The Protein Environment Surrounding Tyrosyl Radicals D$^{\bullet}$ and Z$^{\bullet}$ in Photosystem II: A Difference Fourier-Transform Infrared Spectroscopic Study

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ABSTRACT Photosystem II contains two redox-active tyrosine residues, termed D and Z, which have different midpoint potentials and oxidation/reduction kinetics. To understand the functional properties of redox-active tyrosines, we report a difference Fourier-transform infrared (FT-IR) spectroscopic study of these species. Vibrational spectra associated with the oxidation of each tyrosine residue are acquired; electron paramagnetic resonance (EPR) and fluorescence experiments demonstrate that there is no detectable contribution of Q_A^- to these spectra. Vibrational lines are assigned to the radicals by isotopic labeling of tyrosine. Global ^{15}N labeling, ^{2}H exchange, and changes in pH identify differences in the reversible interactions of the two redox-active tyrosines with N-containing, titratable amino acid side chains in their environments. To identify the amino acid residue that contributes to the spectrum of D, mutations at His^{189} in the D2 polypeptide were examined. Mutations at this site result in substantial changes in the spectrum of tyrosine D. Previously, mutations at the analogous histidine, His^{190} in the D1 polypeptide, were shown to have no significant effect on the FT-IR spectrum of tyrosine Z (Bernard, M. T., et al. 1995. *J. Biol. Chem.* 270:1589–1594). A disparity in the number of accessible, proton-accepting groups could influence electron transfer rates and energetics and account for functional differences between the two redox-active tyrosines.

INTRODUCTION

Photosystem II (PSII) is composed of both hydrophobic and hydrophilic subunits and is responsible for the oxidation of water and production of reduced plastoquinone. Four consecutive charge separations in the PSII reaction center create the oxidizing equivalents necessary for the oxidation of two molecules of H_2O to one molecule of O_2. The redox-active center in the oxygen-evolving system is postulated to be a tetranuclear cluster of manganese atoms (reviewed in Angerhofer and Bittl, 1996; Britt, 1996).

The D1 and D2 polypeptides form the heterodimer core of the PSII reaction center and bind cofactors involved in charge separation and electron transfer, such as the primary chlorophyll (chl) donor (P_{680}) and pheophytin (Nanba and Satoh, 1987). After photoexcitation, P_{680} transfers an electron to a bound quinone, Q_A, via pheophytin. P_{680}^+ is reduced by tyrosine Z. Tyrosine radical Z$^{\bullet}$ is then reduced by the manganese cluster (Babcock et al., 1976; Gerken et al., 1988). Reduction of tyrosine Z$^{\bullet}$ can be retarded by removal of the manganese cluster; under these conditions, an electron paramagnetic resonance (EPR) signal from Z$^{\bullet}$ can be photoaccumulated (Babcock and Sauer, 1975a,b). Z$^{\bullet}$ was identified as a tyrosine radical through EPR and isotopic labeling of *Synechocystis* PSII particles (Boerner and Barry, 1993). Site-directed mutagenesis experiments argue that Z is Tyr^{161} of D1 and is required for oxygen evolution (Debus et al., 1988b; Metz et al., 1989; Noren and Barry, 1992).

A second redox-active tyrosine associated with PSII is the dark stable radical D$^{\bullet}$, which has a dark decay rate with a $t_{1/2}$ of hours (Babcock and Sauer, 1973b). D$^{\bullet}$ was identified as a tyrosine radical through the use of isotopic labeling and EPR spectroscopy (Barry and Babcock, 1987). Site-directed mutagenesis defined D as residue 160 in the D2 polypeptide (Debus et al., 1988a; Vermaas et al., 1988). The function of tyrosine D is unknown, but it is oxidized by the manganese cluster (Babcock and Sauer, 1973a). In the absence of the manganese cluster, tyrosine D is oxidized directly by P_{680}^+ (Buser et al., 1990). Replacement of tyrosine D with either phenylalanine (YF160D2) or tryptophan does not eradicate enzymatic activity, indicating that tyrosine D is not required for oxygen evolution (Debus et al., 1988a; Vermaas et al., 1988; Boerner et al., 1993).

Although these tyrosine residues reside in analogous positions in homologous polypeptides, tyrosine D and Z differ in their oxidation and reduction kinetics. Furthermore, the oxidation potentials of Z$^{\bullet}$ and D$^{\bullet}$ are estimated to be 1 V (Metz et al., 1989) and 760 mV (Boussac and Etienne, 1984), respectively. Our goal is to gain insight into the environmental factors that control the function of these redox-active tyrosines. Fourier-transform infrared (FT-IR) spectroscopy, a vibrational technique that monitors changes in the dipole moment of chemical bonds, can address questions regarding the interaction of each species with the protein environment. Herein we use a method to acquire the difference FT-IR spectra associated with the oxidation of tyrosine D and tyrosine Z from a single PSII sample; we have performed difference infrared measurements on PSII purified from wild-type, isotopically labeled, and mutant

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Synechocystis strains. Use of this difference FT-IR technique distinguishes the tyrosines on the basis of their interaction with amino acid residues in their environment. This distinction has important implications in understanding the function of redox-active amino acid residues.

MATERIALS AND METHODS

Protein purification

Mutants, obtained from Prof. R. Debus (UC Riverside), were produced by methods previously described (Boerner et al., 1993). Glucose-tolerant strains of wild-type and mutant cells were photoheterotrophically grown (Williams, 1988). PSII particles were purified from cyanobacterial cells, manganese-depleted (Noren et al., 1991; MacDonald and Barry, 1992; Bernard et al., 1995), dialyzed against 5 mM HEPES-NaOH (pH 7.5) or 5 mM 2-(*N*-morpholino)ethanesulfonic acid-NaOH (pH 6.0), concentrated in Centricon 100 concentrators (Amicon, Beverly, MA) to 1 mg chl/ml, aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C until use. Chlorophyll determinations were made in methanol (Lichtenthaler, 1987). Steady-state oxygen evolution rates (Barry, 1995) of PSII particles before NH_2OH treatment were (units of μmol O_2/mg chl-h): 1) wild type, 2700; 2), YF160D2, 700; 3) HQ189D2, 1800; 4) HL189D2, 1200; 5) ^{13}C(6)-Tyr-labeled wild type, 2400; and 6) global ^{15}N-labeled wild type, 2300.

Isotopic labeling of cyanobacterial wild-type cultures

Cultures of *Synechocystis* incorporated either tyrosine or ^{13}C(6)-tyrosine (L-4-hydroxyphenyl-$^{13}C_6$-alanine, 99% labeled; Isotec, Miamisburg, OH) (Barry and Babcock, 1987). Cells grown under these conditions required the modifications to the PSII purification protocol previously described (Barry, 1995). ^{15}N global labeling and ^{2}H exchange were performed as previously described (Bernard et al., 1995).

EPR spectroscopy

Manganese-depleted wild-type, HQ189D2, and HL189D2 PSII samples (80 μl @ 1.0 mg chl/ml) were partially dried on mylar strips (MacDonald and Barry, 1992) with 3 mM ferricyanide and 3 mM ferrocyanide. In all of the experiments described here, potassium ferricyanide and potassium ferrocyanide stocks were made up on the same day and added immediately before data acquisition. A Varian E4 spectrometer equipped with a TE cavity was used for tyrosyl EPR measurements at −9 ± 1°C (Ma and Barry, 1996). Illumination was provided with a red-filtered (580-nm cutoff) light from a fiber optic illuminator (Dolan Jenner, Woburn, MA). Spectral conditions were: microwave frequency, 9.1 GHz; power, 1.3 mW; modulation amplitude, 5 G, scan time, 4 min; time constant, 2 s. Spin quantitation on these samples was performed by flash-freezing samples in liquid nitrogen. The spin standard was Fremy's salt, and measurements were performed at 80 K (Babcock et al., 1983; Barry, 1995).

$Fe^{+2}Q_A^-$ EPR spectra at helium temperature were recorded on a Bruker EMX 6/1 X-band spectrometer equipped with an Oxford cryostat. Oxygen-evolving cyanobacterial PSII (200 μl @ 1.3 mg chl/ml) contained 3 moles potassium ferricyanide per mole PSII reaction center. The molar concentration of PSII was calculated from a determination of the chlorophyll concentration (Barry, 1995) and from the previously measured antenna size of this preparation (Patzlaff and Barry, 1996). Manganese-depleted cyanobacterial PSII (200 μl @ 1.3 mg chl/ml), containing 3 mM ferricyanide and 3 mM ferrocyanide, was also used. For some experiments, samples were dried onto mylar strips, as described above. Continuous illumination at 200 K was performed in a glass dewar filled with dry ice-ethanol mixture; a Dolan Jenner illuminator with red (580-nm cutoff) and heat filters was the light source. Spectra were recorded at 4.3 ± 0.2 K. Spectral conditions were: microwave frequency, 9.44 GHz; power, 40 mW; modulation amplitude, 32 G, scan time, 336 s; time constant, 328 ms.

