSII. It is also one of the most stable organic radicals known in protein research and has thus attracted considerable interest from fields other than photosynthesis.

The identification of Tyr$_Z$ and Tyr$_D$ as electron donors on the oxidizing side of PSII has clarified functional aspects of

† This work was supported by the Swedish Natural Science Research Council, The Erna and Victor Hasselblad Foundation, and the Carl Trygger Foundation and by a research grant from the National Science Foundation (DMB 90-19248). W.V. was also supported by a travel grant from the Swedish Natural Science Research Council. This is publication number 137 of the Arizona State University Center for the Study of Early Events of Photosynthesis. The Center is funded by U.S. Department of Energy Grant DE-FG-88ER13969 as part of the USDA/DOE/NSF Plant Science Centers Program.

* Correspondence should be addressed to this author.

‡ Stockholm University.

§ Arizona State University.

[1] Abbreviations: Chl, chlorophyll; D1 and D2 proteins, the D1 (D2) reaction center proteins in PSII; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PSI, photosystem I; PSII, photosystem II; P_{680}, primary electron donor in PSII; P_{700}, primary electron donor in PSI; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Tyr$_D$, accessory electron donor in PSII; Tyr$_Z$, electron transport component between the water-oxidizing system and P_{680}.

[2] The amino acid numbering used in this manuscript refers to the primary sequence in *Synechocystis* 6803. In higher plants the amino acid numbering of the D2 protein differs by one as compared to *Synechocystis* 6803. Thus, Gln164$_{6803}$ corresponds to Gln165$_{plant}$, Tyr160$_{6803}$ corresponds to Tyr161$_{plant}$, His189$_{6803}$ corresponds to His190$_{plant}$, etc. In some earlier publications (Svensson et al., 1990, 1991; Andersson & Styring, 1991) we have used the numbering in higher plants.

0006-2960/93/0432-5436$04.00/0

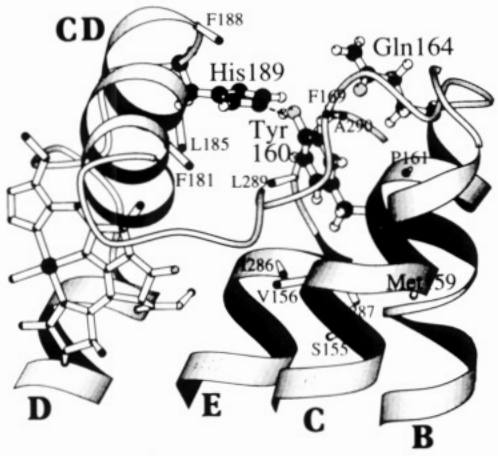

FIGURE 1: Structural model for the environment of Tyr$_D$ in wild-type *Synechocystis* 6803. The figure illustrates part of the D2 protein drawn with the plane of the thylakoid membrane perpendicular to the paper plane. Shown with ball-and-stick representation are the side chains of Tyr160 (Tyr$_D$) and the positions of His189 and Gln164. The proposed hydrogen bond between Tyr$_D$ and His189 is shown with a dashed line. In the protein domain around Tyr$_D$, the position of the C_α and C_β carbons from the neighboring residues is shown to indicate the approximate positions of the side chains. In addition, the figure shows the C_α and C_β carbons of Met159, which has also been mutated (Vermaas et al., 1988). Part of the special pair chlorophyll dimer is shown to the left. The protein backbone, represented by ribbons for the helical regions and wires for the loop regions, is placed identically to the corresponding regions in the structure of the bacterial reaction center from *Rhodopseudomonas viridis*. B, C, D, and E denote the four central transmembrane helices in the D2 protein. The figure also shows the CD helix in the lumenal loop between helices C and D. The figure was created with the program MOLSCRIPT (Kraulis, 1991).

the PSII donor side considerably (Debus, 1992). However, our knowledge is limited by the lack of a three-dimensional structure of PSII. To increase our understanding of the nature, orientation, and three-dimensional positioning of the components on the oxidizing side of PSII, we carried out molecular modeling of this part of the complex (Svensson et al., 1990), utilizing the three-dimensional structure of the reaction center from photosynthetic purple bacteria (Deisenhofer et al., 1985) as a starting point. The molecular modeling was used to determine possible molecular interactions between the tyrosines and other residues in the PSII complex and to analyze the surroundings of Tyr$_Z$ and Tyr$_D$ with respect to hydrophobicity and amino acid conservation (Svensson et al., 1991). Figure 1 shows the environment of Tyr$_D$ as determined by molecular modeling (Svensson et al., 1990).

