A Difference Fourier Transform Infrared Study of Tyrosyl Radical Z· Decay in Photosystem II

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ABSTRACT Photosystem II (PSII) contains a redox-active tyrosine, Z. Difference Fourier transform infrared (FTIR) spectroscopy can be used to obtain structural information about this species, which is a neutral radical, Z·, in the photooxidized form. Previously, we have used isotopic labeling, inhibitors, and site-directed mutagenesis to assign a vibrational line at 1478 cm^{-1} to Z·; these studies were performed on highly resolved PSII preparations at pH 7.5, under conditions where Q_A^- and Q_B^- make no detectable contribution to the vibrational spectrum (Kim, Ayala, Steenhuis, Gonzalez, Razeghifard, and Barry. 1998. *Biochim. Biophys. Acta.* 1366:330–354). Here, time-resolved infrared data associated with the reduction of tyrosyl radical Z· were acquired from spinach core PSII preparations at pH 6.0. Electron paramagnetic resonance spectroscopy and fluorescence control experiments were employed to measure the rate of Q_A^- and Z· decay. Q_B^- did not recombine with Z· under these conditions. Difference FTIR spectra, acquired over this time regime, exhibited time-dependent decreases in the amplitude of a 1478 cm^{-1} line. Quantitative comparison of the rates of Q_A^- and Z· decay with the decay of the 1478 cm^{-1} line supported the assignment of a 1478 cm^{-1} component to Z·. Comparison with difference FTIR spectra obtained from PSII samples, in which tyrosine is labeled, supported this conclusion and identified other spectral components assignable to Z· and Z. To our knowledge, this is the first kinetic study to use quantitative comparison of kinetic constants in order to assign spectral features to Z·.

INTRODUCTION

Photosystem II (PSII) is a multisubunit protein complex that performs oxygen evolution upon photoexcitation in plants, algae, and cyanobacteria. The majority of the prosthetic groups involved in charge separation are bound to the D1 and D2 polypeptides. These groups are the specialized chlorophyll P_{680}, pheophytin, and the quinones, Q_A and Q_B. Upon light excitation, an electron is transferred in successive steps from P_{680} to Q_A and then to Q_B. P_{680}^+ is reduced by a redox-active tyrosine Z, which is tyrosine 161 in the D1 polypeptide. Tyrosyl radical Z· is subsequently reduced by the catalytic site, which contains four manganese atoms. Four oxidation steps are required to release molecular oxygen from water. The catalytic site cycles through intermediate states, called the S_n states, where the number of oxidizing equivalents stored, *n*, is 0–4. In addition to tyrosine Z, there is another redox-active tyrosine, D, on the donor side of PSII; tyrosine D is residue 160 in the D2 polypeptide. Tyrosyl radical D· is stable in the dark for hours; tyrosine D is oxidized by P_{680}^+ (reviewed in Barry, 1995; Britt, 1996).

Difference (light-minus-dark) Fourier transform infrared (FTIR) spectroscopy can be used to identify structural changes that accompany light-induced processes in proteins. The use of difference infrared technique facilitates a detailed examination of structure and/or dynamics (Braiman and Rothschild, 1988). We have employed FTIR spectroscopy to study the redox-active tyrosines in PSII (MacDonald and Barry, 1992; MacDonald et al., 1993; Bernard et al., 1995; Kim et al., 1997, 1998; Kim and Barry, 1998a,b). The vibrational spectrum associated with tyrosine Z oxidation in highly resolved, cyanobacterial preparations has been obtained; site-directed mutagenesis and isotopic labeling were used to assign the spectrum (MacDonald et al., 1993; Bernard et al., 1995; Kim et al., 1997, 1998; Kim and Barry, 1998a). These data were obtained under conditions where there is no detectable Q_A^- contribution to the spectrum. The Z·-minus-Z spectrum, measured in these preparations, exhibits an intense spectral feature at 1478 cm^{-1}. This vibrational feature has been assigned to the C-O vibration of tyrosyl radical, Z·, based on incorporation of three different isotopomers of tyrosine into PSII (Kim et al., 1998; Kim and Barry, 1998a). These data were obtained under continuous illumination. Here, we report kinetic experiments that provide additional support for this assignment.

MATERIALS AND METHODS

Preparation of spinach PSII core complexes

Photosystem II membranes were purified from market spinach (Berthold et al., 1981). Chlorophyll (chl) determination was performed in acetone (Lichtenthaler, 1987). Oxygen rates of isolated PSII membranes were 1000–1100 μmol O_2 (mg chl-hr)$^{-1}$ under conditions previously described (Barry, 1995; Kim and Barry, 1998b). PSII core preparations, with average oxygen rates of 2400 μmol O_2 (mg chl-hr)$^{-1}$, were purified from these PSII membranes by a method previously described (Ghanotakis and Yocum, 1986). The chl antenna size of this preparation has been determined (Patzlaff and Barry, 1996). PSII core samples were manganese-depleted

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using 0.8 M Tris (tris(hydroxyl-methyl)aminomethane)-HCl, pH 8.0, and 2 mM EDTA (Kim and Barry, 1998b). These samples were dialyzed against 5 mM MES (2-(*N*-morpholino)ethanesulfonic acid)-NaOH, pH 6.0, for a period of approximately 20 h. The final buffer (Buffer A) consisted of 5 mM MES-NaOH, pH 6.0, and 0.03% (w/v) dodecyl maltoside (Anatrace, Maumee, OH). The final concentration was 1 mg chl/mL. Samples were frozen in liquid nitrogen and stored at −80°C.