Fluorescence spectroscopy

Spectra were recorded on an Opti-Sciences OS-500 modulated fluorometer (Haverhill, MA). Samples were identical to those employed for $Fe^{2+}Q_A^-$ EPR measurements and contained the same amount of chlorophyll. Samples were partially dehydrated onto a glass window and placed in the temperature control cell used for FT-IR measurements. Temperature control at −9°C was performed with the system described below for FT-IR data acquisition. Red-filtered light was the source of illumination. Spectral conditions were: modulation intensity, 180 μW; saturation intensity, 180 μW; scan time, 3 s.

Difference FT-IR spectroscopy

Data were obtained on partially dehydrated cyanobacterial PSII samples in a manner similar to methods described previously (MacDonald and Barry, 1992; Bernard et al., 1995). Spectra were recorded on a Nicolet (Madison, WI) Magna 550 II spectrometer with a MCT-A detector. Resolution was 4 cm^{-1}, double-sided interferograms were collected, and the mirror velocity was 2.53 cm/s. A Happ-Ganzel apodization function and a single level of zero filling were employed. Manganese-depleted PSII samples, containing 25–30 μg chlorophyll @ 1 mg chl/ml, 3 mM potassium ferricyanide, and 3 mM potassium ferrocyanide, were spread in a 10-mm (diameter) circle on a 25 (diameter) × 2 (thickness) mm Ge window, dried for 25–30 min with a dry nitrogen stream, and then sandwiched with a CaF_2 window. The Ge window blocked illumination of the sample by the He-Ne laser. Temperature was maintained and illumination was provided as described (Barry, 1995). Heat and red (580 nm cutoff) filters were employed. Data acquisition was synchronized with the illumination system through the methods previously described, except that Visual Basic programs were used (Barry, 1995). Each interferogram is the sum of 425 mirror scans, taken in 4 min. Data recorded in the light were ratioed directly to data recorded immediately before in the dark.

Absorbance at 1655 cm^{-1} (the amide I band) was always less than 0.4; the amide I absorbance is smaller than absorbances used in previous reports from this laboratory (MacDonald and Barry, 1992; Bernard et al., 1995). This is due to the higher signal-to-noise ratio of the Magna 550 II infrared spectrometer and the smaller antenna size of the cyanobacterial reaction center. Moreover, this is desirable because of the difficulty of isolating PSII from some site-directed mutants, which produce PSII in low yield, and because of the expense of labeling PSII in cyanobacteria. Difference spectra were normalized to the amide II absorbance and/or to the total amount of protein, which give equivalent results; this indicates that the path length and sample surface area were reproducible. Averages of 8–23 difference and double-difference spectra are shown.

RESULTS

EPR and fluorescence control experiments

In Fig. 1 *A*, we present EPR control experiments on manganese-depleted PSII samples from *Synechocystis* sp. PCC 6803. Samples are maintained at pH 7.5, which enhances the reduction rate of $D^{\bullet}$ (Vass and Styring, 1991). Samples, containing a mixture of 3 mM potassium ferricyanide and 3 mM potassium ferrocyanide, are partially dehydrated on solid mylar substrates and are illuminated for 4 min in the cavity, with red- and heat-filtered white light. The cavity and sample are maintained at −9°C with a stream of cold nitrogen. These conditions closely parallel the conditions employed for FT-IR spectroscopy (see below).

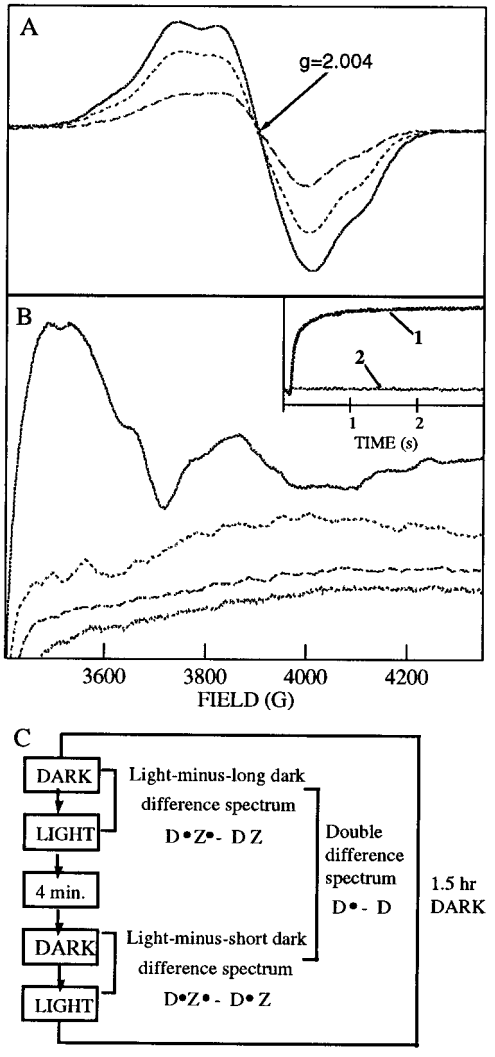

FIGURE 1 (*A*) EPR spectra of tyrosyl radicals in manganese-depleted wild-type *Synechocystis* PSII preparations at pH 7.5. The solid line was recorded under illumination, the dotted line was recorded after a 4-min dark adaptation, and the dashed line was recorded after 90 min. Samples were illuminated in the cavity, which was maintained at −9°C. (*B*) EPR spectra of $Fe^{+2}Q_A^-$ in *Synechocystis* PSII preparations at pH 7.5. Oxygen-evolving PSII preparations with 3 equivalents of potassium ferricyanide (3 moles/mole PSII reaction center) were dark-adapted for 4 min, and a spectrum was recorded (*dotted line*). Samples were then illuminated at −9°C for 4 min, and a spectrum was recorded (*solid line*). Manganese-depleted PSII preparations with 3 mM potassium ferricyanide and potassium ferrocyanide were treated similarly, i.e., dark-adapted for 4 min (*hatched line*) and illuminated at −9°C (*dashed line*). Inset shows fluorescence data on oxygen-evolving PSII (*trace 1*) and manganese-depleted PSII (*trace 2*). Note that *A* and *B* are not on the same scale. (*C*) Schematic of FT-IR data collection method. This method generates difference and double-difference spectra that reflect the oxidation and reduction of tyrosines Z and D, respectively. The boxes labeled "light" and "dark" correspond to 4 min of data acquisition.

Under illumination (Fig. 1 *A*, *solid line*), both tyrosyl radicals are formed. $Z^\bullet$ decay is complete 4 min after illumination (Fig. 1 *A*, *dashed line*), because spin quantitation 2 min after illumination shows 0.9 spin per reaction center (Patzlaff and Barry, 1996). Spin quantitation gives a light-induced increase, attributed to $Z^\bullet$ formation, in 50% of the PSII reaction centers (Fig. 1 *A*, compare *solid* and *dotted lines*).

At −9°C, ~50% of the PSII reaction centers produce $Z^\bullet$ (Fig. 1 *A*). At room temperature, we have previously reported the production of $Z^\bullet$ in 60–90% of cyanobacterial PSII (for example, Noren and Barry, 1992; Boerner and Barry, 1993). Because the specific activity of oxygen evolution is high in the preparations employed here (2700 μmol O_2/mg chl-h), we attribute the decrease to the known temperature dependence for the formation of the $Z^\bullet$ species (Warden et al., 1976).

Because of the slow decay of tyrosyl radical $D^\bullet$, ~60% of the $D^\bullet$ EPR signal has decayed in 90 min (Fig. 1 *A*, compare *dotted* and *dashed lines*). The *g* value of the radicals is 2.004, and the linewidth is ~20 G (reviewed in Miller and Brudvig, 1991).

Cryogenic EPR spectroscopy was then used to examine potential contributions of Q_A^- under these conditions (Fig. 1 *B*). A semiquinone-iron EPR signal can be detected at $g = 1.9–1.82$ at 4 K in PSII samples (reviewed in Miller and Brudvig, 1991). As a positive control, we used a PSII sample that contained 3 moles potassium ferricyanide per mole PSII reaction center; these are conditions in which the maximum yield of Q_A^- should be trapped by a 4-min illumination and flash-freezing outside of the EPR cavity. Comparison of spectra recorded before (Fig. 1 *B*, *dotted line*) and after (Fig. 1 *B*, *solid line*) illumination at −9°C shows that a $Fe^{+2}Q_A^-$ EPR signal is generated in this cyanobacterial PSII preparation. However, under the conditions used for infrared spectroscopy, when samples contain an equimolar mixture of 3 mM potassium ferricyanide/potassium ferrocyanide, no detectable $Fe^{+2}Q_A^-$ signal is formed under the same illumination condition (Fig. 1 *B*, *dashed line*); partially dehydrated samples give consistent results. Furthermore, in the same spectrum, the $g = 2.0$ region shows that this illumination regime traps tyrosyl $Z^\bullet$ (data not shown). This experiment argues that Q_A^- is not stably reduced under the conditions employed for the infrared measurements described here. Additional experiments show that the length of dark adaptation did not change the amount of Q_A^- formed. It should be noted that Q_B is not functional in this preparation (data not shown).

