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Electron Paramagnetic Resonance Characterization of Tyrosine Radical, M^+, in Site-Directed Mutants of Photosystem II[t]

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ABSTRACT Photosystem II contains two well-characterized tyrosine radicals, $D^\bullet$ and $Z^\bullet$. Z is an electron carrier between the primary chlorophyll donor and the manganese catalytic site and is essential for enzymatic function. On the other hand, D forms a stable radical with no known role in oxygen evolution. $D^\bullet$ and $Z^\bullet$ give rise to similar, but not identical, room temperature electron paramagnetic resonance (EPR) signals, which can be distinguished by their decay kinetics. A third room temperature EPR signal has also been observed in site-directed mutants in which a nonredox active amino acid is substituted at the D or Z site. This four-line EPR signal has been shown to have a tyrosine origin by isotopic labeling (Boerner and Barry, 1994, *J. Biol. Chem.* 269:134–137), but such an EPR signal has never before been observed from a tyrosyl radical. The radical giving rise to this third unique signal has been named M^+. Here we provide kinetic evidence that this signal arises from a third redox active tyrosine, distinct from tyrosine D and Z, in the photosystem II reaction center. Isotopic labeling and EPR spectroscopy provide evidence that M is a covalently modified tyrosine.

INTRODUCTION

Photosystem II, the photosynthetic oxygen evolving complex, contains redox active tyrosine residues. This enzyme catalyzes the oxidation of water and the reduction of plastoquinone. After photoexcitation, the primary chlorophyll donor, P_{680}, transfers an electron from its excited state to a pheophytin molecule. The electron is then transferred to quinone acceptor molecules, Q_A and Q_B. $P_{680}{}^+$ is reduced by a tyrosine residue, Z, which is the intermediate electron carrier between $P_{680}{}^+$ and a manganese cluster. This manganese cluster is the catalytic site of water oxidation. The oxidized form of Z, $Z^\bullet$, is a neutral tyrosine radical. Ordinarily, the reduction of $Z^\bullet$ is in the microsecond time regime. However, if the manganese cluster is removed, this reduction time is slowed, and an electron paramagnetic resonance (EPR) signal from $Z^\bullet$ can be observed under steady-state illumination. Photosystem II also contains a stable tyrosine radical, $D^\bullet$, which gives rise to a dark stable EPR signal. The physiological importance of D is not known (for reviews, see Barry, 1993; Debus, 1992).

The D1 and D2 polypeptides are believed to form the heterodimer core of the reaction center (Nanba and Satoh, 1987). Site-directed mutagenesis experiments suggest that redox active tyrosine D is tyrosine 160 of the D2 polypeptide (Debus et al., 1988a; Vermaas et al., 1988) and that tyrosine Z is tyrosine 161 of the D1 polypeptide (Debus et al., 1988b; Metz et al., 1989; Noren and Barry, 1992). Replacement of tyrosine 160-D2 with either a phenylalanine (YF160D2) or a tryptophan (YW160D2) results in the loss of the $D^\bullet$ EPR signal (Debus et al., 1988a; Vermaas et al., 1988). In photosystem II preparations from either the YF160D2 or YW160D2 site-directed mutants, a new EPR signal can be detected under illumination (Boerner et al., 1993). The organic radical that gives rise to this new signal has been named M^+. Deuteration of tyrosine has shown that the M^+ radical has significant hyperfine couplings to tyrosine protons (Boerner and Barry, 1994).

Interestingly, M^+ has also been detected in the YF161D1 mutant (Noren and Barry, 1992), in which a nonredox active phenylalanine residue has been substituted at the putative Z site. A $D^\bullet$ signal is exhibited by this mutant (Debus et al., 1988b; Metz et al., 1989; Noren and Barry, 1992); in the light, the EPR signal of the M^+ radical can be observed (Noren and Barry, 1992).

Although isotopic labeling has clearly established a tyrosine origin for M, M^+ has an EPR lineshape never before observed for a tyrosine radical (Barry, 1993). Here we present a specific labeling experiment that supports the suggestion (Boerner and Barry, 1994) that M^+ is a covalently modified tyrosine radical. The role of M^+ in electron transfer in photosystem II is also of interest. To investigate this question, we have performed kinetic EPR experiments. Our data are consistent with the conclusion that M is a third redox active tyrosine in photosystem II and that the M site is photo-oxidized with low quantum yield. We suggest that covalently modified tyrosine M may play a structural role in photosystem II.

MATERIALS AND METHODS

Growth and labeling of *Synechocystis sp.* PCC 6803 cells

The wild-type, YF160D2, and YF161D1 strains have been described and characterized previously (Boerner and Barry, 1994; Boerner et al., 1993; Debus et al., 1988a,b; Noren and Barry, 1992). Cells of the wild type, the YF160D2 mutant, and the YW160D2 mutant were grown photohetero-

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trophically in BG-11 media supplemented with 5 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid-NaOH (TES-NaOH), pH 8.0, and 5 mM glucose (Rippka et al., 1979; Williams, 1988). Cultures of the YF161D1 mutant also contained 5 μg/ml kanomycin. Cells were harvested after approximately 1 week; the cultures had an absorbance of 1.0–1.5 at 730 nm. Labeling of cells with methylene deuterated tyrosine (L-tyrosine 3,3-d$_2$ 98% labeled) was performed as previously described (Barry and Babcock, 1987; Boerner and Barry, 1994).

Purification of photosystem II particles from *Synechocystis sp.* PCC 6803

Photosystem II particles from photoheterotrophically grown cells were purified by the procedures previously described (Noren et al., 1991; Barry, 1995).

Oxygen evolution assays

Assays were conducted as described (Noren et al., 1991). For cells and thylakoid membranes, each assay contained 20 μg of chlorophyll. For photosystem II particles, assays contained 10 μg of chlorophyll. The assay volume was 1.5 ml. Oxygen evolution rates are presented in Table 1.

Removal of manganese

Purified photosystem II samples were Tris-washed in the light (Noren et al., 1991; Yocum et al., 1981) or hydroxylamine-treated in the dark (Bernard et al., 1995; MacDonald and Barry, 1992; Yocum et al., 1981) to remove manganese. After Tris washing, samples were precipitated with polyethylene glycol (Noren et al., 1991), washed to remove Tris, and then resuspended in buffer A, containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH (MES-NaOH), pH 6.5, 20 mM CaCl$_2$, 25% (v/v) glycerol (Ultrapure; GIBCO BRL, Gaithersburg, MA), 0.05% (w/v) lauryl maltoside (Anatrace, Maumee, OH), and 15 mM NaCl. After hydroxylamine washing, samples were bound to an ion exchange column (Mono Q 5/5; Pharmacia) and washed to remove hydroxylamine, as previously described (Bernard et al., 1995; MacDonald and Barry, 1992). The sample was then concentrated by inverting the column and eluting with NaCl. The sample was dialyzed. If necessary, hydroxylamine samples were further concentrated using Centricon-100 microconcentrators (Amicon, Beverly, MA).

Sodium dodecyl sulfate denaturing gel electrophoresis of photosystem II particles from *Synechocystis sp.* PCC 6803

Sodium dodecyl sulfate (SDS) denaturing gel electrophoresis was performed by the Neville discontinuous method previously described (Piccioni et al., 1982).

TABLE 1 Oxygen evolution rates[*] for wild-type and mutant strains of the cyanobacterium *Synechocystis sp.* PCC. 6803

Strain	Cells[#]	Membranes	Photosystem II particles
Wild type	550–670	470–710	2600–2800
YW160D2	580	460	790
YF160D2	360–620	310–630	600–840
YF160D2[§]	550–670	630	1200
YF161D1	<50	—	—

[*] Oxygen evolution rates in μmol O$_2$/mg chl-h.

[#] All cultures were grown photoheterotrophically.

[§] Cultures grown in the presence of 0.5 mM phenylalanine, 0.25 mM tryptophan, and 0.25 mM tyrosine 3,3-d$_2$.

