Difference FT-IR Study of a Novel Biochemical Preparation of Photosystem II†

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Received March 26, 1992; Revised Manuscript Received August 4, 1992

ABSTRACT: There are two redox-active tyrosines in photosystem II, the water-splitting complex, that form neutral tyrosine radicals. One of these tyrosine radicals, D•, is stable and has an unknown function. The other redox-active tyrosine, Z, acts to transfer oxidizing equivalents from the primary chlorophyll donor of photosystem II to the manganese cluster, where water oxidation occurs. In an attempt to obtain more information about Z and its interaction with its environment, we have begun a study using Fourier-transform infrared (FT-IR) vibrational spectroscopy. To facilitate these studies, we have developed a procedure to isolate spinach photosystem II complexes with an antenna size of approximately 100–110 chlorophylls per reaction center. These complexes show an approximately 2-fold increase in the specific activity of oxygen evolution over the activity of the starting material, photosystem II membranes. Although fully solubilized in detergent, these complexes retain the 24- and 18-kDa extrinsic proteins and exhibit no calcium chloride requirement for optimal oxygen evolution. In manganese-depleted photosystem II samples, Z• can be accumulated in the light. In the dark, the tyrosine radical is reduced and reprotonated to form the neutral tyrosine. Since this process is reversible and light-dependent, we have used light-minus-dark difference FT-IR spectroscopy to observe the vibrational difference spectrum that is associated with the oxidation of this residue. As a control, EPR spectra were measured under identical conditions to assess the amount of Z• that accumulated in the light. We also hope to use difference FT-IR to identify the amino acid with which Z may form a hydrogen bond. As a first step, we have obtained the difference FT-IR spectrum associated with protonation of the imidazole ring of histidine in vitro. This spectrum allows us to assess the contribution that such a protonation would have on the in vivo difference spectrum.

Photosystems I and II are two chlorophyll-containing reaction centers that are found in the chloroplast thylakoid membrane; these reaction centers participate in the electron transfer events of oxygenic photosynthesis. PSII[1] catalyzes the light-induced oxidation of water and reduction of bound quinone. The oxidizing equivalents obtained from one-electron photochemistry in the reaction center are stored at a manganese metal cluster; oxygen is generated through the four-electron oxidation of water at this catalytic site. Oxygen-evolving PSII complexes are composed of both integral membrane proteins and one or more extrinsic polypeptides. The integral membrane proteins, D_1 and D_2, are thought to form the heterodimer core of the reaction center and to bind most of the prosthetic groups that are involved in electron transfer. The 47- and 43-kDa membrane proteins function as light-harvesting proteins, while the 33-kDa extrinsic protein stabilizes the manganese catalytic site. Two other extrinsic proteins of 18 and 24 kDa are also present in the complex as isolated from the plant chloroplast. When these proteins are moved from the reaction center, for example by NaCl washing, complexes are active in oxygen evolution only in the presence of millimolar concentrations of calcium and chloride [for reviews, see Babcock (1987), Yocum (1991), and Debus (1992)].

The prosthetic groups that are involved in electron transfer include the primary chlorophyll donor, P_{680}, and the electron acceptors, pheophytin and plastoquinone. After light absorption, P_{680}* transfers an electron to the primary acceptor, a bound pheophytin molecule. The reduced pheophytin in turn transfers an electron to a bound plastoquinone, which functions as the single electron acceptor, Q_A. Q_A^- reduces a second plastoquinone, Q_B, which acts as a two-electron gate [for reviews see Babcock (1987) and Debus (1992)]. The chlorophyll cation radical, P_{680}^+, oxidizes a tyrosine residue, Z, which in turn is reduced by the manganese catalytic site (Boska et al., 1983; Gerken et al., 1988). The oxidized form of Z can be identified by its characteristic EPR signal (Babcock & Sauer, 1975; Blankenship et al., 1975; Hoganson & Babcock, 1988). The *g* value of this signal has been used to argue that the oxidation of the phenol ring is accompanied by deprotonation of the phenol OH group, giving rise to the neutral tyrosine radical, Z• (Barry & Babcock, 1987). If the manganese cluster is removed, a high concentration of Z•, up to one spin per reaction center, can be accumulated in the light (Babcock & Sauer, 1975). Under these conditions, the EPR spectrum of oxidized form of Z is easily obtained. In the dark, Z• is reduced by either exogenous or endogenous reductants and reprotonated, and its EPR signal is not observed.

Z• was suggested to be a tyrosine radical by comparison of its EPR line shape to that of another redox-active tyrosine in PSII (Barry & Babcock, 1987). This tyrosine, known as D, gives rise to a stable radical of unknown function (Debus et al., 1988a; Vermaas et al., 1988). Unlike the EPR spectrum of Z•, the EPR spectrum of D• is observable in the dark for several hours after illumination (Babcock & Sauer, 1973). D•, like Z•, is believed to be a neutral radical (Barry & Babcock, 1987). The identity of the stable radical, D•, was established by a combination of isotopic labeling and EPR spectroscopy (Barry & Babcock, 1987). Because Z• has a similar EPR line shape to that of D•, it was proposed that Z•

† Supported by NIH Grant GM 43272 and NIH Molecular Biophysics Training Program GM 08277 (to G.M.M.). Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the U. M. McKnight-Land Grant program for partial support of this research.

[1] Abbreviations: chl, chlorophyll; DCBQ, 2,6-dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; FT-IR, Fourier-transform infrared spectroscopy; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PSII, photosystem II; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/92/0431-9848$03.00/0

is also a tyrosine radical (Barry & Babcock, 1987). Further experimental support for this idea comes from time-resolved UV absorption studies of PSII (Gerken et al., 1988). Site-directed mutagenesis suggests that D is tyrosine-160 of the D_2 polypeptide and that Z is tyrosine-161 of the D_1 polypeptide (Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989; Noren & Barry, 1992).

ENDOR and spin-echo EPR studies of $D^{\bullet}$ have shown that $D^{\bullet}$ is a hydrogen-bonded radical (Rodriguez et al., 1987; Evelo et al., 1989a,b). $D^{\bullet}$ is also thought to be sequestered from water, since the hydrogen-bonded proton exchanges slowly (Rodriguez et al., 1987). Therefore, it has been suggested that D is hydrogen-bonded to a nearby amino acid residue that is reversibly protonated and deprotonated when the redox state of the tyrosine is altered (Babcock et al., 1989). Further, it has been proposed that this nearby base may be a histidine (Babcock et al., 1989; Svensson et al., 1990, 1991). A similar interaction has been proposed for $Z^{\bullet}$ (Eckert & Renger, 1988; Svensson et al., 1990, 1991), although there is less experimental information concerning the hydrogen-bonding characteristics of this radical.