To determine the relative yield and rise time of Q_A^- formed under the employed infrared conditions, we have also monitored the variable fluorescence yield from PSII. In the absence of fluorescence quenchers, such as P_{680}^+, the yield of variable fluorescence is linearly proportional to the amount of Q_A^- produced in cyanobacterial PSII (see discussion in Boerner et al., 1992). Whereas the positive control, identical to the above EPR control sample and partially dehydrated, shows a fluorescence yield increase under constant illumination consistent with reduction of Q_A (Fig. 1 *B*, *inset, trace 1*), no increase in fluorescence yield is observed in manganese-depleted, partially dehydrated PSII in the presence of potassium ferricyanide and potassium ferrocyanide (Fig. 1 *B*, *inset, trace 2*). Four minutes (data not shown)

and 3 s (*inset*) of illumination give the same results (Kim et al., 1998).

The results of EPR and fluorescence measurements allow us to conclude that there is no detectable Q_A^- formed in these PSII preparations under conditions employed for infrared spectroscopy; instead, the electron reduces potassium ferricyanide to potassium ferrocyanide.

Data acquisition scheme for FT-IR studies of tyrosine D and Z

Difference infrared spectra of tyrosine D and tyrosine Z can be acquired by a single series of data acquisitions on a single PSII sample by exploiting the clearly distinguishable decay rate of the radicals (Fig. 1 *C*). This method allows us to obtain 1) a difference spectrum associated solely with tyrosine Z oxidation and the accompanying changes in the surrounding protein matrix, and 2) a double-difference spectrum associated solely with tyrosine D oxidation and the accompanying changes in the surrounding protein matrix.

A light-minus-short dark spectrum, reflecting $Z^\bullet - Z$, is constructed with a 4-min dark adaptation between the light and dark spectrum. Unique vibrational modes of $Z^\bullet$ will make a positive contribution to the spectrum; unique modes of Z will make a negative contribution (Fig. 2 *B*). A light-minus-long dark difference spectrum is constructed with a 90-min dark adaptation between the light and dark spectra, which will reflect contributions from both $D^\bullet - D$ and $Z^\bullet - Z$ (Fig. 2 *A*). Construction of the double-difference spectrum from these data isolates contributions from tyrosine D and its dark stable radical (Fig. 2 *C*). Unique modes of $D^\bullet$ will make positive contributions to the double-difference spectrum; unique modes of D will make negative contributions.

The acceptor side will be reoxidized by a ferricyanide/ferrocyanide couple; spectra exhibit a differential feature at 2112 cm^{-1} (positive) and 2036 cm^{-1} (negative) consistent with such oxidation and reduction (Fig. 2). The intensity of the derivative feature at 2112/2036 cm^{-1} correlates approximately with the amount of charge separation in the PSII reaction center. For example, note that the intensities of the positive feature at 2112 cm^{-1}, arising from the ν(C≡N) stretching mode of ferricyanide, are equivalent in the light-minus-short dark ($Z^\bullet - Z$) and double-difference ($D^\bullet - D$) spectra (compare Fig. 2, *B* and *C*, respectively). EPR data show that, under the conditions and dark incubation times employed, tyrosine Z is redox-active in ~50% of PSII centers, and tyrosine D is redox-active in ~60% of the centers (Fig. 1 *A* and discussion above). However, the intensities of the negative line at 2036 cm^{-1}, arising from the ν(C≡N) stretching mode of potassium ferrocyanide, are not equivalent, showing a 35% decrease in the $D^\bullet - D$ spectrum, as compared to the $Z^\bullet - Z$ spectrum (compare Fig. 2, *B* and *C*). Because the standard error in our EPR spin quantitations is 10% (for example, see Noren and Barry, 1992; MacDonald and Barry, 1992; Boerner and Barry, 1993), this difference may be significant and may be due to an additional spectral contribution in this region. Such a spectral contribution could arise from a combination mode arising from the tyrosine radical 7*a* and 6*a* modes (Tripathi and Schuler, 1984), or the S-H stretch of a cysteine (Rath et al., 1994). These possibilities are under investigation. The data in Fig. 2 are obtained on a detergent-solubilized, monodisperse PSII preparation from cyanobacteria. In spectra obtained on plant PSII membranes, the sign of the derivative-shaped signal from ferricyanide and ferrocyanide can be reversed from the sign of this spectral feature in Fig. 2 (for example, Zhang et al., 1997). Such an inversion is possible because two different processes, oxidation at the donor side and reduction at the acceptor side, can give rise to each vibrational feature.

The difference and double-difference spectra are recorded as alternate spectral collections on the same sample.

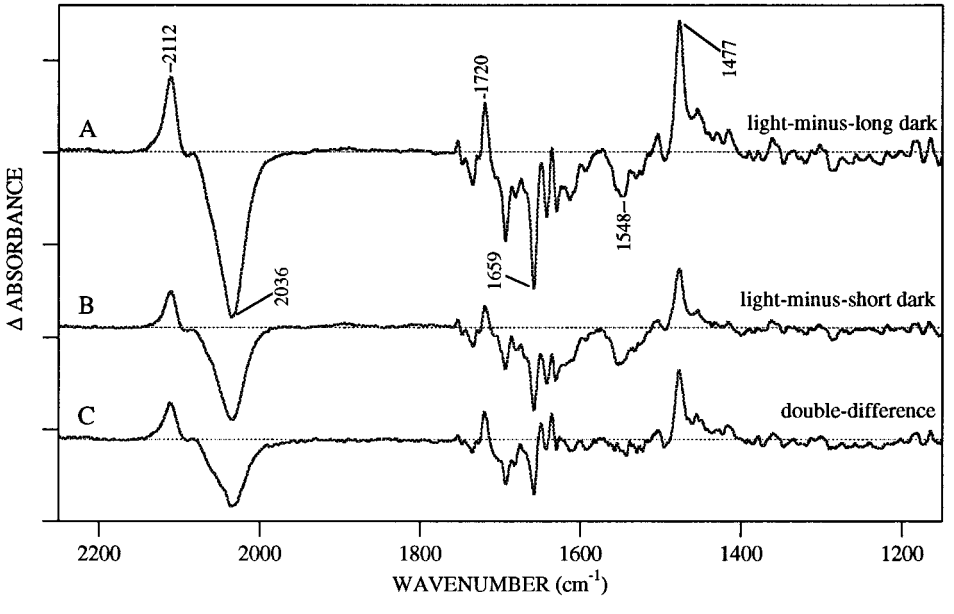

FIGURE 2 Difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations at pH 7.5. Shown are (*A*) a light-minus-long dark spectrum, reflecting $Z^\bullet D^\bullet - Z D$; (*B*) a light-minus-short dark spectrum, reflecting $Z^\bullet - Z$; and (*C*) a double-difference spectrum, reflecting $D^\bullet - D$. The tick marks on the *y* axis correspond to 2.0×10^{-4} absorbance units.

Difference ($Z^{\bullet} - Z$) and double-difference ($D^{\bullet} - D$) spectra are reproducible in intensity and frequency from sample to sample; multiple spectra are averaged to achieve a good signal-to-noise ratio. Examination of the difference FT-IR spectra from 2210–1150 cm^{-1} (Fig. 2, *solid lines*) indicates that the spectra have a high signal-to-noise ratio and a flat baseline (Fig. 2, *dotted lines*) throughout the 2200–1200 cm^{-1} spectral region.

Presentation of difference and double-difference spectra of $Z^{\bullet} - Z$ and $D^{\bullet} - D$

In Fig. 3 *A*, we present the $Z^{\bullet} - Z$ difference FT-IR spectrum, recorded at pH 7.5 on *Synechocystis* PSII. As a negative control, we present data recorded in the presence of hydroxylamine (Fig. 3 *B*), which prevents the oxidation of tyrosine Z (Ghanotakis and Babcock, 1983) and which, in our samples, generates a chlorophyll radical in a fraction of reaction centers (MacDonald et al., 1995). The presence of hydroxylamine changes the amplitude and frequencies of lines throughout the spectrum, consistent with a contribution from the oxidation of tyrosine Z to the control spectrum. Notice that the intensity of a line at 1478 cm^{-1} is decreased upon the addition of hydroxylamine. At pH 6.0, the remaining intensity at 1478 cm^{-1}, observed in the presence of hydroxylamine, has been shown to exhibit a 2 cm^{-1} downshift upon ^{15}N labeling. The frequency of the vibrational feature, the magnitude of the downshift, the extent of ^{15}N incorporation into chlorophyll, and the observation of an infrared-detectable amount of chlorophyll cation radical led to the assignment of this remaining intensity at 1478 cm^{-1} to a chlorophyll macrocycle vibration (MacDonald et al., 1995). Taken together, these results (MacDonald et al., 1995) and previous results obtained from tyrosine labeling (MacDonald et al., 1993) suggested that there are two overlapping contributions in this region: one from tyrosyl $Z^{\bullet}$ and one from chlorophyll (MacDonald et al., 1993, 1995; Bernard et al., 1995). The data presented here are consistent with these previous conclusions.