Room temperature X-band EPR measurements

EPR measurements were performed through the use of a Varian E4 EPR spectrometer equipped with a TE101 Varian cavity. Continuous illumination was provided by a Dolan-Jenner 180 fiber optic illuminator equipped with red and heat filters. Data acquisition was either through the method described previously (Noren and Barry, 1992) or through the use of Macintosh IIcx computer equipped with a National Instruments NB-A2000 A/D board (Austin, TX). In the latter case, the data acquisition program was written with Lab View software (National Instruments). The spacing between the points after digitization of the spectrum was 0.4 G. Other spectral conditions are given in the figure legends.

Kinetic analyses were carried out through the use of the Varian E4 EPR spectrometer and a frequency doubled, pulsed Nd-YAG laser (Continuum, Santa Clara, CA). The magnetic field was held constant in this experiment (see figure legends). The 532-nm excitation pulse was 10 mJ, and the repetition rate was 0.33 Hz. Laser power was measured with a Scientech power meter (Boulder, CO). The TTL pulse from the laser flash lamps was used to synchronize data acquisition 130 ns before the laser flash. The transients from 200 flashes were averaged. The static sample was contained in a quartz flat cell. Data acquisition employed a Macintosh IIcx computer equipped with a National Instruments NB-A2000 A/D board. The data acquisition program was written with Lab View software (National Instruments). The spacing between the points after digitization was 1 ms. Spectral conditions are given in the figure legends. Fits to the experimental data were performed with Kaleidagraph software (Albelbeck Software, Reading, PA).

Simulations of EPR spectra were carried out through the use of the program developed and described by Hoganson and Babcock (1992).

RESULTS

In Fig. 1, we present examples of EPR spectra recorded in the dark (dashed line) and under continuous illumination (solid line) for wild-type (Fig. 1 *A*) and YW160D2 (Fig. 1, *B* and *C*) samples. The mutant photosystem II sample was purified and then split into two aliquots. The aliquots were either Tris- or hydroxylamine-treated to remove manganese. The wild-type sample was Tris treated. Photosystem I contamination of mutant samples is greater than the contamination found in wild-type cyanobacterial samples (Boerner and Barry, 1994; Boerner et al., 1992, 1993; Noren and Barry, 1992; Noren et al., 1991) (see also Fig. 3). This is due to the fact that photosystem II mutants in the cyanobacterium *Synechocystis sp.* PCC 6803 generally produce less photosystem II. This contamination is observable in the EPR spectra recorded under illumination (Fig. 1, *B* and *C*, solid lines), where the YW160D2 samples show increased relative intensity near $g = 2$ (approximately 3380 G), when compared to wild type (Fig. 1 *A*, solid line).

In wild-type preparations, the dark signal (Fig. 1 *A*, dashed line) is due to a stable radical, D$^\bullet$. In the YW160D2 mutant, the D$^\bullet$ signal is not observed (Fig. 1, *B* and *C*, dashed line), in agreement with previous characterizations (Boerner et al., 1993; Debus et al., 1988a; Vermaas et al., 1988). In this mutant, a small, dark stable radical is present after either Tris washing (Fig. 1 *B*, dashed line) or hydroxylamine treatment (Fig. 1 *C*, dashed line). This result is also in agreement with our earlier characterization of intact cells and photosystem II particles from this mutant (Boerner et al., 1993). The origin of this low-amplitude signal is still unknown.

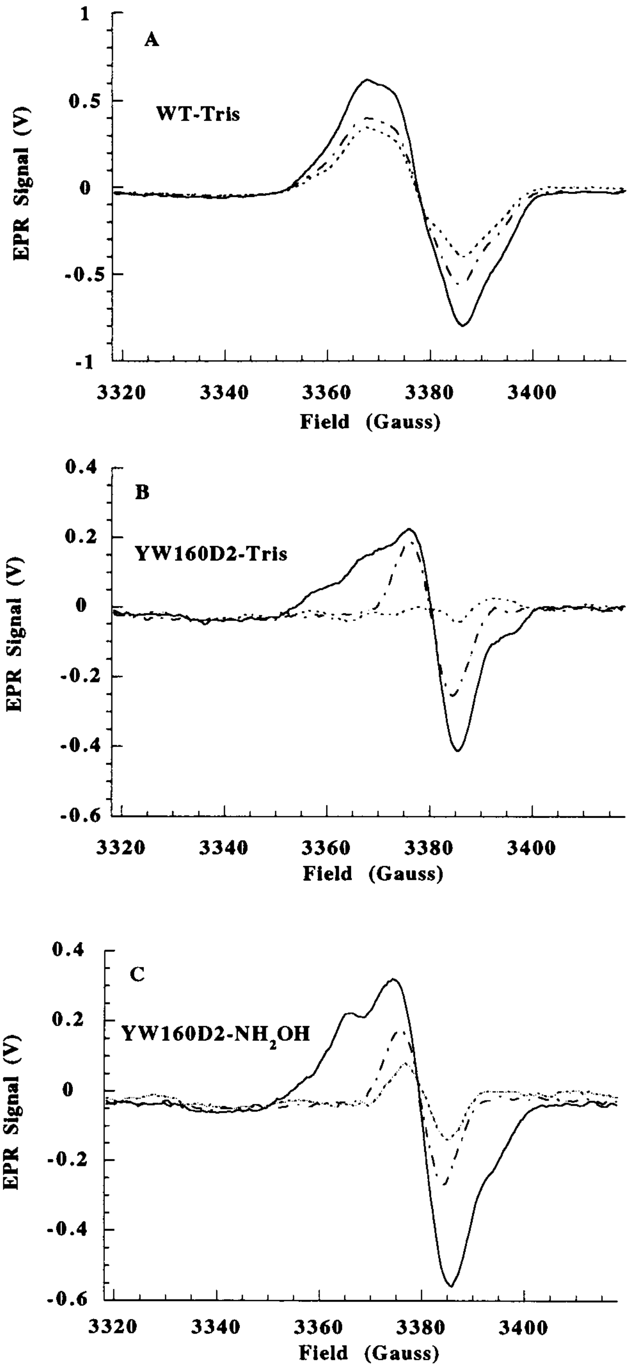

FIGURE 1 Room temperature EPR spectra of photosystem II preparations from the cyanobacterium *Synechocystis sp.* PCC 6803. Photosystem II preparations were from wild-type (*A*) and the YW160D2 mutant (*B* and *C*). In *A* and *B*, the preparation was Tris washed; in *C*, the preparation was hydroxylamine treated. In each panel, the solid line shows the spectrum obtained under illumination, the dot-dashed line shows the spectrum obtained under illumination in the presence of 800 μM benzidine, and the dashed line shows the spectrum obtained after illumination in the dark. Spectra from the YF160D2 mutant were similar (data not shown). Potassium ferricyanide (1.0 mM) was added as an electron acceptor. The chlorophyll concentrations were 0.7 mg chl/ml (*A*) and 0.6 mg chl/ml (*B* and *C*). Gain values were 2.0×10^4 (*A*) and 2.5×10^4 (*B* and *C*). Conditions were as follows: modulation frequency, 100 kHz; microwave frequency, 9 GHz; microwave power, 20 mW; field modulation, 6.3 G; time constant, 2 s; scan time, 4 min.

Spectra shown in the dot-dashed lines in Fig. 1 were recorded on each sample after addition of the photosystem II donor, benzidine. We have previously shown that benzidine reduces light-generated radicals in mutant photosystem II preparations (Boerner et al., 1993; Noren and Barry, 1992). The intensity of the room temperature EPR signal (under continuous illumination) from contaminating photosystem I is not changed by this treatment (Noren and Barry, 1992) (Fig. 1, *B* and *C*, dot-dashed lines). In the wild type, 800 μM benzidine seems slightly less effective at the reduction of light-generated radicals, although most of the light-induced $Z^{\bullet}$ signal is reduced by benzidine treatment (Fig. 1 *A*, dashed and dot-dashed lines). The reproducibility of the field sweeps is demonstrated by the data in Fig. 1 *A*, which show that the zero crossing is not changed upon the addition of benzidine to the EPR flat cell.