Because Z is an electron carrier between the catalytic site and the primary donor, it plays an important role in electron transfer in PSII. An increasing number of enzymes have now been found to contain redox-active tyrosine residues [for a review, see Stubbe (1989)]. Details of the structure of these redox-active species, including their spin density distribution, and of their interaction with the protein environment are of interest in understanding their functional differences. For example, hydrogen-bonding interactions between the phenol OH group and nearby residues could play an important role in control of the redox potential of a tyrosine residue. Oxidation of the phenol ring dramatically decreases the pK_a of this group (Dixon & Murphy, 1976). Therefore, thermodynamic considerations suggest that interactions that shift the pK_a of the phenol OH group will, in turn, affect the oxidation potential of the tyrosine. Since the rate of electron transfer is sensitive to redox potential, such an interaction provides a mechanism for control of electron transfer rate [for example, see Marcus and Sutin (1985) and McLendon and Miller (1985)].

Difference FT-IR has the potential to provide detailed structural information about tyrosine Z and about its interactions with its protein environment. This technique has already proven to be useful in elucidating structure/function relationships in other membrane proteins [for a review, see Braiman and Rothschild (1988)]. The vibrational spectrum is extremely sensitive to changes in geometry and environment, and the high signal-to-noise ratio obtainable in FT-IR means that difference techniques can be used to detect very small changes in the vibrational spectrum. For example, FT-IR has been used extensively in the study of the mechanism of proton transport in bacteriorhodopsin, where changes in the spectrum caused by protonation of a single amino acid residue are observable [for a review, see Braiman and Rothschild (1988)]. FT-IR difference studies of photosynthetic reaction centers from purple, non-sulfur bacteria have also been reported [for a review, see Mantele (1992)]. However, there are relatively few difference infrared studies of light-induced electron transfer mechanism in PSII, perhaps because of the structural complexity of this large protein complex (Tavitian et al., 1986; Nabedryk, 1990a; Berthomieu et al., 1990a,b).

We describe here our use of FT-IR difference (light-minus-dark) spectroscopy to obtain the first vibrational difference spectrum that reflects contributions from the oxidation of tyrosine Z. The difference spectrum was generated by subtracting a vibrational spectrum recorded in the dark from a vibrational spectrum recorded in the light; these data were obtained through the use of a manganese-depleted PSII preparation where $Z^{\bullet}$ can be photoaccumulated. EPR spectroscopy was used as a control in order to assess the contribution of $Z^{\bullet}$ to the FT-IR spectrum.

In addition to yielding structural information about the redox-active tyrosine, these FT-IR difference spectra will also contain contributions from any other moiety that undergoes a change in geometry, protonation state, or environment when tyrosine Z is oxidized. Such alterations are likely to be important in facilitation of electron transfer. For example, if the imidazole ring of a histidine residue is reversibly protonated and deprotonated upon alteration of the redox state of the tyrosine, the vibrational difference spectrum of the histidine may also contribute to the difference spectrum. In order to assess the contribution that protonation of an imidazole ring would make to a difference infrared spectrum, we have obtained the infrared spectrum of histidine in aqueous solution at pH 5.3 and 6.9.

To facilitate these FT-IR studies on PSII, we have developed a novel method to purify PSII complexes from spinach. Our starting material is the preparation of PSII membranes originally described by Berthold et al. (1981). In the purification procedure that we will describe, these membranes are completely solubilized in nonionic detergent, and non-photochemically active chlorophyll-containing antenna proteins are separated from functional PSII complexes in a simple one-step chromatographic purification. The resulting complex exhibits an increase in specific activity for oxygen evolution of approximately a factor of 1.8 and a decrease in antenna size of a factor of 2.6, when compared to the starting material. Interestingly, the oxygen-evolving activity of these PSII complexes exhibits no requirement for calcium chloride, and we will show that these complexes have retained the 24- and 18-kDa extrinsic proteins. In addition to facilitating difference FT-IR studies of PSII, this preparation should be valuable in biophysical investigations of the role of calcium and chloride in the water-oxidizing complex.

MATERIALS AND METHODS

Purification of Photosystem II Complexes. PSII membranes from spinach were prepared by the method described by Berthold et al. (1981). These membranes were resuspended in solubilization buffer containing 50 mM MES–NaOH (pH 6.0), 15 mM NaCl, 25% (w/v) glycerol, and 1% (w/v) lauryl maltoside (Anatrace, Maumee, OH). The final chlorophyll concentration was 1 mg/mL. The mixture was incubated with gentle mixing in the dark for 20 min at 0 °C and then centrifuged at 120000*g* for 30 min to pellet any unsolubilized material.

The solubilized protein was filtered through a Gelman (0.45 μm) Acrodisk filter. A Pharmacia HR5/5 Mono Q column was washed with 6 mL of low-salt buffer containing 25% glycerol, 50 mM MES–NaOH (pH 6.0), 7.5 mM $CaCl_2$, and 0.05% (w/v) lauryl maltoside. Approximately 2 mg of chlorophyll was applied to the washed column, and an 11-mL linear salt gradient, up to 360 mM NaCl, was used for protein elution. The flow rate was 0.5 mL/min, and 0.5-mL fractions were collected. The column profile was monitored as described by Noren et al. (1991) and showed two well-resolved peaks. The first peak, containing light-harvesting proteins, eluted at 140 mM NaCl, and the second peak, containing PSII complexes, eluted at 260 mM NaCl. Peak 2 fractions were

assayed for specific activity, and fractions with rates of oxygen evolution greater than 1000 μmol of O_2 (mg of chlorophyll)$^{-1}$ h^{-1} were pooled. Samples were then frozen at −80 °C. The column profile was reproducible; therefore, fractions from different columns, run on the same day, could be pooled for large-scale purifications. Between purifications, the column was washed with 4 mL of 1 M NaCl (in the buffer described above) and then reequilibrated with 6 mL of low-salt buffer.

For experiments on salt-washed reaction centers, PSII complexes were resuspended in 2 M NaCl (in low-salt buffer) for 30 min at 0 °C at a chlorophyll concentration of approximately 0.25 mg/mL (Kuwabara & Murata, 1983), precipitated by addition of an equal volume of 30% PEG buffer (30% PEG-8000, 20 mM MES–NaOH, pH 6.0, and 20 mM $CaCl_2$), and then pelleted by centrifugation for 30 min at 19 000 rpm in a SS-34 rotor. The precipitated complexes were resuspended in low salt buffer and then stored at −80 °C.