A very important difference between the two tyrosine radicals is their different stability. Signal II$_{slow}$ is extremely stable in the dark (on the order of hours), although the Tyr$_D$/Tyr$_D^{ox}$ couple is highly oxidizing ($E_m \approx +720$–760 mV at pH ≈ 6) (Boussac & Etienne, 1984; Vass & Styring, 1991). This indicates that Tyr$_D^{ox}$ is highly "insulated" from redox-active groups in the PSII complex as well as from the medium. Contrary to this, Tyr$_Z^{ox}$ decays on the microsecond to millisecond time scale (Babcock et al., 1976, 1989). According to the structural model, a possible explanation for the different kinetics might be that the environment of Tyr$_D$ is more hydrophobic than that of Tyr$_Z$ (Svensson et al., 1991).

One of the specific predictions is that His189 in the D2 protein is within hydrogen-bonding distance to the hydroxyl group of Tyr$_D$ (Figure 1). The possible existence of a hydrogen bond between the tyrosyl side chain and a neighboring side chain is highly interesting and might have significant implications for the function of Tyr$_D$ (Babcock et al., 1989). Furthermore, this interaction might be important to properly interpret the Signal II$_{slow}$ spectrum. In addition, the model can be used to suggest other side chains that are close to the tyrosines. In particular, we have focused our attention on Gln164 in the D2 protein, which is predicted to interact closely with Tyr$_D$ (Figure 1).

To test essential aspects of this model and to analyze the effects of perturbations in the environment of Tyr$_D$ on the shape and characteristics of Signal II$_{slow}$, selected site-directed mutations were introduced into the D2 protein. This study presents an EPR analysis of these mutants; the results strongly support the structural predictions of the model. In addition, two of the mutants show greatly modified EPR spectra that we attribute to originate from Tyr$_D^{ox}$.

MATERIALS AND METHODS

Growth Conditions. *Synechocystis* 6803 wild type and mutants were grown at 32 °C in BG-11 medium with 5 mM TES (pH 8.0) in 10-L carboys in white light (≈ 100 μE m^{-2} s^{-1}). Even though all mutants are capable of growing photoautotrophically, the mutant H189L (in which His189 of the D2 protein is mutated to Leu) was grown in the presence of 5 mM glucose to optimize the yield of cells. The presence or absence of glucose did not affect any PSII characteristics that are of importance for this study (not shown). Cells in late log phase were harvested by centrifugation, washed twice, and resuspended in a preparation buffer containing 50 mM MES-NaOH, pH 6.5, 30 mM $CaCl_2$, 10 mM $MgCl_2$, 1 M sucrose, 20 % (v/v) glycerol, and 1 mM EDTA.

Site-Directed Mutagenesis. In this study, three strains with site-directed mutations in the D2 protein are analyzed. These are Q164L (Gln164 mutated to Leu), H189L (His189 mutated to Leu), and H189Y (His189 mutated to Tyr). Transformation protocols and methods used for site-directed mutagenesis of the D2 protein in *Synechocystis* 6803 essentially have been described earlier (Vermaas et al., 1990b). Oligonucleotide-directed mutagenesis was performed using single-stranded DNA from the bacteriophage M13mp18 containing part of the *Synechocystis* 6803 *psb*DI/C operon as a template. Mutants were identified by sequencing, and part of the *psb*DI/C operon containing the mutation was cut out by *Sfi*I/*Eco*RI digestion and ligated into a similarly digested, complementary plasmid denoted pDICK (Vermaas et al., 1990b). The resulting plasmid carries the entire *psb*DI/C operon (with the site-directed mutation) with native flanking sequences and a kanamycin-resistance cartridge inserted downstream of the *psb*C gene. This plasmid construct was used for transformation of the *psb*DI/C/DII deletion mutant of *Synechocystis* 6803, and kanamycin-resistant transformants were selected. After segregation, genomic DNA was isolated from the mutants, and a region of the *psb*DI gene carrying the desired mutation was amplified by the polymerase chain reaction. Amplified DNA was sequenced, and the presence of the desired mutations was confirmed. The control strain, derived from transformation of the *psb*DI/C/DII deletion mutant with a pDICK construct carrying the wild-type *psb*DI/C operon, was used as reference strain in all experiments and is referred to as wild type in this paper.

Quantitation of PSII on a Chlorophyll Basis. The amount of PSII in intact cells of the wild-type and mutant strains on a chlorophyll basis was determined by ^{14}C-atrazine binding. This was performed as described in Vermaas et al. (1990a), except that ^{14}C-atrazine (specific activity 7.3 mCi/mmol; Sigma) rather than ^{14}C-diuron was used and that the concentration of cells in the assay corresponded to 200 μg of Chl mL^{-1}. Unspecific binding of ^{14}C-atrazine to cells was measured in the presence of 20 μM unlabeled diuron.