The EPR spectra presented in Fig. 1, *B* and *C*, show that the light-induced radicals, produced in Tris- and hydroxylamine-treated YW160D2 PSII particles, have different lineshapes. Note that the positions of the two low-field shoulders are shifted to higher fields in the Tris-washed (Fig. 1 *B*) as compared to the hydroxylamine-treated (Fig. 1 *C*) sample. Moreover, differences are observed in spectral lineshape on the high-field sides of the two signals.

An accurate lineshape of light-induced photosystem II radicals can be obtained by the subtraction of a spectrum recorded under illumination in the presence of benzidine from a spectrum recorded under illumination in the absence of the donor. In Fig. 2, we present the results of subtraction of such EPR data. In the case of wild type, this procedure gives a $Z^{\bullet}$ lineshape (Fig. 2, *A* and *B*). As expected, the lineshape is not affected by the method of removal of manganese. In the YW160D2 mutant, samples prepared by our Tris washing procedure give the M^+ lineshape (Fig. 2 *E*), in agreement with our earlier results (Boerner et al., 1993). However, samples prepared by hydroxylamine treatment give a $Z^{\bullet}$ lineshape (Fig. 2 *F*). The EPR spectrum obtained under continuous illumination in the presence of benzidine is not altered by this treatment (see example in Fig. 1). Therefore, the change in EPR lineshape must be due to the observable spectral change in the absence of benzidine (see discussion above). A similar subtraction result is obtained in the YF160D2 mutant (Fig. 2, *C* and *D*). The *g* values of both the $Z^{\bullet}$ and M^+ signals are identical (as assessed by the zero crossing of the signal), in agreement with earlier characterizations (Boerner and Barry, 1994; Boerner et al., 1993; Noren and Barry, 1992). Our previous characterization of the M^+ spin content at nonsaturating microwave powers has shown that the yield is variable, but that M^+ can be produced in up to one spin per reaction center (Boerner and Barry, 1994; Boerner et al., 1993; Noren and Barry, 1992).

In the wild-type sample, the *g* value of the $D^{\bullet}$ and $Z^{\bullet}$ radicals is 2.0046 (Miller and Brudvig, 1991). Thus, signals recorded under illumination, before illumination, and after the addition of benzidine show identical zero crossings, as expected with accurate field sweeps. In mutant samples, the

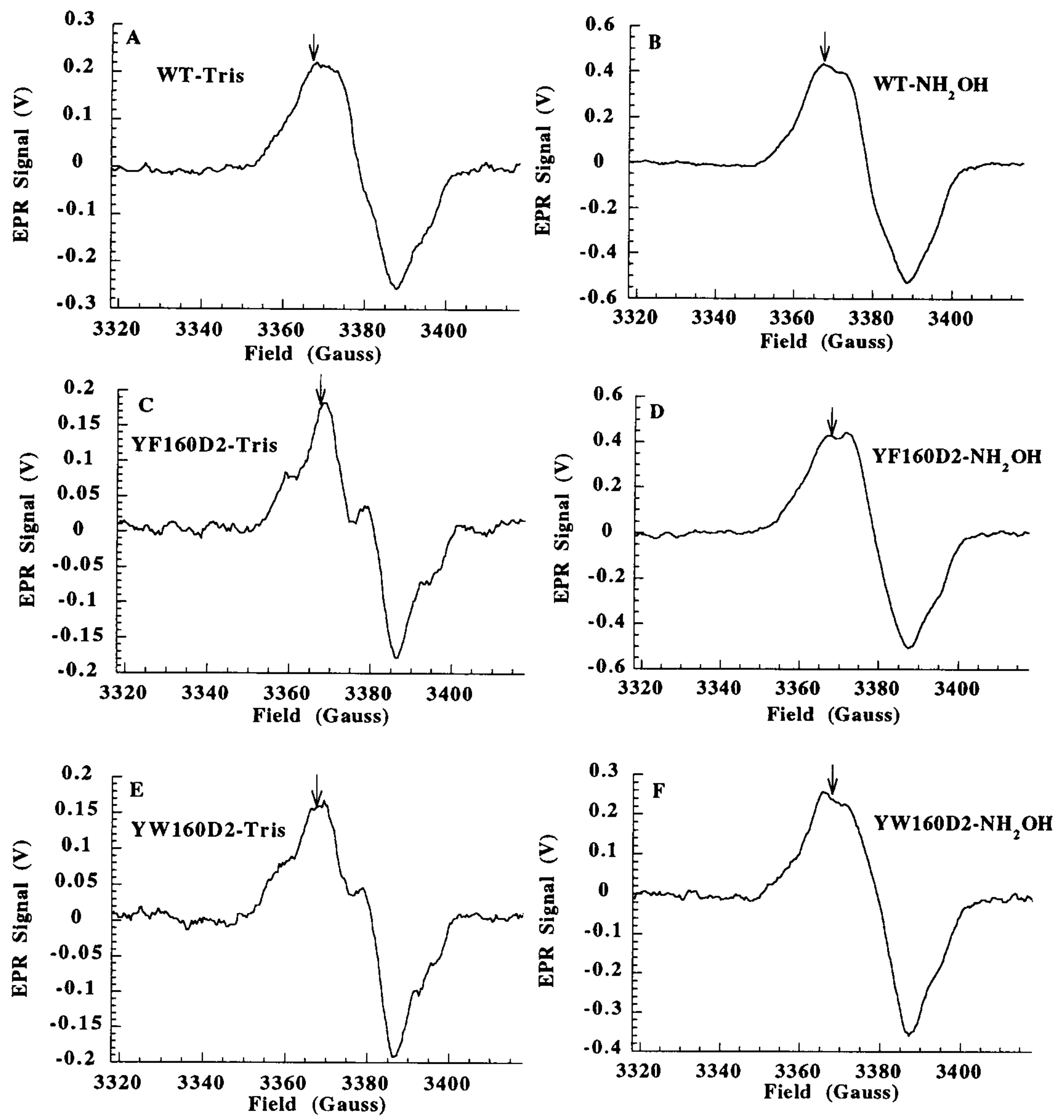

FIGURE 2 Room temperature EPR spectra of light-induced radicals in photosystem II preparations from the cyanobacterium *Synechocystis sp.* PCC 6803. The data show the Z$^{\bullet}$ (*A*, *B*, *D*, and *F*) and the M^{+} (*C* and *E*) lineshapes. In each case, a spectrum obtained in the presence of 800 μM benzidine under illumination was subtracted from a spectrum obtained under illumination in the absence of benzidine. In *A*, *C*, and *E*, the wild-type (*A*), YF160D2 (*C*), and YW160D2 (*E*) photosystem II preparations were Tris washed. In *B*, *D*, and *F*, the wild-type (*B*), YF160D2 (*D*), and YW160D2 (*F*) photosystem II preparations were hydroxylamine treated. The chlorophyll concentrations were 0.7 mg chl/ml for *A*, *B*, and *C* and 0.6 mg chl/ml for *D*, *E*, and *F*. The gain was 2.0 × 10^4 for *A* and 2.5 × 10^4 for *B–F*. Other spectral conditions are given in Fig. 1. The arrow shows the field setting at which transient EPR data (see Fig. 5) were obtained.

signal produced under illumination, which is due to both the tyrosine radical, M^{+}, and a chlorophyll radical from photosystem I, is lower. The zero crossing of this composite signal is shifted to higher fields by approximately 3–4 G, when compared to the wild-type signal produced under illumination. This shift is caused by the addition of the chlorophyll radical signal, which has a *g* value of 2.0025 (Miller and Brudvig, 1991). Such a shift has been observed previously in EPR studies of Tris-washed samples from the YF160D2 and YW160D2 mutants (Boerner et al., 1993). A shift to a lower *g* value was also observed upon simultaneous production of tyrosine radical D$^{\bullet}$ and a photosystem I chlorophyll radical (Barry and Babcock, 1987).