For experiments on manganese-depleted reaction centers, protein samples were incubated in the presence of 10 mM NH_2OH at 0 °C for 1.5 h in darkness with gentle mixing (Yocum et al., 1981). The chlorophyll concentration was approximately 0.4 mg/mL. The 50 mM NH_2OH stock solution was made up in low-salt buffer, and the pH was adjusted to pH 6.0 with NaOH. This stock solution was made up shortly before use. After hydroxylamine treatment, the sample was diluted 1:1 (v/v) with the low-salt buffer that is described above. Hydroxylamine was removed, and the protein was concentrated by the method described by Peiffer et al. (1990). The concentrated sample was then dialyzed against a buffer containing 5 mM MES–NaOH (pH 6.0).

EPR Spectroscopy. Room-temperature EPR spectra were recorded at X-band on a Varian E-4 spectrometer equipped with a Varian TE cavity. The spectrometer was interfaced to a Macintosh IIcx computer via a digital voltmeter (Keithley, Model 195A, Cleveland, OH) and a Mac488A bus controller (Iotech, Cleveland, OH). The data acquisition program was a gift from Dr. John Golbeck (University of Nebraska). Data analysis, including double integration, was performed on the Macintosh computer using the graphics software program Igor (Wavemetrics, Lake Oswego, OR). Fremy's salt was used as a standard for spin quantitation of PSII radicals (Babcock et al., 1983; Wertz & Bolton, 1986).

Low-temperature EPR spectra were recorded on the instrument that is described above, except that it was equipped with a dewar insert for the TE cavity. Cavity temperature was regulated at −9 °C by the flow of cold nitrogen. The temperature was monitored continuously by a chromel constantan thermocouple inserted below the cavity (Cole-Parmer, Chicago, IL). The temperature in the cavity itself was also measured before and after data acquisition and was found to vary a maximum of ±2 °C over the specified interval. The appearance of the EPR spectrum and PSII radical content were not significantly affected by this amount of temperature variation. EPR samples, 87 μg of chlorophyll, were prepared by drying protein samples on mylar sheets for 30 min with a stream of dry nitrogen of defined flow rate. Samples contained either 3 mM ferricyanide or 3 mM ferrocyanide, which was added immediately before drying. Samples were not oriented by dehydration under these conditions, since there was no change in the line shape of the D$^{\bullet}$ radical with a 90° change in orientation of the mylar strips with respect to the magnetic field (O'Malley et al., 1984; Brok et al., 1985; Rutherford, 1985). Illumination in the cavity was performed with a Dolan-Jenner Model 180 illuminator that was equipped with a fiber-optic light guide, a heat filter, and a red filter with a cutoff at 580 nm (Dolan-Jenner, Woburn, MA). There was a 10-min time delay between the light and dark spectra. Other spectral conditions are given in the figure legend.

Difference FT-IR Spectroscopy. Spectra were recorded on a Nicolet 60-SXR spectrometer equipped with a MCT-B liquid nitrogen-cooled detector. The spectral resolution was 4 cm^{-1}, double-sided interferograms were collected, and a Happ–Ganzel apodization function was used. One thousand scans were coadded for each interferogram. Sample temperature was maintained at −9 °C by use of a Harrick constant temperature cell and a temperature controller (Ossining, NY). The temperature was controlled to ±1 °C. Samples, 44 μg of chlorophyll, were deposited onto a germanium window, dried for 30 min with a stream of dry nitrogen, and then sandwiched with a CaF_2 window. Conditions for drying of EPR and FT-IR samples were identical, except for the change in the form of the solid substrate (mylar versus germanium). The absorbance at 1655 cm^{-1} in the amide I band was always less than 0.75 OD and for the data shown here (Figure 3) was approximately 0.7 OD. Samples contained either 3 mM potassium ferricyanide or 3 mM potassium ferrocyanide, which was added immediately before drying. The sample was illuminated by a Dolan-Jenner 180 illumination system that was equipped with an annular fiber-optic light guide, heat filter, and red filter (same as described above). An Alpha Products (Darien, CT) SA-129 RS-232 to A-BUS adapter and a ST-143 digital output driver card were used to interface the Nicolet computer with the illumination system. The germanium window blocked He–Ne laser illumination of the sample from the spectrometer optical bench.

Spectra recorded in the light were ratioed directly to spectra recorded in the dark; 12 of these light-minus-dark spectra were then averaged to obtain the final difference spectrum. There was a 10-min time delay between the light and dark spectra. Dark-minus-dark spectra showed no reproducible vibrational lines but did exhibit baseline variation in the region of the amide I and II bands. The infrared spectra of potassium ferricyanide and potassium ferrocyanide show no fundamental vibrations between 1750 and 1300 cm^{-1} (Griffith & Turner, 1970). Furthermore, light-minus-dark FT-IR spectra of buffer plus ferricyanide alone exhibited no vibrational lines between 1750 and 1300 cm^{-1}.

To obtain the double-difference (ferricyanide minus ferrocyanide) spectrum, light-minus-dark difference spectra were taken on aliquots from the same pooled sample of PSII complexes, so that the reaction center to protein ratio would be constant. To correct for small (~5%) remaining differences in protein concentration, each light-minus-dark spectrum was corrected by the amplitude of the amide II band in the infrared absorption spectrum. This gives a stoichiometric subtraction, corrected only for protein concentration.