In Fig. 4 *A*, we present the $D^{\bullet} - D$ spectrum recorded on *Synechocystis* PSII at pH 7.5. As a negative control, we present data acquired under the same conditions on the YF160D2 mutant, in which a nonredox-active phenylalanine has been substituted for tyrosine D (Fig. 4 *B*). This mutant lacks the $D^{\bullet}$ EPR spectrum (Debus et al., 1988a; Vermaas et al., 1988), but exhibits a small contribution from a narrow, long-lived radical (Debus et al., 1988a), which may be a chlorophyll cation radical. Removal of redox-

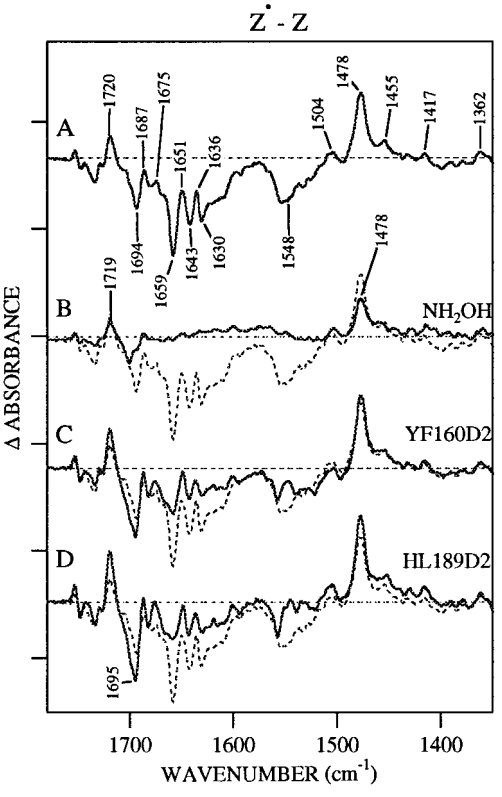

FIGURE 3 Difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations at pH 7.5, reflecting $Z^{\bullet} - Z$. The wild-type spectrum is shown in a solid line in *A* and is superimposed as a dashed line in *B–D*. Also shown are: (*B*) PSII with hydroxylamine; (*C*) YF160D2 PSII; and (*D*) HL189D2 PSII. The tick marks on the *y* axis correspond to 2.0×10^{-4} absorbance units.

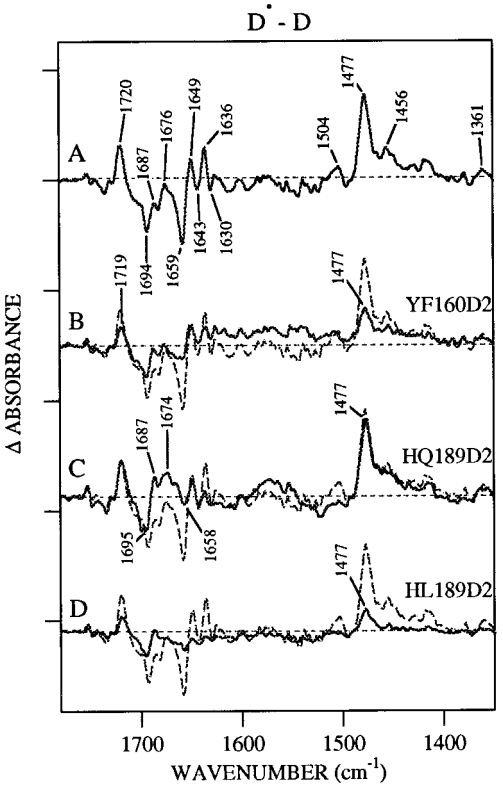

FIGURE 4 Double-difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations at pH 7.5, reflecting $D^{\bullet} - D$. The wild-type spectrum is shown in a solid line in *A* and is superimposed as a dashed line in *B–D*. Also shown are: (*B*) YF160D2 PSII; (*C*) HQ189D2 PSII; and (*D*) HL189D2 PSII. The tick marks on the *y* axis correspond to 2.0×10^{-4} absorbance units.

active tyrosine D changes frequencies and intensities throughout the $D^{\bullet} - D$ spectrum (Fig. 4 *B*) but, as expected, has less influence on the $Z^{\bullet} - Z$ spectrum (Fig. 3 *C*). This result supports the conclusion that our data acquisition method can distinguish the two tyrosines. Remaining intensity at 1477 and 1720 cm^{-1} is consistent with a small chlorophyll radical contribution to the YF160D2 $D^{\bullet} - D$ spectrum (MacDonald et al., 1995).

Previously, the production of a novel, light-induced EPR signal from a posttranslationally modified tyrosine has been observed in Tris-treated preparations from the YF160D2 mutant (Boerner et al., 1993; Boerner and Barry, 1994). However, this signal is not observed in hydroxylamine-treated preparations similar to the preparations employed here; the $Z^{\bullet}$ EPR signal is observed instead (Ma and Barry, 1996). Thus the small effects of the YF160D2 mutation on the $Z^{\bullet} - Z$ infrared spectrum (Fig. 3 *C*) are not due to the production of another amino acid radical. These small effects are reproducible and may be indicative of conformational interactions between the D and Z sites. These effects will be described in more detail in the Discussion.

$^{13}C(6)$ labeling of tyrosine

Experimental identification of tyrosyl radical and tyrosine contributions to these difference spectra requires the use of isotopic labeling. Accordingly, the effect of $^{13}C(6)$ labeling of tyrosine on the $Z^{\bullet} - Z$ spectrum (Fig. 5 *A*) and $D^{\bullet} - D$ spectrum (Fig. 5 *B*) was assessed. $^{13}C(6)$ labeling broadens the EPR spectra of both tyrosyl radicals (data not shown); tyrosine labeling does not result in isotopic incorporation into plastoquinone or chlorophyll (Barry and Babcock, 1987; Bernard et al., 1995; MacDonald et al., 1995).

First we examine the effect of $^{13}C(6)$-tyrosine labeling on positive vibrational features, which arise from unique vibrational modes of the radicals. In the 1780–1350 cm^{-1} region of the $Z^{\bullet} - Z$ spectrum, the most dramatic effect of ^{13}C labeling is the decrease in intensity of the vibrational feature at 1478 cm^{-1} (Fig. 5 *A*). A new positive mode appears at 1423 cm^{-1}. In the 1780–1350 cm^{-1} region of the $D^{\bullet} - D$ spectrum, ^{13}C labeling produces a parallel effect, in which a positive vibrational mode at 1477 cm^{-1} decreases in intensity and a new positive mode appears at 1423 cm^{-1} (Fig. 5 *B*). These results provide a definitive assignment of spectral features at 1478/1477 cm^{-1} to vibrational modes of $Z^{\bullet}$ and $D^{\bullet}$. We continue to favor assignment of the 1478/1477 cm^{-1} peak to the C-O (υ7a) vibrations of the radicals (MacDonald et al., 1993), but this assignment will be discussed in more detail in future work (Kim et al., 1998). This 1478/1477 cm^{-1} peak apparently downshifts to 1423 cm^{-1} and broadens in both the $Z^{\bullet} - Z$ and $D^{\bullet} - D$ difference spectra; the downshift of 50 cm^{-1} is in reasonable agreement with the $\Delta\upsilon$ of 40 cm^{-1} predicted for a harmonic, isolated C-O vibration and predicted by density functional calculations of isotope-induced shifts in tyrosyl radical vibrational modes (Qin and Wheeler, 1995a,b). However, the frequency of this vibrational mode at 1478/1477 cm^{-1} is ~20–30 cm^{-1} downshifted, compared to phenoxyl radicals in vitro (Tripathi and Schuler, 1984, 1987), tyrosyl radicals in vitro (Johnson et al., 1986), and the tyrosyl radical in ribonucleotide reductase (Backes et al., 1989).