The data presented in Figs. 1 and 2 show that this subtraction produces an accurate lineshape for the PSII light-induced radicals. The mutant photosystem II sample was purified and then split into two aliquots for Tris or hydroxylamine treatment. Thus, the Tris- and hydroxylamine-treated samples have the same amount of photosystem I contamination. This is consistent with the size of the signal observed after the addition of benzidine to mutant PSII samples (Fig. 1, *B* and *C*). The amplitudes of these signals, which arise from the primary chlorophyll donor of PSI, are identical. Thus, observed differences between Tris- and hydroxylamine-treated mutant samples do not arise from a different amount of photosystem I contamination.

To summarize, when either a nonredox active phenylalanine or a nonredox active tryptophan is substituted at the site of the stable tyrosine radical in the D2 polypeptide, Tris washing of the preparation results in the generation of the M^+ tyrosine radical under continuous illumination in the presence of the electron acceptor, potassium ferricyanide. However, if the sample is hydroxylamine treated, $Z^{\bullet}$ is produced instead. The method of manganese removal has no effect on wild-type preparations, as expected. The result that observation of the M^+ radical depends on Tris washing explains the failure to observe the signal in the studies described by Metz et al. (1989). In this work, a cyanobacterial "core" photosystem II preparation was employed. This preparation loses manganese at some undefined point during the purification protocol, so Tris washing of the material was not performed.

In an effort to understand the difference between Tris and hydroxylamine treatment in the mutant preparations, we have so far considered two possibilities. One is that the Tris- and hydroxylamine-treated mutant preparations differ in polypeptide content and that the subunit that contains the M site is removed upon hydroxylamine treatment. In Fig. 3 we show a Coomassie-stained SDS denaturing gel of photosystem II preparations from wild type and YF160D2 mutant. In lanes 2 and 3, the wild-type preparation was Tris and hydroxylamine treated, respectively. In lanes 4 and 5, the mutant preparation was Tris and hydroxylamine treated, respectively. No dramatic differences in polypeptide content in the molecular mass range from 24 to 47 kDa are observed when lanes 4 and 5 are compared. This is the molecular mass range in which many of the core subunits of photosystem II are found (Barry et al., 1994). At lower molecular mass there are only subtle differences in staining intensity. Thus, if the Tris- and hydroxylamine-treated particles differ in polypeptide composition, the difference is a subunit that stains poorly with Coomassie blue and has a molecular mass less than 24 kDa. The functions of *Synechocystis* subunits in this molecular mass range are poorly characterized (Barry et al., 1994).

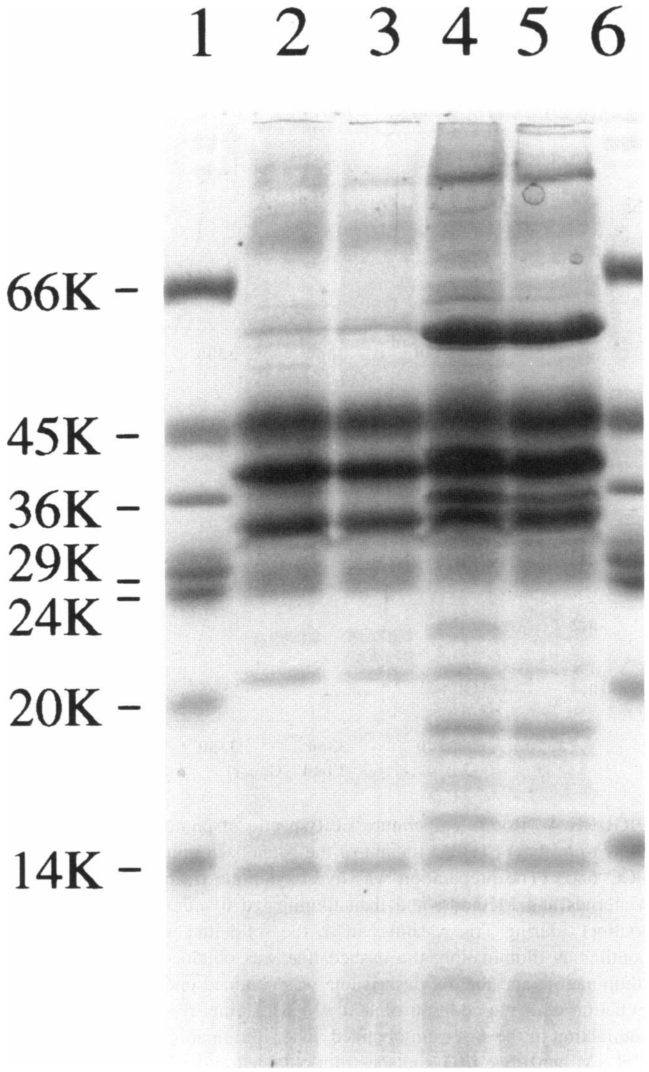

FIGURE 3 SDS-denaturing gel electrophoresis of photosystem II preparations from the cyanobacterium *Synechocystis sp.* PCC 6803. In Lane 2, the wild-type preparation was Tris washed; in Lane 3, the wild-type preparation was hydroxylamine treated; in Lane 4, the YF160D2 preparation was Tris washed; in Lane 5, the YF160D2 preparation was hydroxylamine treated. Eight micrograms of chlorophyll were loaded per lane. The differences in the polypeptide composition near 66 kDa, when the mutant (4 and 5) and wild-type (2 and 3) lanes are compared, are due to contaminating photosystem I in the mutant preparation, as described in the Results. Lanes 1 and 6 show molecular mass standards, as labeled.

Another possible explanation for the differential effects of Tris and hydroxylamine treatment on the mutant preparations is the illumination performed during Tris treatment. Whereas Tris treatment is performed under illumination (Frasch and Cheniae, 1980), hydroxylamine treatment is performed in the dark (Sharp and Yocum, 1981). Perhaps this initial illumination makes the mutant preparation more susceptible to photoinhibition. M^+ might then arise from photodegradation of the complex. It has been suggested previously that photoinhibition of PSII may be associated with the formation of novel protein-based radicals (for a review, see Prasil et al., 1992, and references therein). To investigate this possibility, hydroxylamine-treated mutant preparations were illuminated under conditions similar to those used during Tris treatment. The results are shown in Fig. 4. Even after preillumination of the hydroxylamine-treated YF160D2 preparation, a normal $Z^{\bullet}$ lineshape is observed under continuous illumination in the presence of acceptors (Fig. 4 *B*). These data argue against the idea that M^+ arises because of photodegradation triggered by preillumination.

To investigate the role of M^+ in electron transfer, we have also performed single turnover kinetic assays. In Fig. 5, we present transient EPR data obtained upon flash illumination at a constant low-field position of 3368 G (see position of arrows in Fig. 2). Chlorophyll radicals from

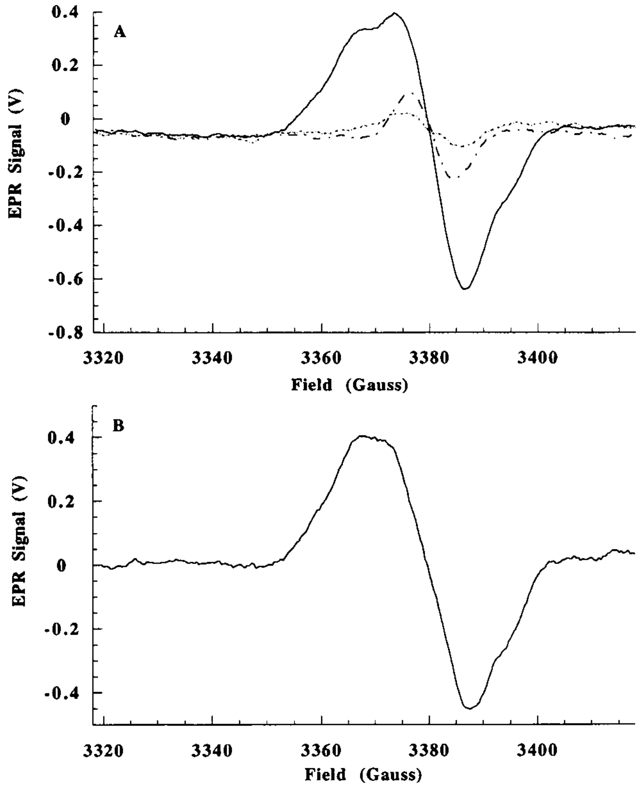

FIGURE 4 Room temperature EPR spectra of photosystem II preparations from the YF160D2 mutant of the cyanobacterium *Synechocystis sp.* PCC 6803. The preparation was hydroxylamine treated as described in Materials and Methods, and then illuminated to mimic conditions used routinely during Tris washing. In *A*, the solid line was obtained under continuous illumination, the dashed line was obtained in the dark after illumination, and the dot-dashed line was obtained under continuous illumination after the addition of 800 μM benzidine. *B* shows the result of subtraction of the spectrum obtained under illumination in the presence of 800 μM benzidine from the spectrum obtained under illumination. Potassium ferricyanide (1.0 mM) was added as an electron acceptor. Conditions were as described in Fig. 1. The chlorophyll concentration was 0.7 mg/ml; the gain was 2.5 × 10^4.