FT-IR Spectrometry on Histidine Model Compounds. L-Histidine monohydrochloride monohydride, 99%, was obtained from Aldrich (Milwaukee, WI). Spectra were recorded using the Nicolet 60-SXR spectrometer described above. The spectral resolution was 4 cm^{-1}, double-sided interferograms were collected, and a Happ–Ganzel apodization function was used. Two thousand scans were coadded for each interferogram. Room-temperature spectra were obtained on 167 mM solutions of histidine that were buffered at either pH 5.3 or 6.9 by 100 mM MES–NaOH. The sample holder was a Micro-Circle cell equipped with a ZnSe crystal (Spectra-Tech, Stamford, CT), and the sample compartment was purged with dry air for 20 min before beginning data collection. A reference spectrum, collected on 100 mM MES–NaOH buffer

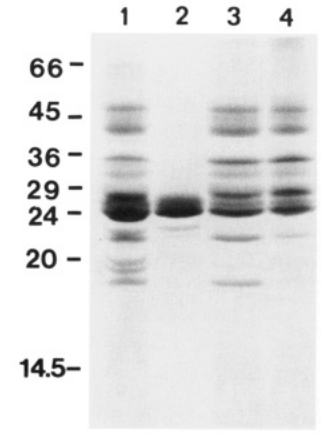

FIGURE 1: Coomassie-stained SDS denaturing gel showing the polypeptide composition of PSII complexes. The lanes show (1) BBY membranes, (2) peak 1 from the Mono Q column, (3) PSII complexes eluting in peak 2 from the Mono Q column, and (4) NaCl-washed PSII complexes. Each lane contains 4 μg of chlorophyll.

at either pH 5.3 or 6.9, was subtracted from the histidine spectrum that was recorded at the same pH. This subtraction removed contributions from water and buffer from the final histidine spectrum and is possible because of the short path length of the Micro-Circle cell.

Oxygen Evolution, Manganese, and Chlorophyll Assays. These assays were performed as described in Noren et al. (1991), with the following modifications. The oxygen assay buffer was 0.4 M sucrose, 50 mM MES, pH 6.0, and 10 mM NaCl, unless otherwise noted, and 20 μg of chlorophyll was used per assay. Chlorophyll assays were performed in 80% acetone, and the extinction coefficients for chlorophyll *a* and *b* in this solvent were taken from Lichtenthaler (1987).

Denaturing SDS–PAGE. Gel electrophoresis and Coomassie staining were performed as described by Piccioni et al. (1982) and Noren et al. (1991). The gel contained 12% acrylamide and 6 M urea.

RESULTS

Solubilization of spinach PSII membranes in the nonionic detergent, lauryl maltoside, and subsequent anion-exchange chromatography yield two well-resolved, chlorophyll-containing peaks. In Figure 1, we present a Coomassie-stained SDS denaturing gel that shows the polypeptide composition of the starting material (lane 1), peak 1 from the anion-exchange column (lane 2), and peak 2 from the anion-exchange column (lane 3). These data show that the major components of peak 1 are components of the light-harvesting complex with molecular masses of 29 and 28 kDa (Ghanotakis et al., 1987). Peak 2 contains polypeptides with predicted molecular masses of 46, 41, 36, 33, 30, 29, 28, 24, and 18 kDa; this is the preparation that we will refer to as "photosystem II complexes". The 46-, 41-, 33-, and 29-kDa polypeptides correspond to the 47-kDa, 43-kDa, D_2, and D_1 polypeptides, as identified by Ghanotakis et al. (1987). The "33-kDa" extrinsic polypeptide runs with a higher apparent mass in our gel system (Noren et al., 1991) and corresponds to the 36-kDa protein. Two additional polypeptides, which migrate with predicted molecular masses of 30 and 28 kD, are present in this preparation. These polypeptides may be components of the chlorophyll *a*/*b* light-harvesting complex, since the preparation retains chlorophyll *b* (see below). In addition, polypeptides with predicted molecular masses of 24 and 18 kDa are present in PSII complexes (lanes 1 and 3). When the preparation is treated with 2 M NaCl, both the 24- and 18-kDa polypeptides are removed, demonstrating that these polypeptides are extrinsic proteins (lanes 3 and 4).

Table I: Oxygen Evolution Activity of PSII Complexes[a]

	oxygen evolution [μmol of O_2 (mg of chl)$^{-1}$ h^{-1}]		
	no additions	3 mM $CaCl_2$	1 μM DCMU
BBY membranes[b]	600	600	100
PSII complexes[c]	1100	1100	120
salt-washed PSII complexes[c]	600	940	

[a] Samples contain 1 mM potassium ferricyanide. The error in oxygen evolution measurements was 10%. [b] 0.4 mM DCBQ. BBY membranes were solubilized in 1% lauryl maltoside. [c] 0.2 mM DCBQ.

Table II: Characterization of PSII Complexes

	yield (%)	chlorophyll *a*/*b* ratio	chlorophyll per 4 Mn[a]	chlorophyll per D• [b]
BBY membranes	100	2.1	270	270
PSII complexes	30	3.0	110	100

[a] The error in manganese quantitation was 7%. [b] The error in D• spin quantitation was 8%.

Table I shows that these PSII complexes exhibit an increase in oxygen evolution activity of a factor of 1.8 when compared to that of the starting material, PSII membranes. Also, there is no calcium requirement for oxygen evolution in the complexes, as expected if the 24- and 18-kDa extrinsic proteins are bound. However, washing with 2 M NaCl induces a calcium requirement (Table I). This preparation does not contain Q_B (MacDonald, Debus, and Barry, unpublished results). However, DCMU inhibits 90% of oxygen evolution activity in the presence of DCBQ (Table I). When potassium ferricyanide is used as an acceptor in the absence of DCBQ, only 10% of the optimal activity is obtained (data not shown).

Table II presents a further characterization of these PSII complexes. The yield is approximately 30% on a chlorophyll basis. D• spin and manganese quantitation indicate that the chlorophyll antenna size of the complex is approximately 100–110 chlorophylls per reaction center. This is a factor of 2.6 reduction from the chlorophyll antenna size of BBY membranes (Table II). Therefore, the approximately 2-fold increase in specific activity, when PSII complexes are compared to the starting material, is due to removal of nonphotochemically active light-harvesting protein from the photosystem II reaction center. Furthermore, the chlorophyll *a*/*b* ratio is increased from 2.1 to 3.0 upon purification of the complexes, consistent with the loss of light-harvesting complex and its associated chlorophyll *b* [for a review, see Green et al. (1991)]. Optical assays (Noren et al., 1991) for the primary donor of photosystem I, P_{700}^+, show that there is no detectable photosystem I contamination in this preparation (data not shown).