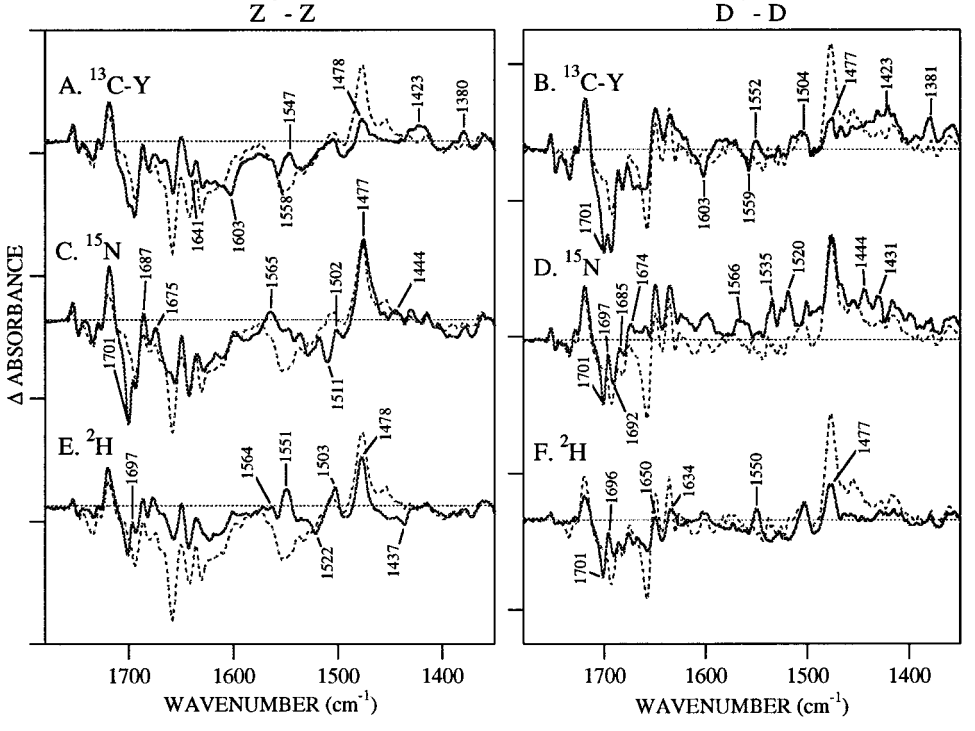

FIGURE 5 Difference and double-difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations at pH 7.5, corresponding to the oxidation of tyrosines Z (*left*) and D (*right*), respectively. The control $Z^{\bullet} - Z$ spectrum is shown as the dashed line in *A*, *C*, *E*; the control $D^{\bullet} - D$ spectrum is shown as the dashed line in *B*, *D*, *F*. (*A* and *B*) (*solid lines*) $^{13}C(6)$ tyrosine was incorporated into PSII. (*C* and *D*) (*solid lines*) ^{15}N was globally incorporated into PSII. (*E* and *F*) (*solid lines*) PSII was exchanged with $^{2}H_2O$ buffer. The tick marks on the *y* axis in the left and right panels correspond to 2.0×10^{-4} absorbance units.

In the $Z^{\bullet} - Z$ spectrum, ^{13}C labeling of cyanobacterial PSII also results in the appearance of new positive intensity at 1547 and 1380 cm^{-1} (Fig. 5 *A*); in the $D^{\bullet} - D$ spectrum, ^{13}C labeling results in the appearance of new intensity at 1552 and 1381 cm^{-1} (Fig. 5 *B*). Thus these downshifted vibrational features are also assignable to the tyrosyl radicals. However, the origin of these downshifted lines at 1547/1552 and 1380/1381 cm^{-1} is still under investigation. The 1380/1381 cm^{-1} feature may be a downshifted C-C stretching (υ19b) mode (but see Kim et al., 1998), whereas the feature at 1547 cm^{-1} may be the downshifted C-C stretch (υ8a) of the tyrosyl radical ring (Tripathi and Schuler, 1984, 1987; Johnson et al., 1986; Qin and Wheeler, 1995a,b). In the $D^{\bullet} - D$ spectrum (Fig. 5 *B*), but not as noticeably in the $Z^{\bullet} - Z$ spectrum (Fig. 5 *A*), isotopic labeling of the tyrosine alters spectral contributions at 1700 cm^{-1}; these changes are consistent with a downshift of a positive vibrational mode. Oxidation of phenol and tyrosine in vitro does not produce a species with infrared absorption in the 1700 cm^{-1} region (Tripathi and Schuler, 1984, 1987; Johnson et al., 1986). Thus this spectral feature may be a perturbation of the vibrational spectrum due to the influence or a unique conformation of the peptide backbone. Model compound studies are under way to investigate this phenomenon.

Next, we discuss the alterations in negative vibrational features upon $^{13}C(6)$-tyrosine labeling; downshifted negative lines arise from unique vibrational modes of the neutral tyrosine. For both tyrosine Z and D, $^{13}C(6)$-tyrosine labeling results in the appearance of new negative spectral features at 1603 cm^{-1} (Fig. 5, *A* and *B*). The origin of the downshifted 1603 cm^{-1} line appears to be the 1659 cm^{-1} vibrational feature, which decreases markedly in intensity upon ^{13}C labeling. This mode may arise from a ring C-C stretching mode of tyrosine, such as υ8a. The υ8a vibration is observed at 1609 and 1617 cm^{-1} for phenol (Tripathi and Schuler, 1984) and for tyrosine (Dollinger et al., 1986; Johnson et al., 1986), respectively, although it should be noted that, to our knowledge, there is no published normal coordinate analysis of tyrosine. Thus this set of assignments implies that the C-C stretching modes of tyrosines D and Z absorb at higher energy, when compared to the C-C stretching mode of tyrosine in vitro. In the 1600–1400 cm^{-1} spectral range, three additional ring vibrations of tyrosine should be infrared observable: υ8b, υ19a, and υ19b (Tripathi and Schuler, 1984; Dollinger et al., 1986; Johnson et al., 1986); however, the intensities of these lines may make them undetectable.

Previous work leads to the expectation that the difference infrared spectrum should contain the C-OH stretching (υ7a) mode of tyrosine between 1240 and 1260 cm^{-1} (Tripathi and Schuler, 1984, 1987). The exact frequency depends on the nature and strength of hydrogen bonding (Takeuchi et al., 1989). Upon $^{13}C(6)$-tyrosine labeling, we observe a downshifted 1229/1226 cm^{-1} vibrational feature in the $Z^{\bullet} - Z$ and $D^{\bullet} - D$ spectra of PSII, but the origin of this line has not yet been identified (data not shown).

Note that both the $Z^{\bullet} - Z$ (Fig. 5 *A*) and $D^{\bullet} - D$ (Fig. 5 *B*) spectra have residual intensity at 1478/1477 cm^{-1} upon isotopic labeling of the tyrosine ring. This is consistent with a small contribution in this region from another species. Based on our previous work, we propose tentatively that this other species arises from a chlorophyll molecule. Such a contribution might arise from either a small chlorophyll radical contribution to the spectrum or from a photooxidation-induced influence on the vibrational spectra of neighboring chlorophyll molecules. Previous work has shown that a 5% contamination from a chlorophyll radical, as determined by EPR spin quantitations on hydroxylamine-containing samples, is infrared detectable. Hydroxylamine blocks the oxidation of tyrosine Z and makes the narrow EPR signal from the chlorophyll radical easily detectable. This small amount of chlorophyll oxidation is difficult to detect via EPR spectroscopy in the samples employed in this study, because tyrosyl radical $Z^{\bullet}$ and any oxidized chlorophyll are both generated under illumination. Thus we cannot distinguish between these two possible explanations at the present time.

^{15}N labeling

Global ^{15}N labeling of every N-containing amino acid side chain or pigment distinguishes the interaction of the two redox-active tyrosines with their respective protein environments (Fig. 5, *C* and *D*). In principle, two types of ^{15}N shifts can be observed. First, isotope-induced shifts in tyrosine and tyrosyl radical vibrational modes that involve atomic displacements of the tyrosine amide nitrogen will be observed. Little is known about the nature of these potential nitrogen contributions at the present time. Therefore, we will make the conservative assumption, which is an overestimation of the effect, that all normal coordinates sensitive to $^{13}C(6)$ labeling of the tyrosine ring could also be sensitive to ^{15}N labeling at that amide position. A second type of ^{15}N isotope shift will be observed, if there are N-containing amino acid side chains or pigments that contribute to the difference spectra. ^{15}N sensitive lines in the $Z^{\bullet} - Z$ spectrum at pH 6.0 were previously observed in the 1660–1630 cm^{-1} region, in the amide II region, and at 1456 cm^{-1} (Bernard et al., 1995). Data presented here, obtained at pH 7.5, show that ^{15}N labeling results in changes in the amide II and in the 1700 cm^{-1} regions in spectra of D and Z oxidation (Fig. 5, *C* and *D*). Overall, the effect of ^{15}N labeling on the $Z^{\bullet} - Z$ spectrum is similar at pH 7.5 and 6.0, and the spectral changes observed are not as dramatic as the effects of ^{15}N labeling on the $D^{\bullet} - D$ spectrum.