Preparation of cyanobacterial PSII samples

Cultures of *Synechocystis sp*. PCC 6803 were grown according to published procedures (Barry, 1995), and cultures were labeled with $^{13}C(6)$-tyrosine (L-4-hydroxyphenyl-$^{13}C_6$-alanine, 99% labeled, Isotec, Miamisburg, OH) by procedures previously described (Barry, 1995). PSII particles were purified from cyanobacterial cells (Noren et al., 1991; Barry, 1995), manganese-depleted (Noren et al., 1991; MacDonald and Barry, 1992; Bernard et al., 1995), dialyzed against 5 mM (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid])-NaOH, pH 7.5, concentrated in Centricon 100 concentrators (Amicon, Beverly, MA) to 1 mg chl/mL, aliquoted, flash-frozen in liquid nitrogen, and stored at −80°C. Chlorophyll determination was in methanol (Lichtenthaler, 1987). Steady-state oxygen evolution rates (Barry, 1995) of cyanobacterial PSII samples before manganese depletion were 2700 μmol O_2 (mg chl-hr)$^{-1}$ for control PSII and 2400 μmol O_2 (mg chl-hr)$^{-1}$ for $^{13}C(6)$-tyrosine-labeled PSII.

Electron paramagnetic resonance (EPR) measurements on tyrosyl radicals

EPR spectra were recorded using a Bruker EMX 6/1 spectrometer equipped with a Bruker ST-TE cavity and a Wilmad variable temperature dewar (Kim et al., 1998). The temperature was maintained at −10°C using a stream of cold nitrogen through a dry ice ethanol bath. Red- and heat-filtered light from a fiber optic illuminator (Dolan Jenner, Woburn, MA) was used to illuminate the sample in the cavity. Spinach manganese-depleted PSII core samples in Buffer A at a concentration of 1 mg chl/mL and in a volume of 300 μL were used. Before dehydration, potassium ferricyanide and potassium ferrocyanide were added to the PSII core sample to give final concentrations of 2.5 mM each. The molar ratio was 180 mole potassium ferricyanide (or potassium ferrocyanide) per mole PSII reaction center. In all the experiments described here, potassium ferricyanide and potassium ferrocyanide stocks were made up on the same day and added immediately before data acquisition. The sample was partially dehydrated on mylar strips (MacDonald and Barry, 1992; Bernard et al., 1995; Kim et al., 1998). These samples were identical to those employed for FTIR and fluorescence measurements except for the use of a mylar support. Spectral conditions were: microwave frequency, 9.21 GHz; power, 0.8 mW; modulation amplitude, 3.5 G; scan time, 4 min; and time constant, 1.3 s.

For the kinetic decay of tyrosyl radical Z· the sample was illuminated for 1 s, and the decay of signal amplitude at a constant magnetic field was followed as a function of time. Spectral conditions were: magnetic field, 3273 G; microwave frequency, 9.21 GHz; power, 12.7 mW; modulation amplitude, 5 G; scan time, 10.5 s; and time constant, 5.1 ms. Data are presented as an average of 144 traces. EPR data were analyzed through the use of the program IGOR (Lake Oswego, OR). In analyzing the data, the baseline immediately before illumination was defined as zero. All data points were then normalized to the signal obtained immediately before the light was turned off; this point was taken as 100%.

Fluorescence measurements

Fluorescence spectroscopy was performed on the manganese-depleted spinach PSII core samples in Buffer A at −10°C. An Opti-Sciences OS-500 modulated fluorometer (Haverhill, MA) was used for the fluorescence measurements. Fifty microliters of a manganese-depleted spinach PSII core sample, containing 2.5 mM potassium ferricyanide and 2.5 mM potassium ferrocyanide, were partially dehydrated on a glass window identical in size to the windows used for FTIR data acquisition. An additional control PSII core sample consisted of spinach manganese-depleted PSII, but containing only 2 molar equivalents of potassium ferricyanide per mole of reaction center. The sample was illuminated with light sources from the fluorometer. Spectral conditions for the fluorescence decay measurements were: modulation intensity, 180 (0.4 μE); saturation intensity, 180 (7 kμE); time constant, 50 ms; and light pulse, 1.3 s. Data presented are an average of 60 traces. Fluorescence data were analyzed through the use of the program IGOR.