Figure 2 presents room-temperature EPR spectra of manganese-depleted PSII complexes containing 3 mM potassium ferricyanide. Hydroxylamine treatment was used to release functional manganese from the complex (Yocum et al., 1981); the hydroxylamine was then removed. This treatment, involving inactivation and removal of the manganese cluster, allows the photoaccumulation of the Z• tyrosine radical (Ghanotakis & Babcock, 1983). The dashed line in Figure 2 is a dark spectrum and shows the EPR line shape of the dark-stable radical, D•. The solid line is the light-induced signal, corresponding to the photoaccumulated tyrosine radical, Z•. The line shapes of D• and Z• are similar to each other and to tyrosine radical EPR spectra that have been recorded

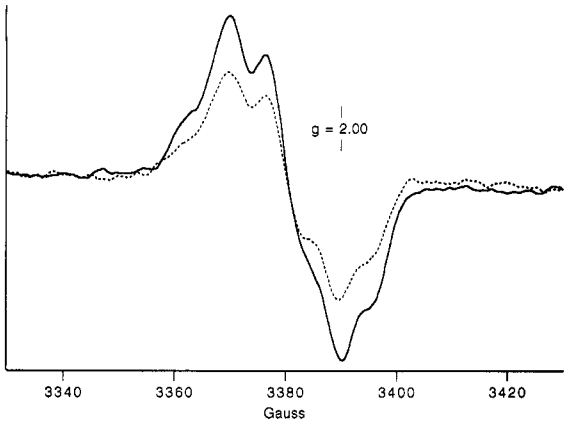

FIGURE 2: Room-temperature EPR spectra of manganese-depleted PSII complexes. The dashed line was recorded in the dark; the solid line was recorded in the light. Conditions were as follows: modulation frequency, 100 kHz; microwave power, 3.5 mW; field modulation, 3.2 G; time constant, 2 s; scan time, 4 min; gain 2×10^4. The spectra were recorded in the presence of 3 mM potassium ferricyanide. The chlorophyll concentration was 2.6 mg/mL. The g value of the tyrosine radical, D•, is known to be 2.0046 (Miller & Brudvig, 1991).

in other PSII preparations (Babcock et al., 1983). Estimates from spin quantitation show a 60% light-induced increase that is attributable to Z•.

Figure 3A is the difference (light-minus-dark) FT-IR spectrum of manganese-depleted PSII complexes at −9 °C in the presence of 3 mM potassium ferricyanide. This spectrum will contain a contribution from the vibrational modes of any cofactor or protein group that undergoes a light-induced change in geometry or environment under these conditions. Therefore, vibrational modes associated with oxidation of tyrosine Z or chlorophyll, reduction of quinone, and protonation or deprotonation of amino acid residues may all contribute to this spectrum. The temperature, −9 °C, was chosen as a compromise in promoting stability of the sample and in obtaining oxidation of tyrosine Z, which is not oxidized at low temperatures (data not shown). The sample was dehydrated in order to obtain a high signal-to-noise difference spectrum, because water has a strong infrared band that overlaps the amide I region of the protein infrared spectrum.

To show that the oxidation of tyrosine Z will make a contribution to the difference FT-IR spectrum that was obtained at −9 °C on a dehydrated sample, we present the data in Figure 4A. The conditions that were used to prepare this EPR sample were identical to those that were used to prepare the sample for the FT-IR experiment shown in Figure 3A; the EPR sample also contains 3 mM potassium ferricyanide. Further, all EPR and FT-IR experiments were performed on aliquots from the same pooled sample of PSII complexes in order to reduce sample-to-sample variation. The dashed line in Figure 4A is the spectrum of the dark-stable tyrosine radical, D•. The solid line shows the spectrum recorded in the light. The line shape of this light-induced signal can be compared with the light-induced signal of Z• that was observed at room temperature (Figure 2). Such a comparison leads to the conclusion that, under dehydrated conditions at −9 °C in the presence of 3 mM potassium ferricyanide, both a tyrosine radical and a chlorophyll radical are generated in the light. Comparison of the amplitudes of the D• and Z• signals at low field indicates that approximately 45% of the centers generate Z• in the light under these conditions. Subtraction of the dark-stable D• signal from the light-induced signal, using this estimate, gives an approximately 8-G line width for the chlorophyll radical (data not

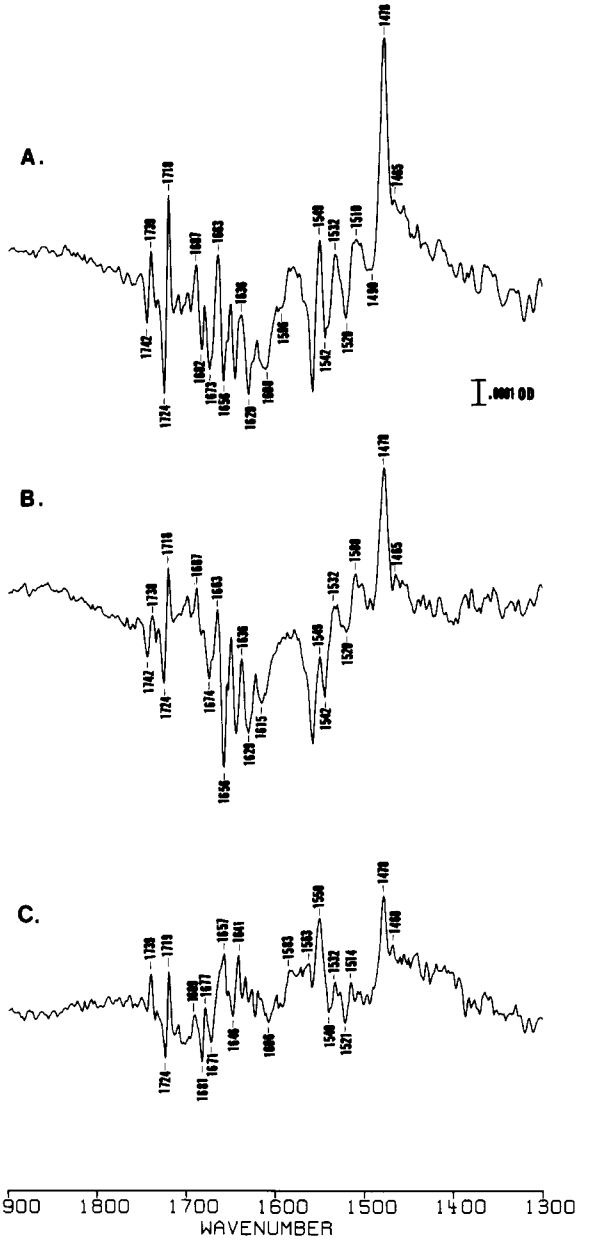

FIGURE 3: Light-minus-dark difference FT-IR spectra of manganese-depleted PSII complexes. PSII complexes were dried on a germanium substrate in the presence of either (A) 3 mM potassium ferricyanide or (B) 3 mM potassium ferrocyanide. Panel C is the double-difference, ferricyanide-minus-ferrocyanide spectrum constructed by subtraction of (B) from (A). Data were recorded at −9 °C.

shown). The chlorophyll radical must be from PSII, since there is no detectable photosystem I contamination of this preparation. This radical is presumably produced in those centers in which the oxidation of Z is blocked by temperature (de Paula et al., 1985) or by dehydration, and the radical may be either P_{680}^+ or another chlorophyll that is oxidized by P_{680}^+ (de Paula et al., 1985). The content of tyrosine and chlorophyll radicals was somewhat variable from sample to sample (data not shown).