Preparation of Thylakoid Membranes and PS II-Enriched Particles. After incubation of the harvested cells at 20 °C for 1 h (Burnap et al., 1989), thylakoid membranes were prepared in the preparation buffer using a Bead Beater (BioSpec Products) to break the cell walls (Kirilovsky et al., 1992; Nilsson et al., 1992). The glass beads were filtered off, and unbroken cells and cell debris were pelleted by centrifugation (2 × 10 min, 4000*g*). The purified thylakoid membranes were pelleted at 100000*g* for 45 min and then dissolved to a concentration of ≈2 mg of Chl mL^{-1} in the preparation buffer but now containing 0.2 M sucrose and 3 mM EDTA. The chlorophyll *a* concentration was determined according to MacKinney (1941). The thylakoids were frozen in liquid nitrogen and stored at −80 °C. For H189Y, a PSII-enriched fraction was isolated from these thylakoids using extraction of thylakoids (1 mg of Chl mL^{-1}) with dodecyl maltoside and octyl glucoside (0.125% and 0.4%, respectively) as described by Kirilovsky et al. (1992). The enrichment of PSII in this preparation was a factor of 4–5 compared to thylakoids, as measured by 77 K fluorescence emission spectra (data not shown).

EPR Spectroscopy. X-Band EPR spectra at room temperature and at liquid He temperature were recorded with a Bruker ESP300 spectrometer equipped with an Oxford Instruments cryostat and temperature controller. The data were processed using the ESP300 software. Decay studies of the radicals at room temperature were done in a flat cell directly in the EPR spectrometer while the EPR spectra were continuously recorded. Light induction of the radical spectra was performed after the decay measurements were completed. Where indicated, thylakoid samples (concentration ≈2 mg of Chl mL^{-1}) were given saturating laser flashes from a Nd–YAG laser (Spectra Physics DCR3G, λ = 532 nm, 8-ns duration, ≈350 mJ, 1-Hz flash frequency) in the EPR cavity. The spectra were recorded 60 s after the end of the flash train to allow reduction of P_{700}^+ from PSI which would otherwise dominate the spectra [there are 5–10 times more PSI than PSII reaction centers in thylakoid membranes of wild-type *Synechocystis* 6803 (Noren et al., 1991; Kirilovsky et al., 1992; Nilsson et al., 1992)].

To estimate the relative radical content in the wild-type and the mutant strains, the radical signals were measured under nonsaturating conditions. The obtained spectra were double-integrated using the ESP300 software, and the radical content was calculated on a chlorophyll basis using Signal II_{slow} in highly functional PSII-enriched thylakoids from spinach (1 Signal II_{slow}/220–240 chlorophylls) as a spin standard, as described by Nilsson et al. (1992).

Chemical reduction of the radicals was achieved by the addition of reductants to samples that had been illuminated with room light for 5 min to oxidize Tyr_D. In experiments with NH_2OH or ascorbate, the reductant was allowed to react for 5–10 min in the dark before the EPR spectra were recorded. In other experiments, dithionite (35-40 mM at pH ≈ 6.3) was added and the samples were incubated for various times at room temperature. After the incubation, oxygen was bubbled slowly through the samples to oxidize excess dithionite, which would otherwise interfere with the spectroscopic measurements. After oxidation of the dithionite, the samples were transferred to the spectrometer and EPR spectra were recorded. All sample handling was performed in darkness.

RESULTS AND DISCUSSION

The molecular model of the environment of Tyr_Z and Tyr_D (Svensson et al., 1990) allows formulation of several hypotheses that can be tested experimentally to evaluate the model's validity. An important outcome of the modeling is a possible hydrogen bond between Tyr_D and His189 of the D2 protein. The close contact between Tyr_D and His189 is not obvious from examining the primary structure of the D2 protein because the residues are 29 positions apart. However, the existence of a hydrogen bond fits with the proposal of a "locked-in" rocking proton between the tyrosines (Tyr_Z and Tyr_D) and neighboring side chains (Babcock et al., 1989). Therefore we set out to experimentally verify the presence of His189 in the close vicinity of Tyr_D. Since Tyr_D is predicted to also interact closely with Gln164 of the D2 protein (Figure 1), we have investigated this possibility as well. To test these hypotheses, formulated on the basis of the molecular model, we utilized EPR spectroscopy to investigate the properties of Tyr_D in strains carrying site-directed mutations in His189 and Gln164 of the D2 protein. These strains are Q164L (Gln164 of the D2 protein mutated to Leu), H189L (His189 mutated to Leu), and H189Y (His189 mutated to Tyr). The three strains are photoautotrophic and evolve oxygen (Table I). However, the rate of oxygen evolution is decreased in the mutants, particularly in the His189L mutant and to a lesser extent in H189Y. The decreased oxygen evolving rates correlate with a decreased PSII content on a chlorophyll basis in these mutants: the number of atrazine-binding sites (one per PSII) is decreased by a factor of 1.5 in H189Y and by a factor of 3 in H189L (Table I). This possibly is related to a decreased PSII stability in the His189 mutants.