We observe three major types of ^{15}N-induced effects in the $D^{\bullet} - D$ spectrum: first, 11–15 cm^{-1} downshifts in the 1540 to 1493 cm^{-1} region and new positive lines at 1444 and 1431 cm^{-1}; second, intensity changes at 1659 and 1535–1520 cm^{-1}; and third, small ($\sim$2 cm^{-1}) downshifts in the 1694–1670 cm^{-1} region. This experiment reveals functional and structural distinctions in the interaction of each

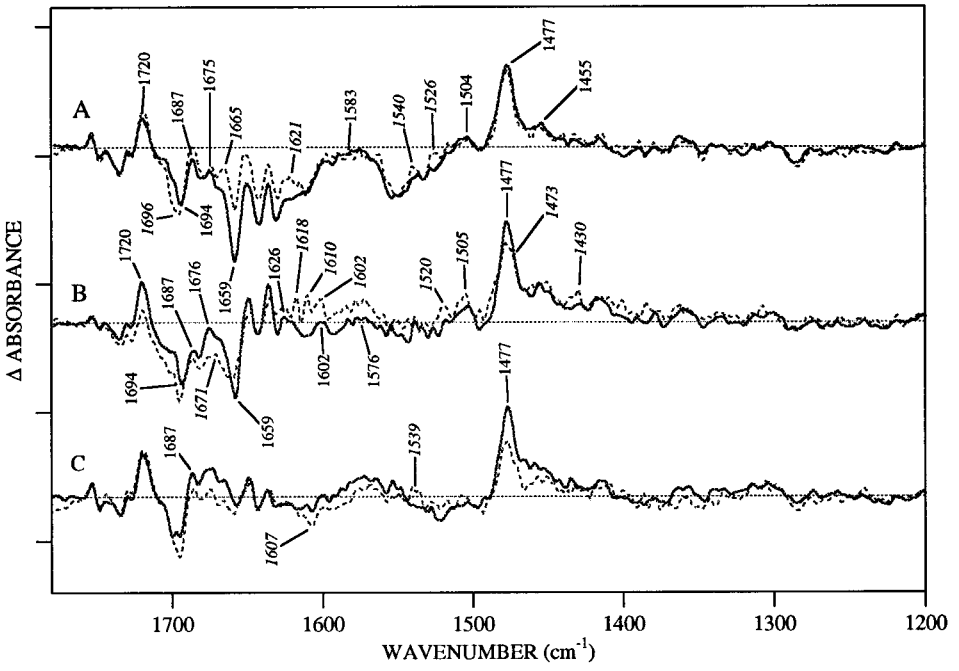

FIGURE 6 Difference and double-difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations, corresponding to the oxidation of tyrosines Z (*A*) and D (*B* and *C*), respectively. In *A*, the $Z^{\bullet} - Z$ spectrum of wild-type PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). In *B*, the $D^{\bullet} - D$ spectrum of wild-type PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). In *C*, the $D^{\bullet} - D$ spectrum of HQ189D2 PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). Frequencies in plain text correspond to features in the spectra taken from PSII samples at pH 7.5, whereas frequencies in *italic* text correspond to features in the spectra taken from PSII samples at pH 6.0. Tick marks on the *y* axis correspond to 2.0×10^{-4} absorbance units.

tyrosine with N-containing amino acid side chains and/or pigments in their environment.

^{2}H exchange

^{2}H exchange also distinguishes tyrosine D and Z (Fig. 5, *E* and *F*). ^{2}H exchange dramatically alters the $D^{\bullet} - D$ spectrum at pH 7.5, but has a much smaller effect on the $Z^{\bullet} - Z$ spectrum. As previously observed at p^{2}H 6.0 in the $Z^{\bullet} - Z$ spectrum (Bernard et al., 1995), ^{2}H exchange resulted in changes in the amide I region and a downshift of intensity from the amide II region (1550 cm^{-1}) to the 1450 cm^{-1} region. At p^{2}H 7.5, a similar set of ^{2}H-induced shifts is observed in the $Z^{\bullet} - Z$ spectrum (Fig. 5 *E*). Note the apparent small decrease in intensity of the 1478 cm^{-1} line upon exchange, which can be attributed to the downshift of a negative amide II band into this region (Bernard et al., 1995). This change was observed at p^{2}H 6.0 as well (Bernard et al., 1995). The positive 1477 cm^{-1} line of $D^{\bullet}$ is modified more than the analogous line of $Z^{\bullet}$ upon this treatment; this change does not seem correlated with a downshift of a more intense amide II band (Fig. 5 *F*). The $D^{\bullet} - D$ spectrum shows more noticeable effects between 1720–1660 cm^{-1} and at 1634 cm^{-1} upon ^{2}H exchange (Fig. 5 *F*). This experiment reveals functional and structural differences in the interaction of each tyrosine with titratable groups, such as amino acid residues, in the environment.

pH effects

Alterations in pH also distinguish the two sites (Figs. 6 and 7). When spectra of $Z^{\bullet} - Z$ are compared at pH 6.0 and 7.5, only small alterations in the spectra are observed (Fig. 6 *A*). Note that the derivative feature consisting of a 1522 (neg-

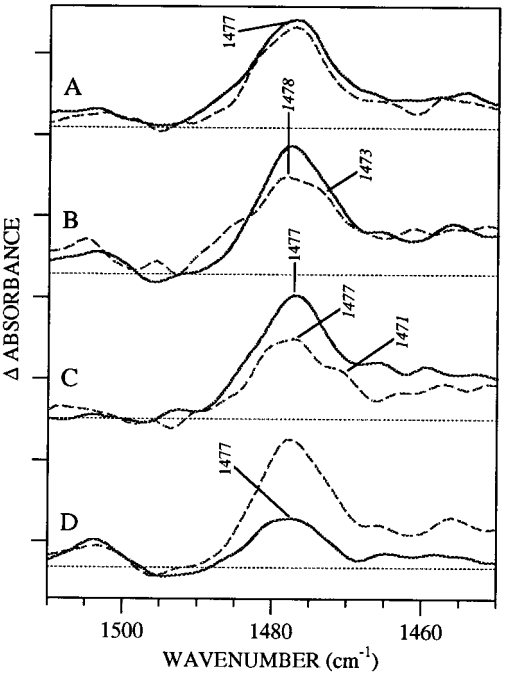

FIGURE 7 Expanded region of the difference and double-difference infrared spectra of manganese-depleted *Synechocystis* PSII preparations, corresponding to the oxidation of tyrosines Z (*A*) and D (*B–D*), respectively. Data in *A–C* are repeated from Fig. 6, *A–C*. Data in *D* are repeated from Fig. 5 *F*. In *A*, the $Z^{\bullet} - Z$ spectrum of wild-type PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). In *B*, the $D^{\bullet} - D$ spectrum of wild-type PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). In *C*, the $D^{\bullet} - D$ spectrum of HQ189D2 PSII is shown at pH 7.5 (*solid line*) and at pH 6.0 (*dashed line*). In *D*, the $D^{\bullet} - D$ spectrum in wild-type PSII (*dashed line*) is compared to ^{2}H exchanged PSII (*solid line*). Frequencies in plain text correspond to features in the spectra taken from PSII samples at pH 7.5, whereas frequencies in *italic* text correspond to features in the spectra taken from PSII samples at pH 6.0. Tick marks on the *y* axis correspond to 1.0×10^{-4} absorbance units.

ative)/1526 (positive) cm^{-1} line is more resolved at pH 6.0 (Fig. 6 *A*, *dotted line*), compared to the spectrum at pH 7.5 (Fig. 6 *A*, *solid line*). A pH shift from 6.0 to 7.5 has no appreciable effect on the C-O vibration of tyrosyl $Z^{\bullet}$ (Fig. 7 *A*). The same pH change upshifts a 1473 cm^{-1} component of the C-O vibration of tyrosyl radical $D^{\bullet}$ (Figs. 6 *B* and 7 *B*); the pH sensitivity of the positive 1473 cm^{-1} line suggests a conformational change, such as a change in hydrogen bonding, between pH 6.0 and 7.5 (MacDonald et al., 1993; Bernard et al., 1995). This change in hydrogen bonding could be due to a pH-induced conformational change in the reaction center.

A decrease in pH produces three other types of alterations in the $D^{\bullet} - D$ spectrum (Fig. 6 *B*): first, a change in the amplitude of a spectral feature at the 1671 cm^{-1} line; second, an increase in the amplitudes of positive features at 1618, 1610, 1520, 1505, and 1430 cm^{-1}; and third, a spectral modification between 1500 and 1485 cm^{-1} (see Fig. 7 *B*). Lines in these regions are also sensitive to ^{15}N labeling and ^{2}H exchange.