Difference FTIR measurements on tyrosine Z

Infrared spectra were obtained using a Magna 550 II spectrometer (Nicolet, Madison, WI) equipped with KBr beamsplitter and a MCT/A detector (Kim and Barry, 1998a). The temperature control and the automated illumination systems were described previously (Kim and Barry, 1998a). Illumination for 1.3 s (spinach PSII) or 4 min (cyanobacterial PSII) was provided by a Dolan Jenner annular illuminator equipped with heat-filter and red- (580 nm cutoff) filter.

In the data collection method employed for spinach PSII core samples, 3 mirror scans, collected in 1.3 s, were coadded for each double-sided interferogram. Spectral conditions were: resolution, 8 cm^{-1}; zero filling, one level; velocity, 2.5 cm/s; apodization function, Happ-Genzel; and temperature, −10°C. Samples containing potassium ferricyanide/ferrocyanide and identical to those used for EPR and fluorescence measurements were used; 50 μl of a manganese-depleted spinach PSII core sample was partially dehydrated and placed between a CaF_2 and a Ge window (MacDonald and Barry, 1992; Barry, 1995; Kim and Barry, 1998a). For the data acquisition scheme employed, see Results and Fig. 1 *A*. In the generation of difference FTIR spectra, data were directly ratioed on a non-interactive basis. Difference spectra, obtained on PSII samples, were normalized to a 0.35 amide II absorbance. Data presented are an average of 143 spectra. Difference FTIR data were analyzed through the use of the program IGOR.

In the data collection method used for cyanobacterial PSII samples, a Z·-minus-Z spectrum was obtained with 4 min of continuous illumination (Kim and Barry, 1998a). Each interferogram was the sum of 425 mirror scans, taken in 4 min. Data recorded under illumination were ratioed directly to data recorded immediately before in the dark; this procedure has been shown to generate a Z·-minus-Z spectrum (Kim and Barry, 1998a). Our previous control experiments have demonstrated that there was no Q_A^- contribution to cyanobacterial PSII spectra under these conditions (Kim et al., 1998). The spectral resolution was 4 cm^{-1}, double-sided interferograms were collected, the mirror velocity was 2.5 cm/s, and the temperature was −9°C. A Happ-Genzel apodization function and a single level of zero filling were employed. Manganese-depleted cyanobacterial PSII samples containing 25–30 μg chlorophyll, 3 mM potassium ferricyanide, and 3 mM potassium ferrocyanide were 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. Difference spectra were normalized to an amide II absorbance of 0.35, as previously described (Kim and Barry, 1998a).

RESULTS

EPR experiments

EPR experiments, performed at −10°C, were used to monitor the decay of the tyrosyl radical (Figs. 1 *B* and 2). At this data acquisition temperature, there is no observed EPR signal from $Fe^{+2}Q_A^-$ on the PSII acceptor side (see discussion in Kim et al., 1998; Miller and Brudvig, 1991). Fig. 2 *A* shows the EPR spectra of the tyrosyl radicals in manga-

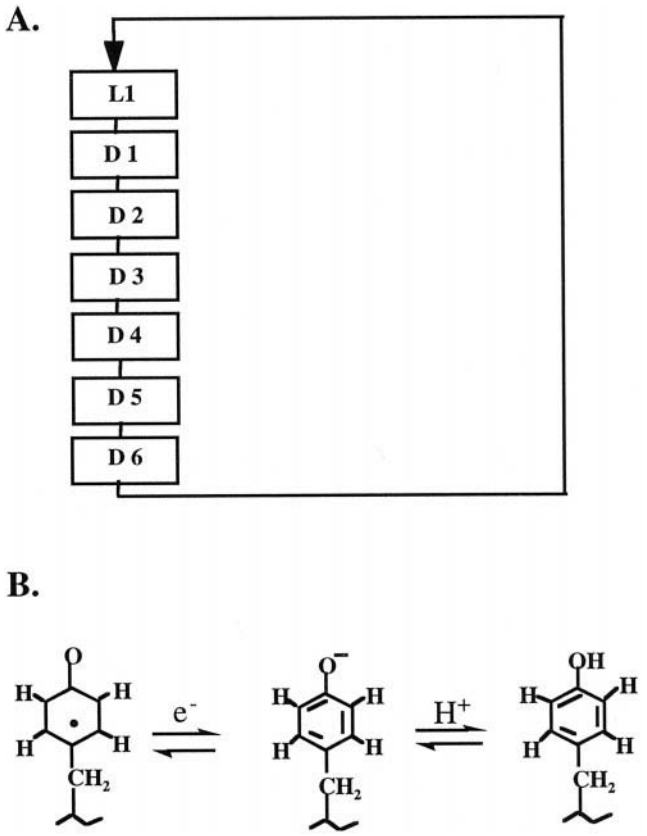

FIGURE 1 (*A*) Data acquisition scheme. The boxes represent 1.3 s of data acquisition. Samples were subjected to a pre-illumination cycle, which was not averaged into the final data set. The cycle was iterated. (*B*) Reduction of tyrosyl radical Z·. The first step is shown as an electron transfer reaction, and the second step involves a protonation reaction.