Table I: Oxygen-Evolving Activity and Quantification of Photosystem II in Whole Cells of Wild Type and Site-Directed Mutants of *Synechocystis* 6803

	wild type	Q164L	H189L	H189Y
oxygen evolution[a]	270	230	100	180
atrazine binding[b]	1/600	1/600	1/2000	1/1000

[a] Measured with 0.6 mM PPBQ and 1 mM $K_3Fe(CN)_6$ as electron acceptors, in 25 mM HEPES buffer, pH 7.5, at 25 °C. O_2 evolution is given in micromoles of O_2 per milligram of chlorophyll per hour. [b] The atrazine binding on a chlorophyll basis is given in atrazine binding sites per chlorophyll. The atrazine dissociation constant was about 250 μM in both wild-type and mutant strains.

EPR Spectra from Wild Type and Mutants. Figure 2 shows the EPR spectra recorded in the dark in thylakoid membranes from the wild type and the mutants. In the wild type the EPR spectrum from Tyr_D^{ox} (Figure 2a) is broad and similar to that published earlier from *Synechocystis* 6803 (Debus et al., 1988a; Kirilovsky et al., 1992; Nilsson et al., 1992; Noren et al., 1991; Vermaas et al., 1988). In contrast, both His189 mutants lack EPR spectra similar to the wild-type Tyr_D^{ox} spectrum. Instead both mutants show narrow, quite structureless EPR spectra with high *g*-values (Figure 2c,d; Table II). The narrow EPR signal found upon modification of His189 is associated with PSII, as a similar narrow radical with an equally high *g*-value is present in a PSII-enriched fraction of H189Y (Figure 2e). On a chlorophyll basis, in the PSII-enriched fraction the signal amplitude is 3.5-fold higher than in thylakoids run under the same conditions, while judging from 77 K fluorescence emission spectra the PSII enrichment is approximately 5-fold (data not shown). PSII-enriched preparations of the H189L mutant were impractical, as the PSII/chlorophyll ratio was low in this mutant (see Table I). The fact that the radical appears to be slightly wider in the PSII-enriched fraction than in thylakoids of H189Y may be attributed to small structural changes induced upon particle preparation. In thylakoids from Q164L we observed a radical EPR spectrum with a high *g*-value (Figure 2b, Table II) but with a shape that was slightly modified compared to the wild-type Tyr_D^{ox} spectrum. In Q164L the EPR spectrum is better

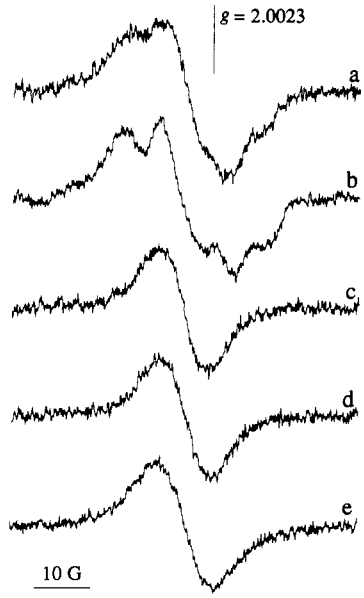

FIGURE 2: Spectral shape of the dark-stable radicals in thylakoid membranes from *Synechocystis* 6803 in the wild type (a), Q164L (b), H189L (c), and H189Y (d) and from a PSII-enriched fraction of H189Y (e). Samples were illuminated with room light and then incubated for 2 min in the dark prior to recording the spectra. Chlorophyll concentration was 1–2 mg of Chl mL^{-1} for thylakoid samples (a–d) and 700 μg of Chl mL^{-1} for the PSII-enriched fraction (e). EPR conditions: microwave power 6.3 mW in a–d and 6.3 μW in e; modulation amplitude 3.3 G; temperature 293 K in a–d and 10 K in e; microwave frequency 9.77 GHz in a–d and 9.24 GHz in e. The spectra presented in a–d were obtained by the addition of recorded spectra from many separate experiments. Thus the gain is not directly comparable between the spectra. Detailed information regarding the relative sizes of the radical signals in the various strains is presented in Table II.