It was previously assumed that the 1477 cm^{-1} line observed in the double-difference spectrum of D at pH 6.0 arose from $Z^{\bullet}$ contributions. The $^{13}C(6)$-tyrosine labeling data presented here (Fig. 5 *B*) are consistent with the assignment of a line at 1477 cm^{-1} to $D^{\bullet}$ at pH 7.5 and imply that $D^{\bullet}$ has multiple C-O lines at pH 6.0, one of which is at 1477 cm^{-1}. This spectral heterogeneity may arise from the heterogeneity of interactions of $D^{\bullet}$ with its environment or from time averaging over several conformational states.

Identification of amino acids interacting with tyrosine D

Models of PSII, based on sequence similarity to the bacterial reaction center, suggest that a histidine interacts with each redox-active tyrosine (Svensson et al., 1990; Ruffle et al., 1992). Mutations at His^{190} in the D1 polypeptide do not affect the difference spectrum of tyrosine Z at pH 6.0 (Bernard et al., 1995). In contrast, data in Fig. 4 *C* show that mutation of the analogous histidine in the D2 polypeptide, His^{189}, to a glutamine (HQ189D2) alters the amplitudes and frequencies of lines in ^{15}N and ^{2}H exchange-sensitive regions of the spectrum at pH 7.5. Our EPR results on partially dehydrated HQ189D2 PSII give 1.0 $D^{\bullet}$ spin per reaction center after a 2-min dark adaptation (Kim et al., 1997), in agreement with previous results (Tang et al., 1993; Tommos et al., 1993; Un et al., 1996). These EPR results indicate that the yield of tyrosyl $D^{\bullet}$ in HQ189D2 PSII is not significantly altered upon mutation; our infrared data show that the infrared C-O vibration of tyrosyl $D^{\bullet}$ is not modified in frequency and intensity at pH 7.5 (Fig. 4 *C*).

Fig. 4 *D* shows that mutation of His^{189} of the D2 polypeptide to a leucine (HL189D2) has a dramatic effect on the $D^{\bullet} - D$ spectrum, producing a spectrum similar to that of the YF160D2 mutant (Fig. 4 *B*). Our EPR results on partially dehydrated HL189D2 PSII give 0.2 radical spin per reaction center after a 2-min dark adaptation (Kim et al., 1997), in agreement with previous characterizations (Tang et al., 1993; Tommos et al., 1993; Un et al., 1996). The chemical identity of this radical is not known. These EPR results indicate that the yield of tyrosyl $D^{\bullet}$ in HL189D2 PSII is significantly modified upon mutation. In agreement with this result, the intensity of the 1477 cm^{-1} feature is significantly smaller in the HL189D2 $D^{\bullet} - D$ spectrum (Fig. 4 *D*, *solid line*), compared to wild type. However, the spectrum of $Z^{\bullet} - Z$ is not affected as dramatically by the HL189D2 mutations, although changes in relative amplitude are observed in the amide I region (Fig. 3 *D*). The spectra shown in Figs. 3 *D* and 4 *D* underscore the point that the two redox-active tyrosines can be distinguished by our data collection protocol. We conclude that His^{189} of the D2 polypeptide is involved in a reversible structural change, which is linked to the oxidation of tyrosine D. In contrast, there is no evidence for such a reversible structural change at His^{190} of D1 when tyrosine Z is oxidized (Bernard et al., 1995).

Analysis of the 1480–1470 cm^{-1} spectral region in wild-type and mutant PSII

$D^{\bullet}$ is known from magnetic resonance studies to be a hydrogen-bonded radical (Rodriguez et al., 1987; Evelo et al., 1989; Tang et al., 1993; Force et al., 1995; Un et al., 1996). Our previous infrared work showed that the 1473 cm^{-1} line of $D^{\bullet}$ downshifted to 1460 cm^{-1} upon ^{2}H exchange. This result was consistent with the conclusion that the 1473 cm^{-1} line of $D^{\bullet}$ arises from a hydrogen-bonded radical (Bernard et al., 1995) and is in agreement with the results of density-functional calculations (see Qin and Wheeler, 1995a,b, and references therein). However, ^{2}H exchange experiments at pH 6.0, on the same exchanged sample, were consistent with the 1478 cm^{-1} line of tyrosyl $Z^{\bullet}$ arising from a non-hydrogen-bonded radical, because no analogous downshift of the 1478 cm^{-1} line was observed. An observed small cancellation of intensity at 1478 cm^{-1} was attributed to the ^{2}H-induced downshift of the amide II band. This conclusion was supported by ^{2}H exchange experiments on ^{15}N-labeled samples (Bernard et al., 1995).

The ^{2}H exchange results at pH 7.5 on $Z^{\bullet}$, which are presented here (Fig. 5 *E*), are in agreement with these earlier observations, because only small changes in amplitude are observed in the 1478 cm^{-1} line of $Z^{\bullet}$ upon ^{2}H exchange, and because no downshifted lines are observed. The small decrease in intensity is consistent with the expected downshift of the amide II band into this spectral region (Bernard et al., 1995). Thus our infrared results are inconsistent with a hydrogen bond to tyrosine $Z^{\bullet}$ in wild-type PSII. It should be noted that magnetic resonance studies of $Z^{\bullet}$ indicate that $Z^{\bullet}$ is involved in a disordered hydrogen bond in freeze-trapped samples of mutant YF160D2 PSII (Force et al., 1995; Un et al., 1996). This difference between the results of our FT-IR experiments and the magnetic resonance studies may be due

to the use of the YF160D2 mutant instead of wild-type PSII in the previous studies, to the difference in sample conditions and trapping procedures employed, or to the different time scales of the vibrational and magnetic resonance techniques.

Magnetic resonance techniques have obtained evidence for a hydrogen bond between His^{189} of the D2 polypeptide and $D^{\bullet}$ (Tang et al., 1993; Tommos et al., 1993; Force et al., 1995; Un et al., 1996; Campbell et al., 1997). At pH 6.0, mutation of His^{189} in the D2 polypeptide to glutamine (HQ189D2) eliminates the C-O mode at 1473 cm^{-1} (Figs. 6 *C* and 7 *C*), assigned to hydrogen-bonded $D^{\bullet}$ (Bernard et al., 1995). This result is consistent with previous magnetic resonance results. Note that there is new intensity at 1471 cm^{-1} in HQ189D2 PSII; the origin of this band must be determined by isotopic labeling.

At pH 7.5, the hydrogen bonding status of tyrosyl $D^{\bullet}$ is ambiguous. On the one hand, ^2H exchange results in a substantial reduction of amplitude of the 1477 cm^{-1} line (Fig. 7 *D*), which does not seem correlated with an intense vibrational feature at 1550 cm^{-1} in unexchanged samples (Fig. 5 *F*). Thus it seems unlikely that the entire reduction of amplitude is due to a downshifted amide II band. On the other hand, there is no obvious appearance of a broad, downshifted spectral feature upon exchange, as observed at pH 6.0 (Bernard et al., 1995). Furthermore, the HQ189D2 mutation does not dramatically affect the 1477 cm^{-1} vibration of $D^{\bullet}$ in intensity or frequency at pH 7.5 (Figs. 6 *C* and 7 *C*), although mutation of histidine to glutamine alters other regions of the spectrum (Fig. 4 *C*). This result is under investigation. Note that if $D^{\bullet}$ is indeed hydrogen bonded at pH 7.5, as well as at pH 6.0, our data suggest that the C-O frequency is not linearly related to hydrogen bond strength when the two tyrosines are compared. Such a lack of correlation may be due to differences in polarity or in electric fields at the two tyrosines.