photosystem II or photosystem I make a negligible spectral contribution at this field setting (see Fig. 1, for example). In Fig. 5 *A*, the wild-type preparation was Tris washed to remove manganese; in Fig. 5 *B*, the preparation was hydroxylamine treated. In both samples the rise time of the radical is faster than the instrument response time. This is expected because the oxidation of Z by P^+ occurs in the microsecond time scale in such manganese-depleted samples (Boska et al., 1983) and because our time constant is 8 ms. The decay kinetics can be fit (Table 2) with a single exponential function. In hydroxylamine-treated samples, the average $t_{1/2}$ is 220 ms. In Tris-washed samples, the average $t_{1/2}$ is 150 ms.

In wild-type preparations, similar kinetics were obtained in the presence of 1 mM ferricyanide and in the presence of a mixture of 1 mM ferricyanide and 1 mM ferrocyanide (Table 2). Because our cyanobacterial preparation contains no functional Q_B, we assign the kinetics observed in wild-type photosystem II to recombination of $Z^{\bullet}$ with Q_A^-. (Q_A^- is EPR silent under normal conditions at room temperature, because of its proximity to the nonheme iron atom of photosystem II.) Such an assignment is consistent with the lack of dependence of the recombination rate on the concentration of the donor, potassium ferrocyanide (Table 2). Similar single exponential kinetics, with a $t_{1/2}$ of 130 ms, were obtained at a high field position (3392 G, Table 2). The kinetics are also unchanged by continuous illumination for 4 min (Table 2), demonstrating that neither the laser flashes nor continuous illumination adversely affects electron transfer rate in a room temperature, stationary wild-type sample.

In Fig. 5 we also present kinetic EPR data on photosystem II particles from the YF160D2 and YW160D2 after Tris washing (Fig. 5, *C* and *E*) and hydroxylamine treatment (Fig. 5, *D* and *F*). The M^+ radical decays irreversibly in 30 min in Tris-washed preparations from the two mutants (Boerner and Barry, 1994). To control for this irreversible decay under continuous illumination, single turnover kinetic assays were performed on dark-adapted samples before continuous illumination. After the kinetic transient (Fig. 5) was obtained, the sample was then illuminated with a fiber optic light source, and a field scan was performed (see Fig. 2, for example).

As shown in Fig. 5, in all four mutant preparations, the rise time is faster than the instrument response time. The decay kinetics are similar to those observed in the wild-type preparations, with hydroxylamine-treated mutant preparations giving an average $t_{1/2}$ of approximately 180 ms and Tris-washed mutant preparations giving an average $t_{1/2}$ of approximately 150 ms. In analogy to the assignment in wild-type photosystem II, we assign these kinetics to recombination of a donor-side radical with Q_A^-. Because the kinetics observed in the mutant preparations are similar to wild type, we conclude that the donor-side radical combining with Q_A^- is $Z^{\bullet}$ in all of the mutant samples. Note that the kinetics are similar in mutant preparations whether M^+ or $Z^{\bullet}$ is produced under continuous illumination after the single turnover study. This result can be obtained if the oxidation of M^+ occurs with low quantum yield.

The kinetic data presented in Fig. 5 suggest that M is a third redox active site in the reaction center and that this redox active site is oxidized slowly. To support this hypothesis, we present the data in Fig. 6, which were obtained on the YF161D1 mutant. Tyrosine 161 of D1 is the putative tyrosine Z site. Thus this mutation substitutes a nonredox active phenylalanine for the photosystem II donor-side electron carrier. The YF161D1 preparation was Tris washed.

Spectra obtained on the YF161D1 mutant under continuous illumination (solid line), in the dark after illumination (dashed), and under continuous illumination in the presence of benzidine (dot-dashed line) are shown in Fig. 6 *A*. As expected, the Tris-washed YF161D1 mutant exhibits the dark stable radical, $D^{\bullet}$ (Debus et al., 1988b; Metz et al., 1989; Noren and Barry, 1992) (Fig. 6 *A*), and produces M^+, not $Z^{\bullet}$, under continuous illumination (Noren and Barry,