Table II: Properties of the EPR Spectra of Tyr$_D^{ox}$ in the Wild Type and in Site-Directed Mutants of *Synechocystis* 6803

	wild type	Q164 L	H189L	H189Y
g-value[a]	2.0050	2.0055	2.0053	2.0052
line width (G)	17 ± 1	20 ± 1	10 ± 1	10 ± 1
radical size[b]	1/700	1/740	1/7200	1/3400
half-time for decay, pH 6.3[c] (min)	270	120	75	65
half-time fo decay, pH 7.7[d] (min)	45	55	15	20
inducible by illumination[d]	yes	yes	yes	yes
reducible[e]	yes	yes	yes	yes

[a] The approximate error in the *g*-value estimation is ±0.0002. [b] The radical size is given in radical spins per chlorophyll. It was estimated by double integration of the signal as described in Materials and Methods. [c] The decay of the radicals was measured at the indicated pH in the dark at 20 °C. [d] The radicals were allowed to decay in the dark. Then the thylakoid membranes were exposed to a train of laser flashes (also see Figure 3). [e] The samples were reduced with ascorbate or NH_2OH (10 mM) in the wild type or with dithionite (35 mM) in the mutants as described in Materials and Methods.

resolved than in wild type, and in fact the shape of the spectrum is almost identical to the spectrum from spinach, although the *g*-value is somewhat higher in Q164L.

The EPR spectra in Figure 2 were recorded in the dark and originate from stable radicals (see below). Our hypothesis is that the EPR spectra from the mutants originate from Tyr$_D^{ox}$ in an environment that is modified due to the point mutations. In both the wild type and the mutants, the radicals giving rise to the EPR spectra have *g*-values (Table II) that are slightly higher than those of Tyr$_D^{ox}$ in higher plants (g =2.0046 in spinach). High *g*-values are characteristic for deprotonated oxidized tyrosyl radicals in proteins (Barry & Babcock, 1988; Bender et al., 1989; Noren & Barry, 1992). Thus, the *g*-values are as expected from radicals representing Tyr$_D$, and in fact there are no other simple radicals reported from the photosynthetic apparatus with equally high *g*-values (Miller & Brudvig, 1991).

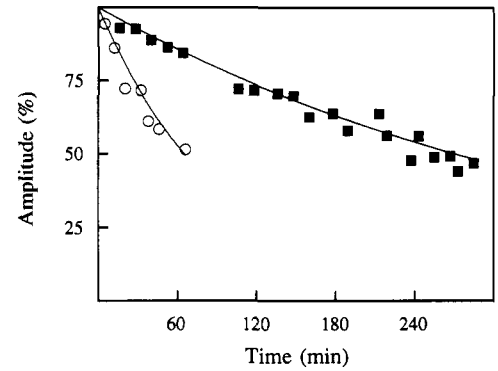

FIGURE 3: Stability of the radical EPR spectra in thylakoids from wild type (squares) and H189Y (circles) at pH 6.3. The samples were illuminated with room light and then incubated at 20 °C in the dark for various times while the EPR spectra were continuously recorded. The decay is fitted with single-exponential curves with decay half-times of 4.5 h for the wild-type signal and 65 min for the radical in the mutant. EPR conditions were as in Figure 2, spectra a–d.

The line width of the EPR spectrum of the dark-stable radical is 17–20 G in the wild type and in Q164L; this value is similar to the line width of 19 G (Miller & Brudvig, 1991) of Tyr$_D^{ox}$ (Signal II$_{slow}$) from higher plants. Thus, the hyperfine couplings are similar between cyanobacteria and higher plants; this is a strong indication that the immediate surroundings of the radicals are almost identical, because even minor changes in the spin-density distribution and geometry of the tyrosyl radicals may give rise to major changes in the hyperfine couplings (Barry & Babcock, 1988; Bender et al., 1989). In Q164L the EPR spectrum of Tyr$_D^{ox}$ is slightly modified as compared to the spectrum in the wild type (Figure 2). This indicates that the mutation has induced changes in the environment of Tyr$_D$, which is supported in the model where the two side chains are within a few angstroms of each other (Figure 1).

In contrast to the spectrum in Q164L, the dark-stable radical EPR spectra in both His189 mutants are only 9–10 G wide (Table II), which is much narrower than in wild type or in higher plants. In addition, the spectra are much less resolved. The EPR spectrum of the radical in H189Y thylakoids lacks resolved hyperfine structure (Figure 2d). In the case of H189L, the EPR spectrum also lacks well-resolved hyperfine structure, but there is a small shoulder on the low-field side of the spectrum (Figure 2c), which might be indicative of weak hyperfine couplings. A similar narrow EPR spectrum (also showing a small shoulder) has been observed in an independently created similar mutant (X.-S. Tang, D. A. Chisholm, P. Nixon, and B. A. Diner, personal communication). The narrow lines and weaker hyperfine structure indicate that the radicals we interpret to be Tyr$_D^{ox}$ (see below) in the His189 mutants reside in very different environments than Tyr$_D^{ox}$ in the wild type.