DISCUSSION

We have presented the vibrational spectra associated with the oxidation of tyrosine D and of Z at pH 7.5. $^{13}C(6)$ labeling of tyrosine identifies spectral contributions from the tyrosine and tyrosyl radical in the $Z^{\bullet} - Z$ and $D^{\bullet} - D$ spectra. In these spectra, a positive line at 1478/1477 cm^{-1} and a negative line at 1659 cm^{-1} are observed to downshift to 1423 and 1603 cm^{-1}, respectively. We have tentatively assigned the 1478/1477 cm^{-1} feature to the vibrational $\upsilon 7a$ mode of $Z^{\bullet}$ and $D^{\bullet}$ and the 1659 cm^{-1} feature to the vibrational $\upsilon 8a$ mode of Z and D; analysis of a larger set of isotopically labeled samples is in progress (Kim et al., 1998; Kim and Barry, unpublished results)

This assignment of the positive 1478 cm^{-1} feature to the C-O stretching vibration of the tyrosyl radicals can be discussed with regard to data presented from previous model compound studies. The C-O stretching mode of tyrosyl radicals has been assigned to a 1510 cm^{-1} peak (Johnson et al., 1986). The corresponding line in phenoxyl radicals has been assigned to a 1505 cm^{-1} spectral feature (Tripathi and Schuler, 1984). In ribonucleotide reductase, a 1498 cm^{-1} feature has been assigned to the C-O stretching mode of a non-hydrogen-bonded tyrosyl radical (Backes et al., 1989). Note, then, that the lines, assigned to tyrosyl radical and tyrosine in PSII, are shifted from the frequencies observed for phenoxyl and tyrosyl model compounds in vitro (for example, see Tripathi and Schuler, 1984, 1987; Dollinger et al., 1986; Johnson et al., 1986). A 20 cm^{-1} downshift of the C-O frequency is also observed when the tyrosyl radicals in PSII are compared to the tyrosyl radical in ribonucleotide reductase. Such frequency shifts are typical for polarizable molecules in proteins (for example, see Barry and Mathies, 1987; Braiman and Rothschild, 1988). The frequency of the C-O stretching mode can be affected by a variety of factors, such as electric field strength, polarity, and hydrogen bonding. For example, the hydrophobicity of the membrane protein environment may be responsible for the upshifts in vibrational modes of the tyrosine. Under illumination, electric field effects could play an important role in determining vibrational frequencies. Such effects are known to shift pigment absorption spectra (Tiede et al., 1996). For example, a 20 cm^{-1} downshift would correspond to only a ~2-mV potential change at the redox-active tyrosyl radical. This potential change is small in magnitude and could occur in the light-induced state of PSII. In addition to frequency shifts, there may be accompanying alterations in the normal modes of vibration.

The data acquisition strategy employed in this study has been used previously (MacDonald et al., 1993). In our previous work, one vibrational feature from tyrosine $Z^{\bullet}$ (1478 cm^{-1}) and one feature from tyrosine $D^{\bullet}$ (1473 cm^{-1}) were identified at pH 6.0 by isotopic labeling. Two modes of tyrosine Z (1657, 1522 cm^{-1}) and one mode of tyrosine D (1534 cm^{-1}) were assigned as well. The spectral results presented here are consistent with the isotope-induced changes observed at pH 6.0 previously (MacDonald et al., 1993), because the 1522 cm^{-1} peak of $Z^{\bullet}$ and the 1473 cm^{-1} peak of $D^{\bullet}$ are not observed at pH 7.5 (see Fig. 5), and because an isotope-induced alteration is observed at 1534 cm^{-1} in the $D^{\bullet} - D$ spectrum. However, the spectra presented here are improved in signal-to-noise ratio, when compared to our previous work, and the 10% PSI contamination, present in the control and isotopically labeled samples previously employed, has been decreased. These improvements allow the identification of more lines of the tyrosyl radicals and the neutral tyrosines and allow the assignment of downshifted lines.

As reviewed recently (Angerhofer and Bittl, 1996), our results are not in agreement with the assignment of a spectral feature at 1478 cm^{-1} to the C-O vibration of Q_A^- (Hienerwadel et al., 1996; Zhang et al., 1997). The assignment of the 1478 cm^{-1} line to the C-O vibration of the semiquinone anion radical alone (Hienerwadel et al., 1996, 1997; Zhang et al., 1997) is premature because of several factors: the use of different illumination conditions, i.e.,

light sources, intensities, and repetition rates for some EPR control experiments and for IR spectroscopy (Hienerwadel et al., 1996; Zhang et al., 1997), the use of high concentrations of phosphate and formate in samples, which increase the difficulty of observing the vibrational features associated with tyrosine D oxidation (Hienerwadel et al., 1996, 1997), incomplete quantitation of chlorophyll radical production (Hienerwadel et al., 1996), and failure to carry out isotopic labeling experiments to identify quinone vibrational modes (Hienerwadel et al., 1996; Zhang et al., 1997). These points will be discussed in more detail in a future publication (Kim et al., 1998).

Our data support the hypothesis that D and Z differ in their interaction with the environment. This conclusion is based on the effects of pH, ^{15}N global labeling, and ^{2}H exchange on these spectra. ^{15}N labeling indicates that the oxidation of tyrosine D has a greater effect on N-containing amino acid side chains or pigments, when compared to oxidation of tyrosine Z. Deuterium exchange shows that there are more exchangeable groups influenced by tyrosine D oxidation. Finally, tyrosine D and its protein environment, but not tyrosine Z, show titration effects between pH 6.0 and 7.5.

The above results suggest a contribution from histidine(s) and/or other N-containing amino acid side chain to the $D^{\bullet} - D$ difference spectrum, which is not observed in the $Z^{\bullet} - Z$ spectrum. We have also shown that mutations at a histidine predicted to be in the environment of tyrosine D and known to be hydrogen bonded to the radical (Tang et al., 1993; Tommos et al., 1993; Force et al., 1995; Un et al., 1996; Campbell et al., 1997) dramatically modify the $D^{\bullet} - D$ spectrum. Because this difference FT-IR method detects alterations in the environment of the tyrosine that are coupled to its oxidation, these data imply that His^{189} undergoes a reversible structural change upon oxidation of D. Data from chemical complementation studies show that the structural change involving His^{189} is a reversible protonation (Kim et al., 1997). Note that we have no information about hydrogen bonding of His^{189} to the neutral form of tyrosine D, because the assignment of the C-OH mode of tyrosine D is still in progress.

In addition to their large effects on the spectra of $D^{\bullet} - D$, mutations at His^{189} and Tyr^{160} in the D2 polypeptide also have a small effect on the $Z^{\bullet} - Z$ spectrum (Fig. 3, *C* and *D*). Most of the $Z^{\bullet} - Z$ spectrum is unaffected in intensity and frequency by such mutations, but alterations, corresponding to a small percentage of the peptide backbone, are observed in the amide I region. In particular, site-directed mutagenesis in PSII, which alters the biochemical properties of one of the redox-active tyrosines (tyrosine D), appears to affect the structural environment of the second redox-active tyrosine. Our observations imply that there are structural interactions between the two tyrosine residues. The possibility of long-range conformational changes upon site-directed mutation must always be taken into account. We have proposed such conformational changes previously, to explain the loss of PSII subunits and observation of novel oxidized species in the YF160D2 and YF161D1 mutants (Noren and Barry, 1992; Boerner et al., 1993). Furthermore, protein conformational changes, induced by mutation at a redox-active amino acid, have been observed in other systems. In cytochrome *c* peroxidase, 2-aminothiazole, an artificial substrate, can bind in a cavity introduced by a W191G mutation; the reaction between 2-aminothiazole and peroxide-oxidized W191G results in covalent modification of Y236 in the enzyme (Musah and Goodin, 1997). This covalent modification is only observed in site-directed mutants of W191 (Musah and Goodin, 1997). Furthermore, binding of protonated benzimidazole in this cavity mutant results in the generation of a large solvent-accessible channel via a surface loop rearrangement (Fitzgerald et al., 1996). Thus mutation of a single aromatic residue in a large complex can lead to structural changes at distant sites.

Two histidines, His^{189} in D2 and His^{190} in D1, are predicted to be in the environment of tyrosine D and Z, respectively (Svensson et al., 1990; Ruffle et al., 1992). Glutamine substitutions at the two sites have different effects on the infrared spectra, reflecting oxidation of each tyrosine. Observed effects on the tyrosine D spectrum are consistent with reversible, linked proton and electron transfer (Kim et al., 1997), whereas observed effects on the tyrosine Z spectrum are not consistent with such a simple interaction between tyrosine Z and His^{190} of D1. These results suggest that these histidines play different roles in proton transfer reactions in PSII. A possible role of His^{190} of D1 in proton transfer away from the tyrosine Z site (Hoganson and Babcock, 1997) is under investigation.

In addition, our vibrational difference spectra of D and Z provide evidence for alterations in the structures of the two tyrosines themselves. Magnetic resonance techniques have also provided evidence for such structural differences (Boerner and Barry, 1993; Force et al., 1995; Tommos et al., 1995; Tang et al., 1996; Un et al., 1996). In our work, ^{2}H effects on the 1477 cm^{-1} region and ^{13}C isotope-induced changes in the 1700 and 1550 cm^{-1} regions distinguish the two radicals. These structural alterations will be discussed further when a complete set of isotopomers has been studied. A detailed characterization of tyrosyl and tyrosine vibrational modes in vitro is required to make further assignments of peaks in these difference FT-IR spectra of PSII. These model compound studies are in progress.

Our results are significant because the observed difference in interaction with amino acids in the environment may be critical in determining the function of the two redox-active sites. If the equilibrium for protonation is linked to the oxidation/reduction reaction, then a difference in the identity and pK_a of protonated groups could have a significant impact on the midpoint potential and electron transfer properties of the redox-active tyrosine.

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