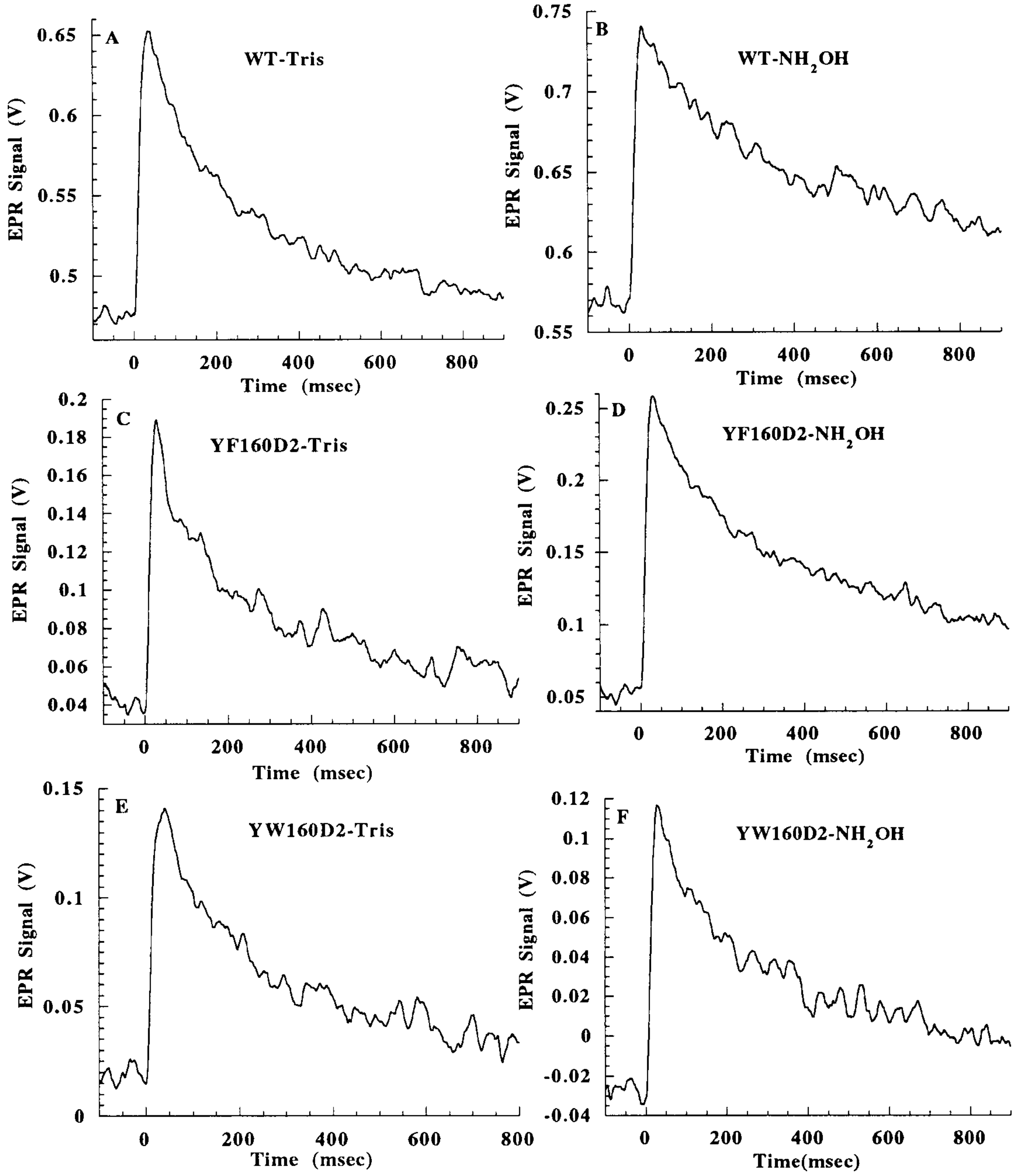

FIGURE 5 Kinetic transients obtained on photosystem II preparations from the cyanobacterium *Synechocystis sp.* PCC 6803, as measured by room temperature EPR spectroscopy. In *A*, *C*, and *E*, the wild-type (*A*), YF160D2 (*C*), and YW160D2 (*E*) preparations were Tris washed. In *B*, *D*, and *F*, the wild-type (*B*), YF160D2 (*D*), and YW160D2 (*F*) preparations were hydroxylamine treated. The chlorophyll concentrations and gains were the same as in Fig. 2. The magnetic field was set at 3368 G, and the time constant was 8 ms. Other conditions are given in Materials and Methods.

1992) (Fig. 6 *B*). Previous analysis has shown that the M^+ signal is still produced after 60 min of repeated illumination at room temperature. However, as a precaution, single turnover assays were obtained before continuous illumination of YF161D1 mutant samples. As shown in Fig. 6 *C*, no kinetic data could be obtained on samples from the YF161D1 mutant with saturating flashes, although subsequent, continuous illumination still generated the M^+ signal (Fig. 6 *B*).

We conclude that M is oxidized in very low yield on a single turnover in the YF161D1 mutant. This result is consistent with the idea that substitution of a nonredox active phenylalanine for tyrosine 161 eliminates redox active Z from the complex and that Z, but not M, is oxidized with high quantum yield.

To investigate the origin of the M^+ lineshape, we have continued our experiments aimed at specific labeling of the radical (see discussion in Boerner and Barry, 1994). We employ the numbering scheme for tyrosine in which carbon 4 is adjacent to the phenol oxygen. In Fig. 7 *C*, we present EPR spectra obtained on the dark stable tyrosine radical, $D^\bullet$,

TABLE 2 Decay half-times, $t_{1/2}$,[*] (ms) from transient, room temperature EPR measurements on photosystem II preparations from *Synechocystis sp.* PCC 6803

	Tris-washed		Hydroxylamine-treated	
	Before illumination	After illumination	Before illumination	After illumination
Wild type	150[#] (0.994)	140[#] (0.992)	220[#] (0.985)	220[#] (0.962)
	160[§] (0.996)	160[§] (0.991)	230[#] (0.985)	
		130[¶] (0.993)		
YF160D2	160[#] (0.981)	Not detected[‖]	190[#] (0.992)	200[#] (0.979)
	140[#] (0.975)	Not detected[‖]		
YW160D2	140[#] (0.979)	Not detected[‖]	170[#] (0.981)	160[#] (0.952)

[*] Each kinetic trace was fit to a single exponential decay, $y = A + Be^{-kt}$, using Kaleidagraph software. $t_{1/2} = \ln 2/k$. The numbers in the parentheses are the correlation coefficients of the fits. The magnetic field was set at 3368 G, unless otherwise noted.

[#] 1.0 mM $K_3Fe(CN)_6$ was used as an electron acceptor.

[§] 1.0 mM $K_3Fe(CN)_6$ and 1.0 mM $K_4Fe(CN)_6$ were used.

[¶] 1.0 mM $K_3Fe(CN)_6$ and 1.0 mM $K_4Fe(CN)_6$ were used, and the magnetic field was set at 3392 G.

[‖] Under continuous illumination, M^+ irreversibly decays in approximately 30 min in the YF160D2 and YW160D2 mutants, as previously described (Boerner et al., 1993). However, its stability in the YF161D1 mutant is similar to that of wild-type $Z^{\cdot}$ (Noren and Barry, 1992).

after specific deuteration of tyrosine at the methylene position. Fig. 8 *C* shows the effect of specific deuteration of tyrosine at the methylene position on M^+. Upon methylene deuteration, M^+ exhibits a doublet spectrum, whereas $D^{\cdot}$ does not (Figs. 7 *C* and 8 *C*). The doublet spectrum observed after methylene deuteration of M^+ can only be simulated by the assumption that the 3 and the 5 hydrogens are not equivalent. By contrast, the spectrum of methylene-deuterated $D^{\cdot}$ is best simulated with 6.9-G couplings to equivalent hydrogens (Hoganson and Babcock, 1992). Large changes in the g_x, g_y, and g_z components of the g tensor fail to give good simulations of M^+ in the absence of the alterations in hyperfine splittings described above.

DISCUSSION

We have presented EPR results that help to elucidate the structure of tyrosine radical, M^+, and the role of M in electron transfer in photosystem II. We will discuss the structural studies first, which are based on specific deuteration of tyrosine.

Previous studies of unmodified tyrosine radicals in vivo (Babcock et al., 1992; Barry et al., 1990; Bender et al., 1989; Hoganson and Babcock, 1992) and in vitro (Barry et al., 1990; Dixon et al., 1974; Dixon and Murphy, 1976; Sealy et al., 1985) have shown that these radicals follow an odd-alternate pattern of spin density arrangement in the aromatic ring, with maximum spin density at the 1, 3, and 5 positions. The isotropic coupling, a_{iso}, to the α-protons on the ring is proportional to the spin density, ρ, at the adjacent carbon (Bender et al., 1989; Wertz and Bolton, 1986). In a powder sample, a rhombic hyperfine tensor is observed for α-protons, because of a dipolar interaction of the proton with spin density delocalized over the aromatic ring. Typically the pattern is 1.5 a_{iso}, 0.5 a_{iso}, a_{iso}, for the A_x, A_y, and A_z hyperfine tensor components, respectively. The y axis is along the C-H bond and the z axis is perpendicular to the plane of the tyrosine ring (Bender et al., 1989; Wertz and Bolton, 1986). By contrast, a_{iso} for β-protons is proportional both to the spin density at the adjacent carbon atom and to the dihedral angle, θ, that the proton makes with respect to the p_z orbital on C_4: $a_{iso} = B_0 + B_1\rho \cos \theta$. B_0 is negligible, and B_1 is approximately 60 G for the tyrosine radical in ribonucleotide reductase (Bender et al., 1989; Wertz and Bolton, 1986). In a powder sample, the hyperfine tensor for β-protons is nearly isotropic (Barry et al., 1990; Bender et al., 1989; Wertz and Bolton, 1986).

The pattern of spectral changes observed upon specific deuteration of $D^{\cdot}$ is in many ways typical of unmodified tyrosine radicals. Couplings to the 2,6 protons are small, and deuteration at these positions has little effect on the spectrum (Barry et al., 1990). Methylene deuteration produces a spectrum characteristic of equivalent splittings to the 3,5 protons. The spectrum produced by 3,5 deuteration is dominated by coupling to one of the β-methylene protons. This coupling is radical specific and reflects environmental effects on the conformation at the C_1-C_β bond. Perdeuteration of the radical produces a narrow singlet spectrum.