Characterization and Origin of the New Radical EPR Spectra in the His189 Mutants. Tyr$_D^{ox}$ (Signal II$_{slow}$) is extremely stable and decays only after several hours at neutral pH (Babcock & Sauer, 1973; Vass & Styring, 1991). In thylakoids from wild-type *Synechocystis* 6803, Tyr$_D^{ox}$ has a decay half-time (at 20 °C) of 4.5–5 h in the dark at pH 6.3 (Figure 3, Table II); this is quite similar to the stability of Tyr$_D^{ox}$ in spinach (Vass & Styring, 1991). The radical in Q164L decays with a half-time of 2 h (Table II), while the half-time of decay for the radicals in the two histidine mutants is 1–1.3 h at 20 °C (Figure 3, Table II). Thus, the radicals

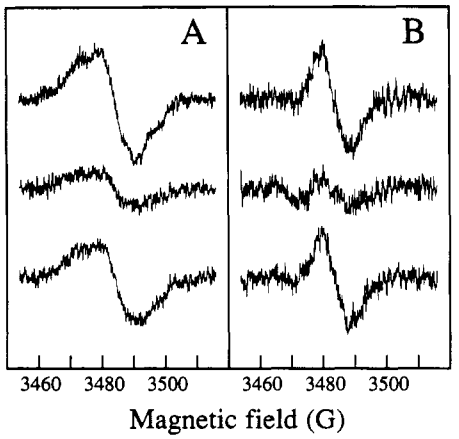

FIGURE 4: EPR spectra showing the light induction of the radical signals in thylakoid membranes from the wild type (A) and from H189Y (B). The top spectra were recorded as in Figure 2. The middle spectra were recorded after dark incubation at room temperature for 6.5 h (A) or 2 h (B). The bottom spectra were recorded 1 min after the dark-incubated samples had been given a train of 11 (A) and 60 (B) saturating laser-flashes. EPR conditions were as in Figure 2, spectra a–d.

presented in Figure 2 are more stable than any other radical of photosynthetic origin except Tyr_D^{ox}. In addition, the dark-stable radicals in all mutants presented here are less stable at alkaline pH (Table II); this feature is also similar to Tyr_D^{ox} from spinach, which is destabilized with a pK about 7.5 (Vass & Styring, 1991). Thus, both the large stability at neutral pH and the decreased stability at higher pH support the assignment of the radicals to Tyr_D^{ox}.

Another feature that is important for the assessment of the origin of the radicals is whether or not they can be induced by illumination. In higher plants, the light-dependent oxidation of Tyr_D occurs via the S_2 and S_3 states of the water-oxidizing complex in reactions that occur on the time scale of seconds (Vass & Styring 1991, and references therein); usually most of Tyr_D becomes oxidized after a few single-turnover flashes. We tested the light induction of the radical signals in thylakoid membranes of the wild type and the mutants of *Synechocystis*. The radicals were allowed to decay in darkness (this takes several hours at room temperature; Table II). Then the samples were exposed to a train of saturating laser flashes and EPR spectra were recorded 1 min after each set of flashes. Figure 4 shows the results of experiments with the wild type (Figure 4A) and with H189Y (Figure 4B). In both strains, the dark-stable radical can be regenerated by flashes. This is also the case for the radicals from Q164L and H189L (Table II); this confirms that these radicals are associated with the photosynthetic systems in the thylakoids. The quantum efficiency for the light induction seems to vary for the different radicals, and preliminary results indicate that the radicals in the His189 mutants are more difficult to induce than Tyr_D^{ox} in the wild type (see below).

Another clue to the origin of the radical EPR spectra is whether they are formed by oxidation, and hence whether they can be reduced chemically. Tyr_D^{ox} in the wild type is easily reduced by ascorbate, hydroxylamine, and dithionite (Table II). The radicals in the mutants can also be reduced, and hence all four radicals are derived from oxidized species, as would be expected if they represented Tyr_D^{ox}. However, in the three mutants the radicals are less susceptible to reductants; we have been able to reduce them only with dithionite and not with weaker reductants (Table II). The reason for their increased resistance toward reduction is presently unknown but may reflect the altered environment of the radical created by the introduction of the point mutations.