We conclude from Figure 4A that, under the conditions that were used to record the difference FT-IR data, tyrosine Z can be oxidized. As a control to show that the FT-IR difference spectrum is sensitive to the concentrations of oxidized and reduced components in the reaction center, we will compare the light-minus-dark infrared spectrum, generated in the presence of the oxidant, potassium ferricyanide, to the light-minus-dark spectrum generated in the presence

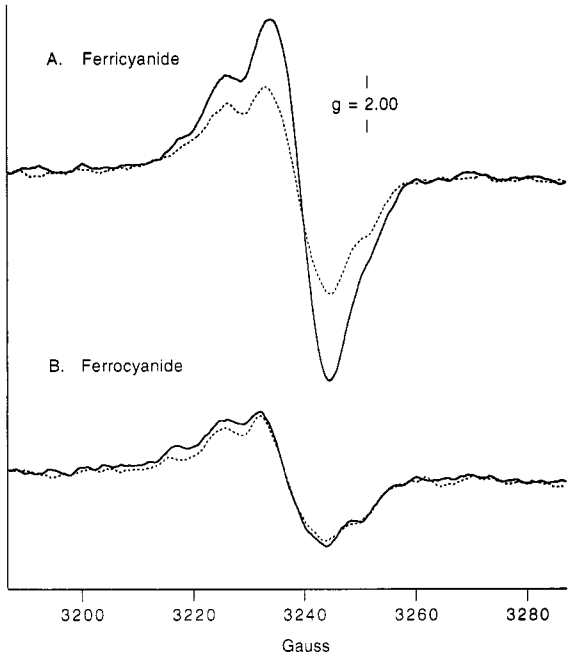

FIGURE 4: Low-temperature EPR spectra of manganese-depleted PSII complexes. In each panel, the dashed line was recorded in the dark; the solid line was recorded in the light. PSII complexes were dried on a mylar substrate in the presence of either (A) 3 mM potassium ferricyanide or (B) 3 mM potassium ferrocyanide. Spectral conditions were the same as in Figure 2 except that the microwave power was 0.7 mW. The temperature was −9 °C. The g value of the tyrosine radical, D•, is known to be 2.0046 (Miller & Brudvig, 1991).

of the reductant, potassium ferrocyanide. Potassium ferrocyanide is known to be a donor to PSII (Yerkes & Babcock, 1980), and, therefore, should lower the concentration of oxidized donor side species in the photosteady state.

Figure 4B shows the EPR spectrum, at −9 °C, recorded in the presence of potassium ferrocyanide in a dehydrated sample. Again, the dashed line represents the dark-stable radical, D•, while the solid line is the light-induced signal. Spin quantitation shows that, in the presence of ferrocyanide, the amplitude of the dark-stable radical is reduced by a factor of 0.35, compared to the magnitude of this signal in the sample that contains potassium ferricyanide (Figure 4A). Spin quantitation also shows that only a 16% light-induced increase is observed in the presence of the reductant. We attribute the light-induced increase mainly to a small population of destabilized D. Comparison of panels A and B in Figure 4 demonstrates that, as expected, the oxidation of tyrosine Z and chlorophyll makes a smaller contribution to the EPR spectrum recorded in the presence of ferrocyanide than to the spectrum recorded in the presence of ferricyanide.

The EPR spectra shown in Figure 4 suggest that comparison of difference FT-IR data that have been recorded in the presence of potassium ferricyanide with data that have been recorded in the presence of potassium ferrocyanide will aid in the identification of vibrational lines due to oxidation of tyrosine Z. In Figure 3B, we present the light-minus-dark difference FT-IR spectrum of manganese-depleted PSII complexes in the presence of 3 mM potassium ferrocyanide. These data were obtained under the same conditions as those used to record the EPR spectrum shown in Figure 4B. Comparison of the two difference FT-IR spectra obtained in the presence of either potassium ferricyanide (Figure 3A) or potassium ferrocyanide (Figure 3B) shows differences in the frequency and intensity of vibrational modes between 1900 and 1300 cm^{-1}.

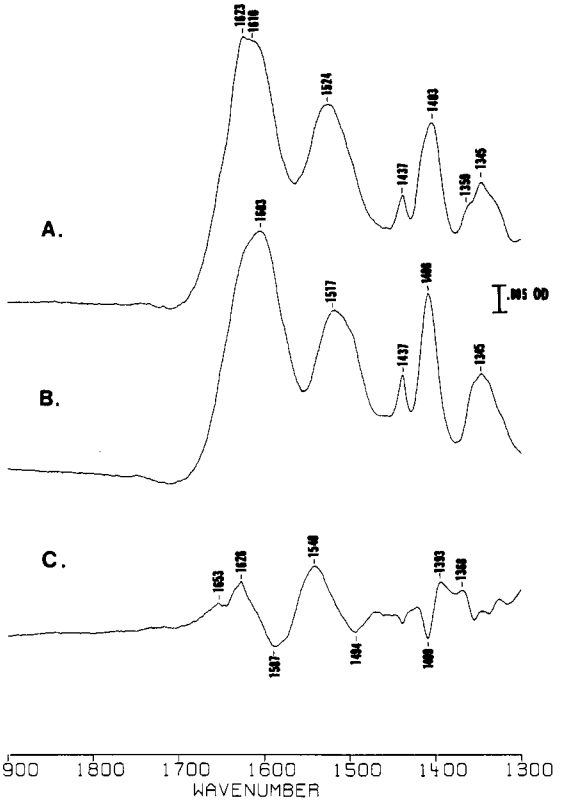

FIGURE 5: FT-IR spectra of L-histidine at (A) pH 5.3 or (B) pH 6.9. Panel C shows the difference infrared spectrum due to the protonation of the imidazole ring: (A) minus (B).