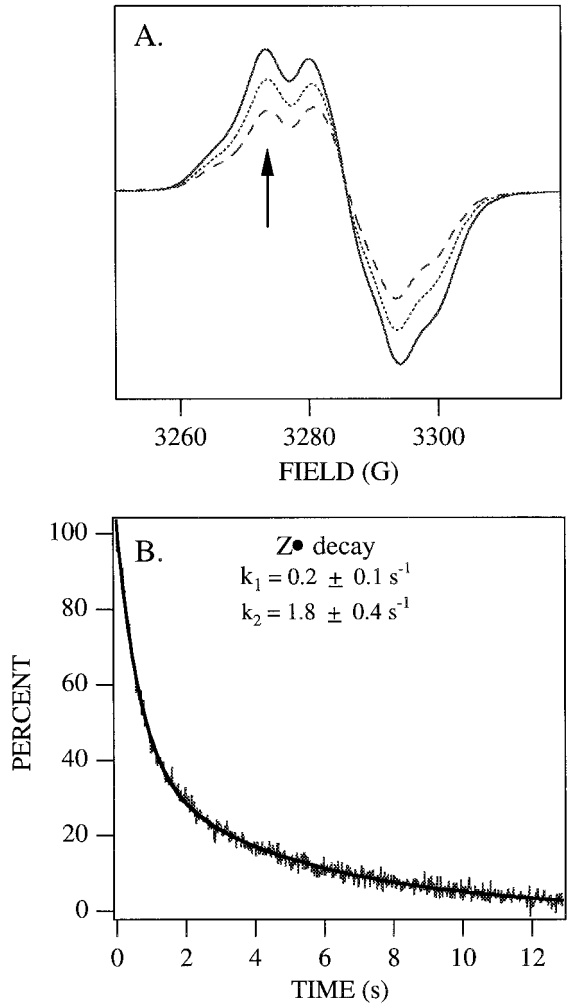

FIGURE 2 (*A*) EPR spectra obtained on manganese-depleted, spinach core PSII samples at pH 6.0 and a temperature of −10°C. Samples contained 2.5 mM potassium ferricyanide and 2.5 mM potassium ferrocyanide and were partially dehydrated. The spectrum shown in the solid line was obtained under illumination and represents the composite spectrum of tyrosyl radicals Z· and D·. The spectrum shown in the dotted line was obtained 4 min after illumination and represents tyrosyl radical D·. The spectrum shown in the dashed line was obtained 90 min after illumination and shows the expected slow decay of D·. The *arrow* indicates the magnetic field employed in order to obtain a light-induced EPR transient. The *g* values of the tyrosyl radicals are 2.004 (Miller and Brudvig, 1991). (*B*) Decay of the light-induced EPR signal of tyrosyl radical, Z·. The data were fit with a bi-exponential function (superimposed) to obtain rate constants. See Materials and Methods for spectral conditions.

nese-depleted spinach PSII core samples. Tyrosyl radical Z· was generated under illumination (Fig. 2 *A, solid line*). The lifetime of Z· is pH- and preparation-dependent (see Dekker et al., 1984, for an example). In our samples, after 4 min of dark adaptation, tyrosyl radical Z· has decayed, leaving only the signal from tyrosyl radical D· (Fig. 2 *A, dotted line*). After 90 min of dark adaptation (Fig. 2 *A, dashed line*), contributions from D· were still observable, reflecting the slow decay kinetics of this species.

Fig. 2 *B* shows the kinetics of the decay of light-induced signals, monitored at a constant magnetic field (Fig. 2 *A*, *arrow*). The data acquisition scheme employed is shown in Fig. 1 *A*. The sample was illuminated for 1 s and the repetition time was 0.06 Hz, which precluded a large contribution from tyrosyl radical D·. Therefore, a plot of the EPR amplitude as a function of time will reflect mainly Z· decay. Such a plot is shown in Fig. 2 *B*. The experimental data were fit well with a bi-exponential function (Fig. 2 *B*, *solid line*). The two rate constants derived from this fitting procedure were 0.2 ± 0.1 s^{-1} and 1.8 ± 0.4 s^{-1} (χ^2 = 1300), with approximately 40% of the amplitude showing the slow phase and the remaining 60% showing the fast phase of decay.