After illumination of the sample, one Tyr_D^{ox} is present per PSII reaction center, and this has become a useful spectroscopic probe to determine the relative concentration of PSII in preparations of different origin (Miller & Brudvig, 1991; Nilsson et al., 1992). Using double integration of the EPR signals, we have calculated the amount of the modified dark-stable radicals per PSII in thylakoid membranes from the site-directed mutants (Table II); under the same conditions in wild type, one Tyr_D^{ox} per 700 Chl was observed (Nilsson et al., 1992). The radical in Q164L is present in amounts approximately equal to those in the wild type, and it seems that all Tyr_D can be oxidized in this mutant. However, it appears that under the conditions used not all Tyr_D is EPR-detectable in the His mutants: the number of spins observed in the His mutants is lower than would be expected from the amount of PSII on a chlorophyll basis (cf. Tables II and I).

In any case, because the EPR signal in the His189 mutants is (1) PSII-associated, (2) very stable (for hours), (3) destabilized at high pH, (4) light-inducible, and (5) reducible, we attribute this signal to originate from Tyr_D^{ox}. All these properties are shared with the normal Tyr_D^{ox} radical, which is the only known photosynthetic radical with these properties. Furthermore, tyrosyl radicals in proteins typically have high g-values (Barry & Babcock, 1988; Bender et al., 1989; Noren & Barry, 1992), which further strengthens our assignment.

One interesting aspect will be to analyze the consequences of the lack of His189 with respect to the function of Tyr_D. The presence of the "locked-in" proton (Babcock et al., 1989) in a hydrogen bond between Tyr_D and a neighboring residue was proposed from external nuclear double resonance (ENDOR) (Rodriguez et al., 1987) and spin-echo EPR measurements (Evelo et al., 1989). In this model the "locked-in" proton is associated mostly with the tyrosyl when this residue is in its reduced state, while upon oxidation of the tyrosyl residue the proton moves toward the hydrogen-bonding partner. Thus, removal of the hydrogen-bonding partner of Tyr_D in the His189 mutants is likely to alter the functional properties of Tyr_D, and our preliminary data indicating a decreased efficiency of Tyr_D oxidation by light indeed are in line with such modifications in the properties of Tyr_D. Moreover, the apparent presence of an analogous hydrogen bond between Tyr_Z and a neighboring side chain also may be of large functional importance with respect to the fast oxidation (and reduction) of Tyr_Z.

At present we do not attempt to explain the narrow radical signals in the histidine mutants theoretically. In all investigated proteins with tyrosyl radicals, the spectra are much wider than the spectra reported here for the histidine mutants (Figure 2c,d). The reason is that the unpaired electron spin to a large extent resides on the aromatic ring where the dominating hyperfine couplings are considered to involve the β-methylene protons and the 3,5-ring (*ortho*) protons on the tyrosyl side chain (Bender et al., 1989; Hoganson & Babcock, 1992). However, changes in various parameters, including the spin distribution, may give rise to narrow spectra with small hyperfine interactions; one likely explanation for the observed spectra in the His mutants is that the unpaired spin is localized mainly on the phenolic oxygen atom in the tyrosyl side chain.

Relevance for the Structural Model. The experimental observations fit well with predictions made on the basis of computer modeling of the Tyr_D environment (Figure 1). In the His189 mutants, the point mutation is predicted to have removed hydrogen-bonding capabilities with the phenolic

oxygen on Tyr_D. This is a major change in the environment of the Tyr_D^{ox} radical that is likely to induce large spectral changes in the EPR spectrum. Indeed, in these mutants, Tyr_D^{ox} appears to be very much affected in its electronic distribution, resulting in very narrow EPR spectra with high g-values (Figure 2c,d). In the case of Q164L, the perturbation of Tyr_D^{ox} was predicted to be much less significant than upon mutation of His189; only a minor alteration in the shape of Signal II_{slow} was observed in this mutant (Figure 2b). In addition, in the M159R mutant (Met159 in the D2 protein is mutated to arginine) the EPR spectrum from Tyr_D^{ox} was similar to that in the wild type (Vermaas et al., 1988), even though the mutation in this case is in the residue next to Tyr_D. This result fits with the predictions since, in an α-helical arrangement, two adjacent residues are at a 100° angle with respect to each other. This is also observed in the computer model (Figure 1), where Met159 is turned away from Tyr_D and thus is not expected to significantly affect the electronic configuration or orientation of Tyr_D.

Other experimental results also are in line with predictions made from the molecular model. In this model Asp170 on the D1 protein is close to Gln165 and Glu189 on the D1 protein and these residues might constitute a metal binding site on the donor side of PSII (Svensson et al., 1991). Indeed, studies on mutants in Asp170 on the D1 protein show that such mutants are modified in the binding of Mn to PSII (Nixon & Diner, 1992; Boerner et al., 1992). Even though the authors were careful not to state firmly that Asp170 was a ligand to the Mn cluster, the residue clearly participates in Mn binding, corroborating the suggestion made from the molecular modeling.