To aid in the identification of these changes, we constructed the double-difference spectrum, ferricyanide (light-minus-dark) minus ferrocyanide (light-minus-dark) shown in Figure 3C. This spectrum is a result of a stoichiometric subtraction on the basis of protein concentration and, therefore, reflects the changes in concentrations of redox intermediates under the two conditions. Although the frequencies of vibrational lines were reproducible, there was some variation in the relative intensities of vibrational modes in the double-difference spectrum. We attribute this variation to small differences in the content of oxidized chlorophyll and tyrosine from one ferricyanide- and ferrocyanide-containing sample to another.

Figure 5 shows the FT-IR spectrum of histidine at pH 5.3 (Figure 5A) and at pH 6.9 (Figure 5B). Contributions from the buffering agent and water have been subtracted. Figure 5C is the difference of the spectra in panels A and B. This difference spectrum reflects the change in protonation state of the imidazole ring. At pH 5.3, 83% of the histidine is in the form where the imidazole ring is protonated; at pH 6.9, 89% of the histidine is in the form where the imidazole ring is unprotonated. This change in pH will have little effect on protonation/deprotonation of the carboxylate or amino groups of histidine, and therefore, the vibrational difference spectra of these functional groups should not contribute significantly to the spectra presented here. To confirm this expectation, spectra of histidine at pH 1.9 and 9.0 were also obtained (data not shown).

DISCUSSION

PSII reaction centers that are active in oxygen evolution contain the 47-kDa, 43-kDa, D_1, and D_2 polypeptides, as well as subunits of cytochrome b_{559} and other low molecular weight proteins [for reviews, see Ghanotakis et al. (1987) and Debus (1992)]. The 33-kDa protein stabilizes the manganese cluster;

however, the reaction center is capable of evolving oxygen in its absence, if the manganese cluster is bound (Ono & Inoue, 1983, 1984; Kuwabara et al., 1985; Bockholt, et al., 1991; Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991; Bricker, 1992). In the absence of the 24- and 18-kDa proteins, plant PSII complexes evolve oxygen, but only in the presence of nonphysiological concentrations of calcium chloride. Under these conditions, the specific activity of oxygen evolution is approximately 80% of wild type (Ghanotakis et al., 1984; Miyao & Murata, 1984).

The antenna size of PSII membranes has been reported to be approximately 250 chlorophylls (Berthold et al., 1981). These membranes contain some Triton X-100 but are not completely solubilized in detergent and still pellet at low centrifugal forces (40000*g*). In addition to photochemically active reaction centers, this preparation contains chlorophyll *a*/*b* light-harvesting antenna proteins. With one exception (Enami et al., 1989), previously reported biochemical procedures that remove these light-harvesting proteins have the side effect of removing the 24- and 18-kDa extrinsic polypeptides [for a review, see Ghanotakis et al. (1987)]. In the preparation of Enami et al., *n*-heptylthioglucoside and high concentrations of magnesium chloride are used to isolate photosystem II complexes by centrifugation. This preparation retains the 18- and 24-kDa proteins in some centers but exhibits a small increase of activity in the presence of calcium chloride.

In contrast, the PSII complexes isolated by our one-step purification are completely solubilized, retain the 24- and 18-kDa as well as the 33-kDa extrinsic polypeptides, show an increase in reaction center content on a chlorophyll basis of a factor of 2.6, and an increase in the specific activity of oxygen evolution of a factor of 1.8. As expected if the 24- and 18-kDa proteins are retained in all centers, oxygen evolution activity in these complexes shows no requirement for calcium chloride. The fact that our preparation retains chlorophyll *b* and reproducibly gives an antenna size of 100–110 chlorophylls may be an indication that there are two pools of chlorophyll *a*/*b* antenna proteins, one set of which is more tightly associated with the reaction center than the other. Such an organization of chlorophyll *b*-containing antenna proteins has been suggested previously (Dunahay & Staehelin, 1986; Camm & Green, 1989).

The small reaction center size of the PSII complexes, isolated by the method we have described, has proven to be useful for FT-IR spectroscopic studies. In all photochemical reaction centers, the protein and cofactor structural features that facilitate forward electron transfer reactions and decrease the rate of back reaction are unknown. X-ray crystallographic analysis, while indispensable in understanding structure at the atomic level, does not easily provide information about structural alterations. Difference FT-IR spectroscopy has the potential to give this type of information.

The light-minus-dark difference FT-IR spectrum, obtained in the presence of the oxidant, potassium ferricyanide, reflects geometric and protonation changes in the reaction center that accompany electron transfer under these oxidizing conditions. From EPR experiments on similar samples, we conclude that $Z^{\bullet}$ and chl^{+} are accumulated in the photosteady state in the presence of the electron acceptor, ferricyanide. To show that the FT-IR spectrum is sensitive to the concentrations of redox intermediates in PSII, we also obtained a light-minus-dark difference spectrum in the presence of a reductant, potassium ferrocyanide. From EPR experiments on similar samples, we conclude that ferrocyanide has the effect of lowering the concentrations of $Z^{\bullet}$ and chl^{+}. Also, the concentration of Q_A^- will be higher in the light in the presence of this electron donor than in the presence of ferricyanide [see, for example, Breton et al. (1991)]. The changes in intensity and frequency that we observe in vibrational modes upon comparison of these two difference FT-IR spectra demonstrate that the infrared spectrum reflects changes in structure that are of interest. Moreover, evaluation of the amplitudes of the vibrational lines in the double-difference spectrum shows that some are of the correct order of magnitude (1×10^{-4} OD) to be caused by a change in a single amino acid residue.[2]

From our EPR experiments, we expect that the double-difference infrared spectrum (ferricyanide-minus-ferrocyanide) will contain positive modes from $Z^{\bullet}$ and chl^{+} and negative modes from Z and chl. A difference infrared study of chlorophyll oxidation in vitro (Nabedryk et al., 1990b) has been reported previously. The difference spectrum associated with reduction of Q_A may also contribute to the double-difference spectrum. An infrared study of quinone reduction in photosystem II (Berthomieu et al., 1990b) has been reported. Since we have no independent experimental confirmation of the size or direction of the contributions from Q_A^- and Q_A to the double-difference spectrum, we will not discuss the quinone contribution at this time. A possible contribution from $D^{\bullet}$-minus-D will be also neglected, since it is small (see Results). Here, we will focus on a preliminary assignment of unique vibrational modes (i.e., modes not observed in the difference spectrum of chlorophyll oxidation (Nabedryk et al., 1990b) or quinone reduction (Berthomieu et al., 1990b)) to $Z^{\bullet}$-minus-Z and an assessment of whether reversible histidine protonation will contribute to a difference infrared spectrum.