Isotopic labeling and EPR spectroscopy show that M^+ has a tyrosine origin, because large hyperfine couplings are sensitive to deuteration of tyrosine. However, the unusual four-line spectrum of M^+ suggests that the structure of this tyrosyl radical is unique. The work presented here shows that the spectral pattern observed upon specific deuteration at the methylene position of M^+ is markedly different from the pattern observed for $D^{\cdot}$. Specific deuteration experiments show that introduction of either 3,5 deuterated tyrosine or methylene-deuterated tyrosine into photosystem II gives a doublet lineshape for M^+. To simulate the 3,5-deuterated spectrum, the hyperfine splitting to the strongly coupled β-proton of M^+ must be larger than the coupling observed in $D^{\cdot}$. This result suggests a rearrangement in spin density or a change in the C_1-C_β conformation when M^+ is compared to $D^{\cdot}$. To simulate the methylene-deuterated spectrum, we must lift the equivalency of the 3 and 5 hydrogens. Again, this result suggests a dramatic reorganization of spin density or a removal of the 3 or 5 hydrogen atom through posttranslational modification. Perdeuteration of tyrosine,

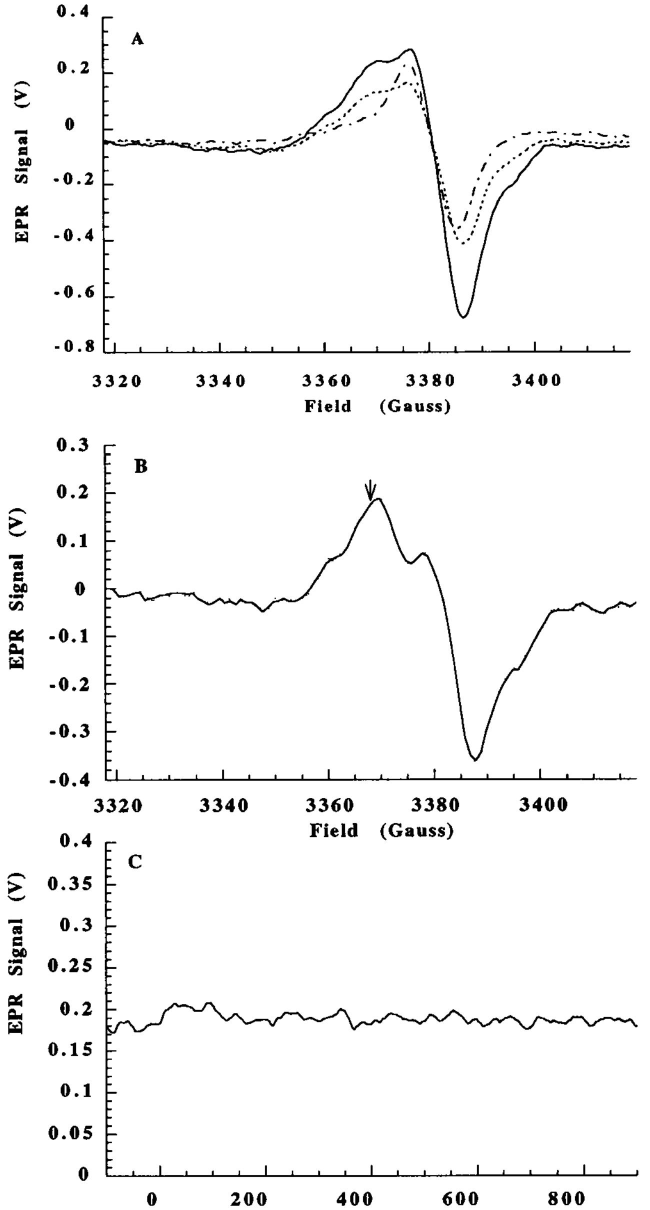

FIGURE 6 Room temperature EPR spectra and kinetic transients obtained on a Tris-washed photosystem II preparation from the YF161D1 mutant of the cyanobacterium *Synechocystis sp.* PCC 6803. In *A*, the solid line was obtained under continuous illumination, the dashed line was obtained in the dark after illumination, and the dot-dashed line was obtained under continuous illumination after the addition of 800 μM benzidine. In *B*, a spectrum obtained in the presence of benzidine under illumination was subtracted from a spectrum obtained under illumination in the absence of benzidine. In *C*, the result of a transient EPR experiment on this preparation is shown. The field was set at 3368 G (see arrow in *B*), and the time constant was 8 ms. The spectral conditions were the same as described in Figs. 1 and 5. The chlorophyll concentration was 0.45 mg chl/ml, and the gain was 2.5×10^4.

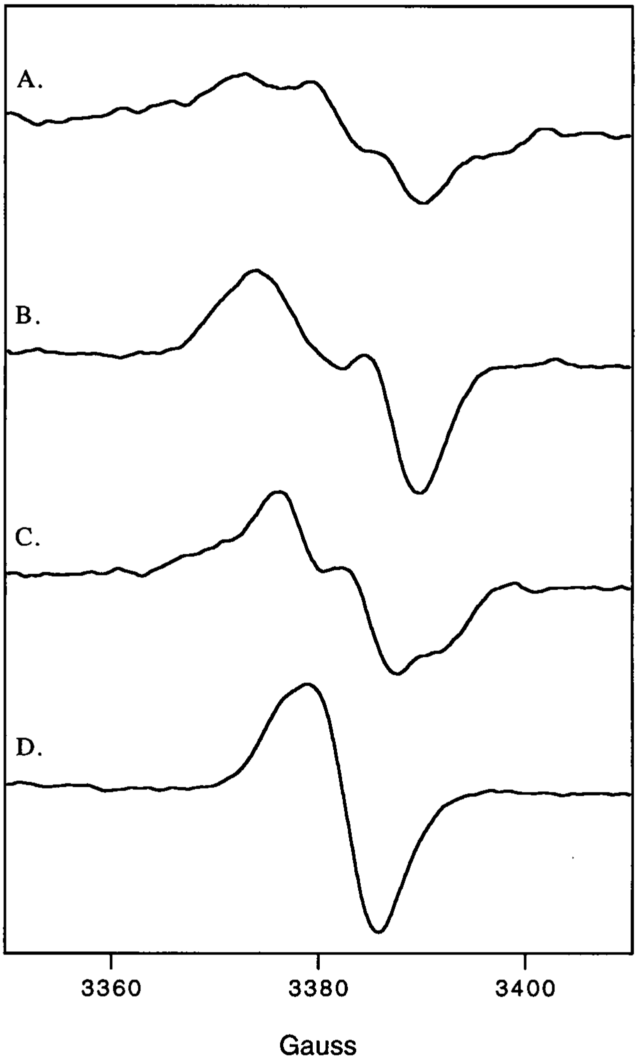

FIGURE 7 Room temperature EPR spectra of D$^\bullet$ in cyanobacteria. A wild-type culture was labeled with methylene deuterated tyrosine (*C*). Growth conditions were used in which tyrosine is required for growth (Barry and Babcock, 1987; Boerner and Barry, 1993, 1994). Spectra obtained after the wild-type culture was supplied unlabeled tyrosine (*A*), 3,5 deuterated tyrosine (*B*), and perdeuterated tyrosine (*D*) are reproduced from Boerner and Barry (1994) for comparison. EPR conditions are the same as Fig. 1, except that the microwave power was 3.5 mW, and the field modulation was 2 G.

although substantially narrowing the spectrum, does not collapse all hyperfine couplings. To simulate the perdeuterated spectrum, we must invoke significant coupling to an exchangeable proton or to a nuclear spin of nontyrosine origin. Such substantial spectral alterations from the labeling patterns observed for unmodified tyrosine radicals suggest that M^+ is a covalently modified tyrosine radical. There is precedence for such an observation, because posttranslationally modified redox active tyrosines are known to exist in other enzymes (see, for example, Babcock et al., 1992; Hartmann and Dooley, 1995; Ito et al., 1991; Janes and Klinman, 1995; Warncke et al., 1994; Whittaker and Whittaker, 1990, and references therein).