Fluorescence experiments

Fluorescence measurements were used to determine the yield and decay of Q_A^- (Boerner et al., 1992; Kim et al., 1998; Kim and Barry, 1998a). In the absence of fluorescence quenchers, such as P_{680}^+, the fluorescence yield serves as an indirect measure of the yield of Q_A^- (see Boerner et al., 1992, and references therein). The partially dehydrated sample, identical to the samples used for the infrared and EPR experiments, was illuminated for 1.3 s with a repetition rate of 0.1 Hz. Fig. 3A shows the decay of Q_A^-, as assessed by

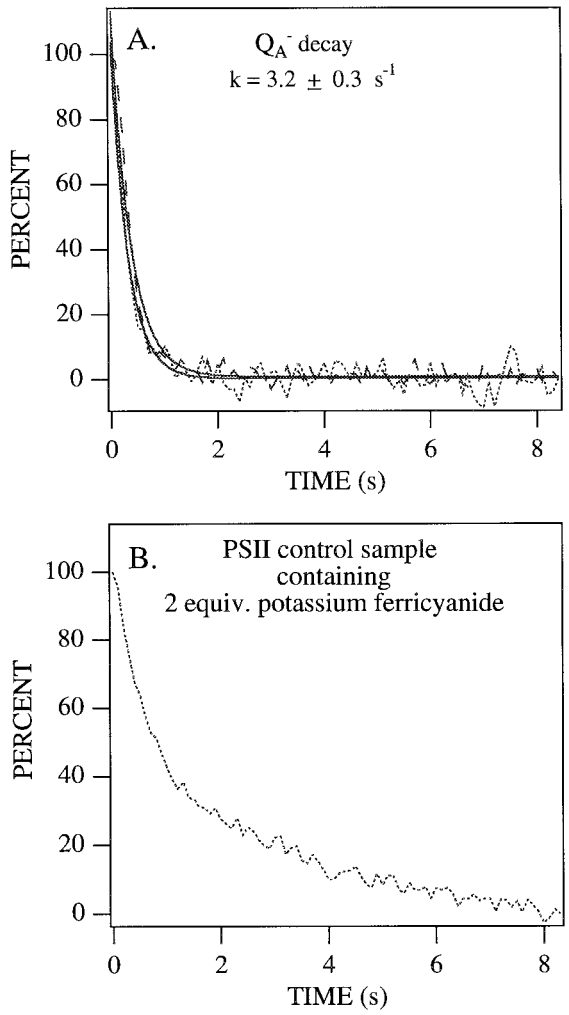

FIGURE 3 The decay of Q_A^-, as assessed by the decay of variable fluorescence. Data were obtained on manganese-depleted spinach core PSII samples at pH 6.0 and a temperature of −10°C. (*A*) Samples contained 2.5 mM potassium ferricyanide and 2.5 mM potassium ferrocyanide (*dotted line*) or 2.5 mM potassium ferricyanide, 2.5 mM potassium ferrocyanide, and 100 mM DCMU (*dashed line*), and samples were partially dehydrated. The data were fit with mono-exponential functions (superimposed) to obtain rate constants. (*B*) The partially dehydrated sample contained two molar equivalents of potassium ferricyanide per mole of PSII reaction center. See Materials and Methods for conditions.

this method. Data obtained in the presence and absence of 3-(3,4-dichlorophenyl)-1,1,dimethylurea (DCMU) were similar (Fig. 3 *A*, *dashed and dotted lines*), indicating that the formation and/or oxidation of Q_B^- is not influencing the Q_A^- decay rate (see discussion in Kim et al., 1998; Kim and Barry, 1998a, and references therein). The data were fit well with a mono-exponential fit (Fig. 3 *A*, *solid line*); the rate constant derived is 3.2 ± 0.3 s^{-1}. The χ^2 value for the mono-exponential fit was 1200; there was no significant improvement if a double exponential function was employed.

The rate of Q_A^- decay was increased by a factor of two compared to the fast phase in Z· decay, as assessed by EPR spectroscopy. However, the rate of Q_A^- decay was increased by a factor of approximately 16 compared to the slow component in Z· decay. These results can be explained by the presence of 2.5 mM potassium ferricyanide in the sample, which can rapidly reoxidize Q_A^-. In this mechanism, 60% of Q_A^- decays by rapid recombination with Z·. We postulate that this recombination corresponds to the 1.8 s^{-1} phase in Z· decay. Another 40% of Q_A^- decays by reduction of potassium ferricyanide, with a rate similar to the recombination rate. Based on the fluorescence-derived rate of 3.2 s^{-1}, the rate of ferricyanide reduction is predicted to be approximately 1.4 s^{-1}. The remaining 40% of Z· is reduced slowly by potassium ferrocyanide, giving rise to the 0.2 s^{-1} phase in the decay of the Z· EPR signal. This model, similar to a mechanism proposed in the literature (Dekker et al., 1984), can be tested by decreasing the concentration of potassium ferricyanide, which should slow the rate of reoxidation of Q_A^- as judged by fluorescence. Fig. 3 *B* shows the decay of Q_A^- in the control spinach manganese-depleted PSII core sample containing only 2 molar equivalents of potassium ferricyanide per mole of PSII reaction center. As expected, in this sample, the rate of Q_A^- decay decreased.