CONCLUSIONS

Despite the inherent limitations of computer modeling studies, the results presented here illustrate both the usefulness and meaningfulness of this technique in PSII research. In the absence of a high-resolution PSII crystal structure, a detailed and dependable model of PSII or regions thereof is highly relevant provided that the theoretical studies are accompanied by experimental work aimed at testing the structural model. As demonstrated here for Tyr_D, the model can be used to put forward testable hypotheses regarding both the structure and function of the protein complex. With a combination of experimental studies and refined computer modeling, it will be possible to determine functional roles of side chains other than the tyrosines and the His189/His190 residues on the D2 and D1 proteins, respectively. Of immediate interest in this respect is the assessment of the nature of the primary donor in PSII, P_{680}, and the identification of ligands to the Mn cluster. The combination of computer modeling and mutant analysis most likely will continue to prove to be a powerful approach in this research.

ACKNOWLEDGMENT

We thank Mr. Torbjörn Astlind for technical assistance with the EPR measurements.

REFERENCES

Andersson, B., & Styring, S. (1991) in *Current Topics in Bioenergetics* (Lee, C. P., Ed.) Vol. 16, pp 1–81, Academic Press, San Diego, CA.

Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 483–503.

Babcock, G. T., Blankenship, R. E., & Sauer, K. (1976) *FEBS Lett. 61*, 286–289.

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.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Barry, B. A., & Babcock, G. T. (1988) *Chem. Scr. 28A*, 117–122.

Bender, C. J., Sahlin, M., Babcock, G. T., Barry, B. A., Chandrashekar, T. K., Salowe, S. P., Stubbe, J.-A., Lindström, B., Petersson, L., Ehrenberg, A., & Sjöberg, B.-M. (1989) *J. Am. Chem. Soc. 111*, 8076–8083.

Boerner, R. J., Nguyen, A. P., Barry, B. A., & Debus, R. J. (1992) *Biochemistry 31*, 6660–6672.

Boussac, A., & Etienne, A.-L. (1984) *Biochim. Biophys. Acta 766*, 576–581.

Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22*, 123–130.

Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269–352.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988b) *Biochemistry 27*, 9071–9074.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature 318*, 618–624.

Evelo, R. G., Dikanov, S. A., & Hoff, A. J. (1989) *Chem. Phys. Lett. 159*, 25–30.

Hoganson, C. W., & Babcock, G. T. (1988) *Biochemistry* 427, 5848–5855.

Hoganson, C. W., & Babcock, G. T. (1992) *Biochemistry 31*, 11874–11880.

Kirilovsky, D. L., Boussac, A. G. P., van Mieghem, F. J. E., Ducruet, J.-M. R. C., Setif, P. R., Yu, J., Vermaas, W. F. J., & Rutherford, A. W. (1992) *Biochemistry 31*, 2099–2107.

Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946–950.

MacKinney, G. (1941) *J. Biol. Chem. 140*, 315–322.

Metz, J. G., Nixon, P. J., Rögner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.

Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1–7.

Miller, A.-F., & Brudvig, G. W. (1991) *Biochim. Biophys. Acta 1056*, 1–18.

Nilsson, F., Gounaris, K., Styring, S., & Andersson, B. (1992) *Biochim. Biophys. Acta 1100*, 251–258.

Nixon, P. J., & Diner, B. A. (1992) *Biochemistry 31*, 942–948.

Noren, G. H., & Barry, B. A. (1992) *Biochemistry 31*, 3335–3342.

Noren, G. H., Boerner, R. J., & Barry, B. A. (1991) *Biochemistry 30*, 3943–3950.

Rodriguez, I. D., Chandrashekar, T. K., & Babcock, G. T. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. I, pp 479–482, Martinus Nijhoff Publishers, The Hague, The Netherlands.

Svensson, B., Vass, I., Cedergren, E., & Styring, S. (1990) *EMBO J. 9*, 2051–2059.

Svensson, B., Vass, I., & Styring, S. (1991) *Z. Naturforsch. 46c*, 765–776.

Trebst, A. (1986) *Z. Naturforsch. 41c*, 240–245.

Vass, I., & Styring, S. (1991) *Biochemistry 30*, 830–839.

Vermaas, W. F. J., & Ikeuchi, M. (1991) in *Cell Culture and Somatic Genetics of Plants* (Bogorad, L., & Vasil, I. K., Eds.) Vol. 7B, pp 25–111, Academic Press, San Diego, CA.

Vermaas, W. F. J., Rutherford, A. W., & Hansson, Ö. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.

Vermaas, W., Charité, J., & Shen, G. (1990a) *Z. Naturforsch. 45c*, 359–365.

Vermaas, W., Charité, J., & Shen, G. (1990b) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol I, pp 231–238, Kluwer Academic Publishers, Dordrecht, The Netherlands.