Aside from the structural studies described above, we have performed experiments to test the hypothesis that M is a third redox active site in the photosystem II. These experiments are based on a measurement of Z$^\bullet$ recombination

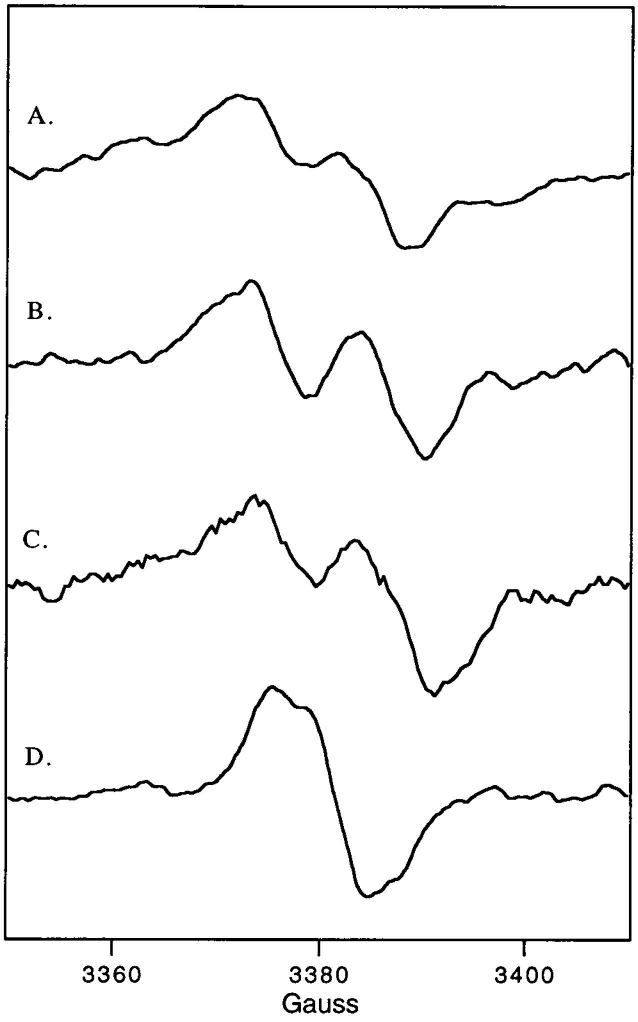

FIGURE 8 Room temperature EPR spectra of M^+ in cyanobacteria. A YF160D2 culture was labeled with methylene deuterated tyrosine (*C*). Growth conditions were used in which tyrosine is required for growth (Barry and Babcock, 1987; Boerner and Barry, 1993, 1994). Spectra obtained after the YF160D2 culture was supplied unlabeled tyrosine (*A*), 3,5 deuterated tyrosine (*B*), and perdeuterated tyrosine (*D*) are reproduced from Boerner and Barry (1994) for comparison. EPR conditions are the same as in Fig. 1, except that the microwave power was 3.5 mW, and the field modulation was 2 G.

kinetics in wild-type cyanobacterial photosystem II. In wild-type preparations, we observe $Z^{\bullet}$ decay kinetics with a $t_{1/2}$ of 220 ms (hydroxylamine treated) and of 150 ms (Tris treated) at pH 6.5. These values are somewhat larger than those derived from previous optical measurements of $Z^{\bullet}$ Q_A^- recombination in *Synechocystis* core particles, which gave a $t_{1/2}$ of 80 ms (Metz et al., 1989). However, the previous measurement was performed at pH 5.9, and the rate of recombination is known to be sensitive to pH (Dekker et al., 1984). Our measurements are in approximate agreement with previous measurements of $Z^{\bullet}Q_A^-$ recombination in spinach photosystem II membranes at pH 6.5 (Dekker et al., 1984).

In previous work, we characterized the rate of Q_A^- decay in the YF160D2 and YW160D2 mutants by monitoring the decay of Q_A^- absorbance at 320 nm (Boerner et al., 1993). The recombination rates so derived were somewhat slower than those presented in this study, which involves directly monitoring the rate of decay of donor-side radicals through the use of EPR. However, our previous experiments were performed at 8°C, whereas the EPR measurements are performed at room temperature. Thus, the observed small differences in rate could be due to a temperature effect. Other than this difference in rate, the conclusions arrived at here are in agreement with the conclusion of this previous study; there is no significant difference in the rate of recombination when the D2 mutants are compared to wild type (Boerner et al., 1993).

A simple scheme that can explain our kinetic experiments is shown in Table 3. In the case of the single turnover assay, charge separation is known to generate the state $P^+Q_A^-$ on the picosecond time scale. Z is then oxidized on the microsecond time scale by P^+. In the absence of a functional manganese cluster, $Z^{\bullet}$ recombines with Q_A^- in the millisecond time scale (reviewed by Renger, 1992).

Our experiments on wild-type preparations are consistent with previous results that show that $Z^{\bullet}$ recombines in the millisecond time scale with Q_A^-. Furthermore, we have shown that M^+ is not observed in a single turnover assay. A reasonable explanation of this observation is that the recombination rate of $Z^{\bullet}$ and Q_A^- is significantly faster than the rate at which M is oxidized.

In the YF161D1 mutant (Table 3), Q_A^- recombines with the donor side with a $t_{1/2}$ of 1 ms (Metz et al., 1989). This rate is again much faster than the rate at which M is oxidized, so we observe no kinetic transient from this radical. We observe no kinetic transient from P^+ in the YF161D1 mutant because the recombination of $P^+Q_A^-$ would not be observable under the data acquisition conditions employed.

On the other hand, when the preparation is continuously illuminated in the presence of acceptors, the oxidation of M via $Z^{\bullet}$ or P^+ is now possible (Table 3). Under these conditions, the negative charge is stabilized on the exogenous acceptor (Table 3), and the large number of turnovers will build up a high concentration of the terminal donor, no matter how slowly this terminal donor is oxidized. Thus,

TABLE 3 Electron transfer schemes

Single turnover assays
 YF160D2 and YW160D2 mutants
 $MZPQ = MZP^+ Q^- = MZ^{\bullet} P Q^-$

 YF161D1 mutant
 $MPQ = MP^+ Q^-$

Continuous illumination in the presence of acceptor (A)
 YF160D2 and YW160D2 mutants
 $MZPQ\ A = MZP^+ Q^-\ A = M Z^{\bullet} P Q^-\ A = M^+ ZPQ\ A^-$

 YF161D1 mutant
 $MPQ\ A = MP^+ Q^-\ A = M^+ PQ\ A^-$

continuous illumination of the D2 and D1 mutants builds up a high photosteady-state concentration of M^+. $Z^\bullet$ is reduced under these conditions by electron transfer from M^+.

These kinetic schemes and our kinetic data are consistent with the hypothesis that M^+ arises from a redox active, covalently modified tyrosine in photosystem II that is slowly oxidized by P^+ via tyrosine $Z^\bullet$. Thus, the EPR data mutant support the hypothesis that the oxidation of M^+ occurs slowly and that M is a third redox active site, different from both tyrosine D and Z, in the photosystem II reaction center.

The observation that formation of the M^+ radical is dependent on Tris washing is intriguing. Because Tris washing is performed in the light, whereas hydroxylamine treatment is performed in the dark, we suspected that this preillumination step might underlie the spectral differences observed. However, preillumination of hydroxylamine-treated samples fails to give the M^+ lineshape. Thus, we do not favor the hypothesis that M^+ arises because of photoinhibition, but are left to consider other possible explanations. One explanation is that the subunit composition of photosystem II is altered by the difference in treatments. To be consistent with our results, this subunit must have a molecular mass of less than 24 kDa and stain poorly with Coomassie blue. Another possible explanation is that our Tris- and hydroxylamine-treated preparations differ in metal content. However, it seems unlikely that this metal is bound, photooxidizable manganese, because the $Z^\bullet Q_A^-$ recombination rates are similar when the two types of preparations are compared (Babcock and Sauer, 1975; Hoganson et al., 1989). A third possible explanation is that the conditions used for Tris washing may allow access of endogenous reductants to the mutant reaction centers. In this scenario, M is normally oxidized and therefore remains EPR silent in hydroxylamine-treated preparations.

We are also left to consider the observation that M^+ has only been observed in site-directed mutants thus far. Access of exogenous reductants may be facilitated in site-directed mutants, because it is known that mutagenesis of photosystem II sometimes has the effect of destabilizing protein-protein interactions (Boerner et al., 1992; Noren and Barry, 1992).

Experiments to further elucidate the structure and function of M are in progress. We suggest that M may play some structural role in photosytem II, perhaps as a ligand to the metal-containing active site.

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