FTIR experiments

Fig. 1 *A* shows the data acquisition scheme employed for FTIR spectroscopy. The sample was pre-illuminated before the beginning of data acquisition. The sample was then illuminated (L1, 1.3 s, Fig. 1 *A*). Following illumination, six sets of data were recorded at 1.3-s intervals (denoted D1, D2, etc.). This data acquisition scheme was iterated at 0.06 Hz.

In Fig. 4, *A–E*, we present difference FTIR spectra acquired as a function of time after illumination by this protocol. The control experiments, described above, show that Z· and Q_A^- will be produced under illumination and will decay after illumination with different kinetics. Also, we have shown that Q_B^- does not influence the rate of decay of Q_A^-. In addition to Z·, tyrosyl radical D· may make a minor contribution to data obtained with a repetition rate of 0.06 Hz. Both D· and Z· contribute intensity to a positive 1478 cm^{-1} line at pH 6.0 (Kim and Barry, 1998a).

The FTIR spectra obtained on these PSII samples exhibited an intense positive line at 1478 cm^{-1}, which decayed as a function of time after illumination (Fig. 4, *A–E*). A derivative shaped feature at 2114 (pos.) and 2033 (neg.) cm^{-1} (Fig. 4, *inset*) was observed in Fig. 4 *A*; this signal also decayed. These spectral features arise from the CN stretching vibration of potassium ferricyanide and potassium ferrocyanide, respectively, and reflect electron transfer events occurring at both the donor and acceptor side of PSII. Because there are two electron transfer processes, one involving tyrosine Z and one involving Q_A, with the potential to give rise to each of these spectral features, the sign of the derivative can be inverted in some preparations and at some concentrations of potassium ferricyanide; see Zhang et al. (1997) for an example.

To identify the origin of the spectral feature at 1478 cm^{-1}, the decay of the 1478 cm^{-1} line was plotted as a