Tyrosine Oxidation. Resonance Raman studies have been performed that have identified vibrational changes associated with the formation of the neutral phenoxyl radical (Tripathi & Schuler, 1984). Upon oxidation, the C–O stretch of the phenol, assigned to 1255 cm^{-1}, is shifted to 1505 cm^{-1}. This large change in frequency is caused by the increased double-bond character of this bond in the radical, where spin density is delocalized upon the oxygen. The frequency of this vibrational mode will be sensitive to spin density distribution and to hydrogen-bonding interaction. There are two ring modes, at 1609 and 1510 cm^{-1}, which are present in phenol and are not observed in the radical (Tripathi & Schuler, 1984). Also, substantial shifts occur in the frequencies of two other modes between 1700 and 1300 cm^{-1}: 1475–1398 cm^{-1} (C–C stretch) and 1339–1331 cm^{-1} (C–H bend) (Tripathi & Schuler, 1984). Resonance Raman studies have also been performed to observe vibrational modes of the tyrosine radical in ribonucleotide reductase (Backes et al., 1989). These data show a vibrational mode at 1498 cm^{-1} that was assigned to the C–O of the oxidized tyrosine. This tyrosine radical is believed to be deprotonated and non-hydrogen-bonded (Backes et al., 1989; Bender et al., 1989).

Although there are likely to be intensity differences between the resonance Raman spectrum and the infrared spectrum and although the vibrational spectrum of tyrosine/tyrosine radical is likely to be more complex than that of phenol/phenoxyl radical, we can use the study of Tripathi and Schuler (1984) to perform a preliminary assignment of modes in the double-difference spectrum to tyrosine oxidation. The tyrosine radical will make a positive contribution to the double-

[2] The intensities of vibrational lines in the bacteriorhodopsin difference FT-IR spectrum that have been assigned to changes in single amino acid residues (Gerwert et al., 1989) were corrected for the respective molecular weights of PSII and bacteriorhodopsin in order to obtain this estimate.

difference spectrum; the tyrosine will make a negative contribution. In Figure 3C, we observe negative lines at 1606 and 1521 cm^{-1} that we tentatively attribute to tyrosine; there is a positive mode at 1514 cm^{-1} that could be the C–O stretch of the tyrosine radical. We favor the assignment of the 1514-cm^{-1} line to the C–O stretch, since this mode is not observed in the vibrational difference spectrum associated with chlorophyll oxidation (Nabedryk et al., 1990b) or with Q_A reduction (Berthomieu et al., 1990b). Such an assignment would predict a different spin density distribution/hydrogen-bonding interaction for tyrosine radical $Z^{\bullet}$ than for either the tyrosine radical in ribonucleotide reductase or phenoxyl radicals in vitro. It is difficult to evaluate this possibility since there are no ENDOR data on $Z^{\bullet}$. The region between 1300 and 1200 cm^{-1} is very complex (data not shown). In particular, at this time we cannot identify a unique negative mode near 1260 cm^{-1} that could be the C–O stretch of tyrosine.

Histidine Protonation. On a basis of a model of the structure of PSII, it has been suggested that Z is hydrogen-bonded to histidine 190 of D1 (Svensson et al., 1990, 1991). Tyrosine radicals are unstable in aqueous solutions (Steenkin & Neta, 1982). Since $Z^{\bullet}$ is reasonably stable, the radical may be sequestered from solvent. Therefore, a residue that is hydrogen-bonded to Z may be reversibly protonated by oxidation of the tyrosine. In such a case, vibrational modes of the imidazole ring that are affected by protonation would be expected to appear in the double-difference spectrum. Vibrational modes of the histidine may also appear in the spectrum, even if the residue is not sequestered from water. This can occur if the oxidation of tyrosine Z causes a pK_a shift in a nearby histidine residue. The pH effect on electron transfer kinetics between Z and P_{680}^{+} may be consistent with this last scenario (Conjeaud & Mathis, 1980; Boska et al., 1983).

While it has been reported previously that the histidine infrared spectrum in water is not affected by pH (Venyaminov & Kalnin, 1990), this is not consistent with expectations from an earlier infrared study of imidazole and imidazolium ion in solid form (Garfinkel & Edsall, 1958). To show which histidine modes might be affected by protonation of the imidazole ring in vivo, we have generated model compound data for histidine in two protonation states in water. Use of the Micro-circle cell allows us to obtain accurate subtractions of contributions from water and buffer. The spectra that we present in Figure 5 show a substantial change in the intensities and frequencies of histidine ring modes with protonation of the imidazole ring. The difference (protonated-minus-unprotonated) spectrum (Figure 5C) shows positive modes at 1653, 1626, and 1540 cm^{-1} and negative modes at 1587 and 1494 cm^{-1}. These data demonstrate that, if it is reversibly protonated and unprotonated, the vibrational modes of a histidine residue will contribute to a difference spectrum. The in vivo double-difference spectrum (Figure 3C) shows vibrational lines with frequencies that are not inconsistent with the protonation of a histidine residue. However, it is premature for us to attempt assignments of histidine modes in the double-difference spectrum, since vibrational modes of the chlorophyll macrocycle may also contribute to the spectrum in this region (Mantele, 1992; Nabedryk et al., 1990b). Future work will clarify this issue.

In conclusion, we have used a new preparation of PSII to obtain the first difference FT-IR spectrum that reflects contributions from the redox-active tyrosine, Z. We have presented a preliminary assignment of this spectrum based on a comparison with existing phenoxyl radical model compound data. Also, we have established through studies of histidine protonation in vitro that reversible protonation of a histidine residue should contribute to a difference infrared spectrum. Definitive assignment of the double-difference infrared spectrum awaits a study of D_2O exchange as a function of pH, isotopic labeling, which can be performed in cyanobacterial PSII (Barry & Babcock, 1987), and the infrared characterization of tyrosine radicals in vitro. Complete assignment of this spectrum will yield detailed information about the structural changes that accompany electron transfer in PSII under these conditions.

ACKNOWLEDGMENT

B.A.B. thanks M. Braiman and A. Kliger for assistance in Nicolet computer interfacing. An attempt to generate the $Z^{\bullet}$ minus Z vibrational spectrum in a less resolved preparation of photosystem II was performed in 1989 by B.A.B. with A. Kliger and M. Braiman at the University of Virginia, Charlottesville, VA. We also thank R. Neuman and C. Schenck for a gift of bacterial reaction centers, which we used as a positive control in our early difference FT-IR experiments.

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