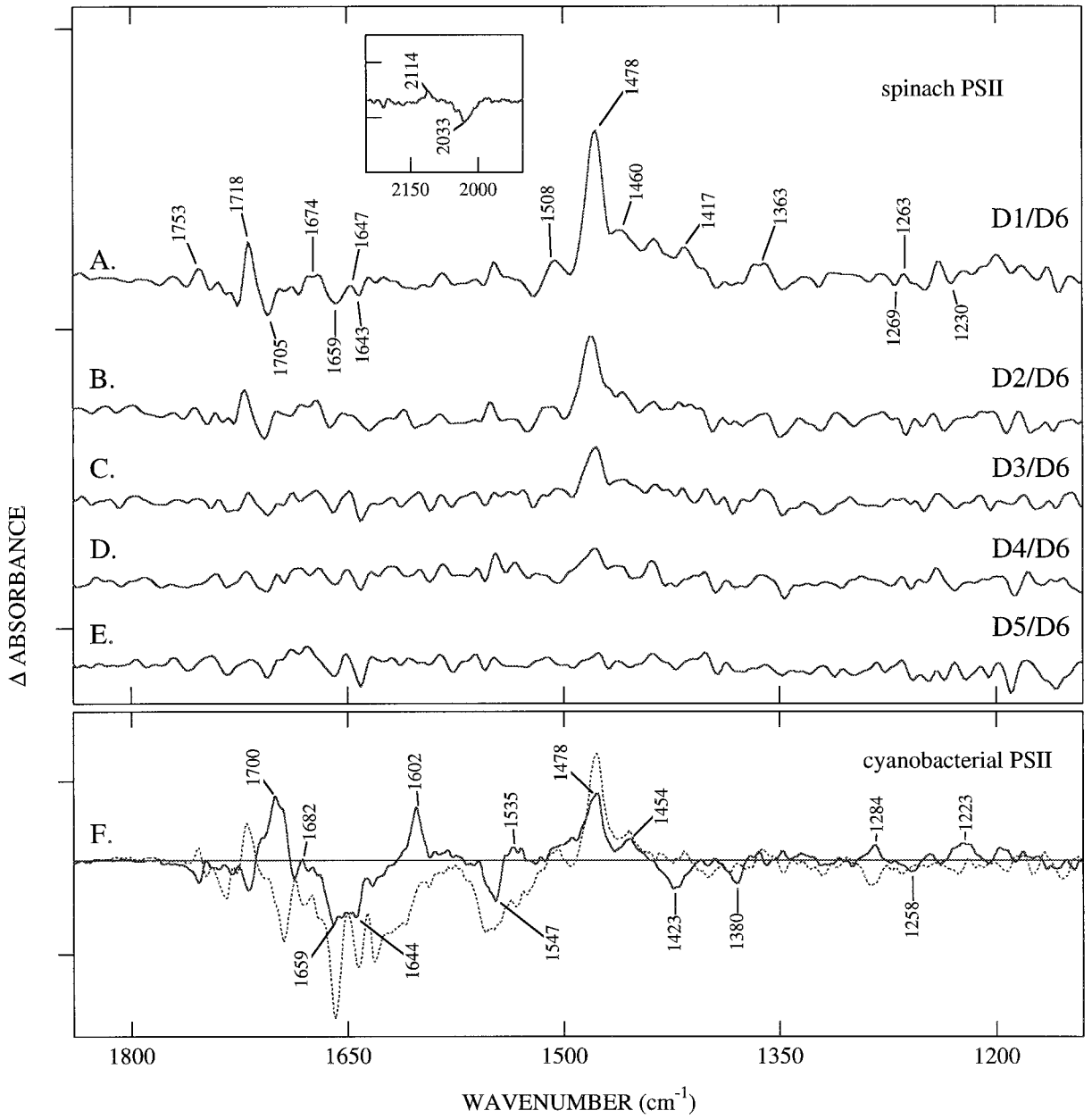

FIGURE 4 Difference FTIR spectra, representing Z·-minus-Z. Data were obtained on manganese-depleted spinach core PSII samples at pH 6.0 (*A–E*). Data corresponding to Z·-minus-Z were also obtained on manganese-depleted, cyanobacterial PSII at pH 7.5 (F). Spectra shown in *A* through *E* correspond to the D1/D6 (D1-minus-D6), D2/D6 (D2-minus-D6), D3/D6 (D3-minus-D6), D4/D6 (D3-minus-D6), and D5/D6 (D5-minus-D6) difference FTIR spectra, respectively (see Fig. 1 *A*). Samples contained 2.5 mM potassium ferricyanide and 2.5 mM potassium ferrocyanide and were partially dehydrated; the temperature was −10°C. In (*F*), solid line, the isotope-edited Z·-minus-Z spectrum is shown. The double difference spectrum is the result of a non-interactive, one-to-one subtraction: control Z·-minus-Z minus $^{13}C_6$ Tyr Z·-minus-Z. In *F*, the dotted line shows a control Z·-minus-Z spectrum alone; this spectrum was replotted from Kim and Barry, 1998a for comparison. Data in *A–F* are an average over multiple PSII preparations. *Inset*: Oxidation-reduction bands of potassium ferrocyanide and potassium ferricyanide in the spectrum shown in Fig. 4 *A*. Tick marks on the *y* axis are 4×10^{-4} absorbance units in *A-F* and 2×10^{-4} absorbance units in the inset. See Materials and Methods for conditions.

function of time at which the mirror scans were initiated (Fig. 5A). The data are fit well with a mono-exponential function (Fig. 5 *A*, *solid line*); the rate constant derived was $0.2 \pm 0.1\ s^{-1}$. The χ^2 value for a mono-exponential fit was 27; there was no significant improvement in the fit if a double exponential function was employed. This rate is indistinguishable from the slow kinetic component in Z· decay, as assessed by EPR spectroscopy.

Isotopic labeling is of assistance in the assignment of vibrational lines. A double difference spectrum, derived from cyanobacterial control PSII and from cyanobacterial PSII in which tyrosine is ^{13}C-labeled at all ring positions, is presented in Fig. 4 *F* (*solid line*). The extent of tyrosine labeling under these conditions has been estimated as close to 100% from an analysis of alterations in EPR lineshapes (Barry and Babcock, 1987). Fig. 4 *F* (*dotted line*) shows the Z·-

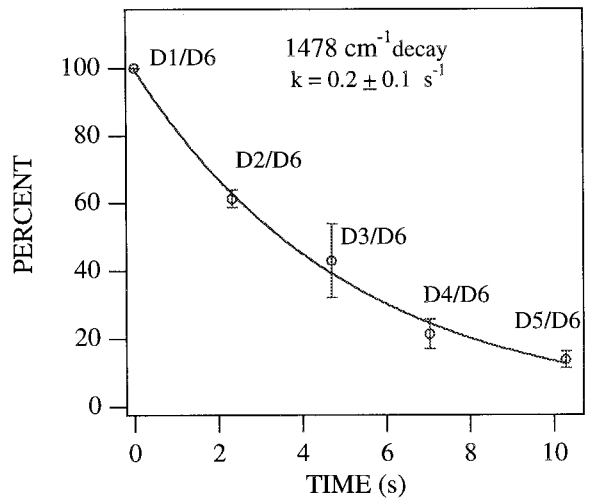

FIGURE 5 Decay of the 1478 cm^{-1} line, derived from the difference FTIR spectra shown in Fig. 4. The change in the amplitude of the 1478 cm^{-1} line was plotted as a function of the time at which the mirror scans began. Amplitudes were obtained from D1/D6 (D1-minus-D6), D2/D6 (D2-minus-D6), D3/D6 (D3-minus-D6), D4/D6 (D4-minus-D6), D5/D6 (D5-minus-D6) difference FTIR spectra. Data were fit with a mono-exponential function to give a rate constant.

minus-Z spectrum, obtained from cyanobacterial PSII through continuous illumination, for comparison (Kim and Barry, 1998a). Because these spectra were normalized on the basis of the total protein concentration and pathlength, the double difference spectrum was constructed by a direct, one-to-one, non-interactive subtraction of Z·-minus-Z spectrum, obtained from $^{13}C_6$ Tyr-labeled samples, from a Z·-minus-Z spectrum, obtained from a control, unlabeled sample. Unique vibrational modes of unlabeled Z· make a positive contribution to the double difference spectrum; unique vibrational lines of labeled Z· make a negative contribution. Unique vibrational modes of unlabeled Z make a negative contribution to the double difference spectrum; unique lines of labeled Z make a positive contribution. We have previously demonstrated that the Z·-minus-Z FTIR spectrum is relatively pH-independent, except for a small difference in the 1540–1520 cm^{-1} region (Kim and Barry, 1998a).

Inspection of the double difference spectrum (Fig. 4 *F*, *solid line*) confirms that a component of the 1478 cm^{-1} line downshifts to 1423 cm^{-1} (negative) upon $^{13}C_6$ tyrosine labeling. However, only a component of this spectral feature is sensitive to tyrosine labeling. Remaining intensity at 1478 cm^{-1} (Fig. 4 *F*, *solid line*) may be assigned to chl^+ (MacDonald et al., 1995; Kim and Barry, 1998a).

Other spectral features observed in time-resolved Z·-minus-Z infrared spectra

In addition to the 1478 cm^{-1} line, the difference spectra associated with Z· reduction exhibit other vibrational features that decay over the time course of data acquisition. For example, time-dependent spectral changes are observed at 1718 (pos.), 1460 (pos.), and possibly 1230 (neg.) cm^{-1} (Fig. 4, *A–E*). Inspection of the double difference spectrum, control-minus-$^{13}C_6$ Tyr labeled (Fig. 4 *F*, *solid line*), indicates that Z· makes a potential spectral contribution at 1700 cm^{-1} (potentially shifting to 1659 cm^{-1}) and 1454 cm^{-1} (potentially shifting to 1380 cm^{-1}) and that Z makes a potential spectral contribution at approximately 1644 cm^{-1} (possibly shifting to 1602 cm^{-1}). Isotope-induced spectral changes in the 1260–1220 cm^{-1} region are observed, as well. A negative line at 1547 cm^{-1} was also observed, which potentially shifts to 1535 cm^{-1} (Fig. 4 *F*). Any corresponding observed vibrational bands in Fig. 4, *A–D*, are therefore assignable to tyrosine and the tyrosyl radical in spinach PSII (Kim and Barry, 1998a; Kim et al., 1998). Other spectral components reflect structural changes in the environment of tyrosine Z (Kim and Barry, 1998a).

DISCUSSION

In this paper, we describe the use of kinetic techniques to assign spectral features to tyrosyl radical, Z·, in PSII. Samples used for EPR, fluorescence, and FTIR measurements were dehydrated and handled in a similar manner, in order to make such a kinetic comparison possible. A quantitative comparison of transient difference FTIR and EPR data can be used in the assignment of vibrational lines to Z·. A comparison of EPR and FTIR kinetic data with the kinetics of Q_A^- decay, as measured by fluorescence, is also possible. If the PSII sample is kinetically heterogeneous, the measuring beam of a modulated fluorometer, which is employed here, has a small actinic effect, which closes reaction centers and biases kinetics to give apparent faster rates (Boerner et al., 1992). However, the magnitude of this effect is expected to be less than a factor of two, for events occurring on the millisecond to second time scale that is relevant here. For example, a half-time of 530 ms, instead of 700 ms, was measured for the $S_2Q_A^-$ recombination rate, when a 1.6 kHz modulated fluorometer was employed (Nixon and Diner, 1990; Boerner et al., 1992). By contrast, the modulation of the monitoring beam employed here is 0.5 kHz (intensity, 0.4 μE). Furthermore, control fluorescence experiments, employing a low concentration of potassium ferricyanide (Fig. 3 *B*), have demonstrated that the modulated fluorometer can detect slower components in the decay rate of Q_A^-.

Therefore, the sixteen fold difference observed between the rate of Q_A^- decay (3.2 $\pm$ 0.3 s^{-1}), as assessed by fluorescence measurements, and the slow component in the rate of Z· decay (0.2 $\pm$ 0.1 s^{-1}), as assessed by EPR spectroscopy, is larger than the difference expected from an actinic effect alone. A comparison of these rate constants with the rate constant for the decay of the 1478 cm^{-1} line (Fig. 5, $k = 0.2 \pm 0.1$ s^{-1}) supports the assignment of a component in the 1478 cm^{-1} line to Z·. Note that components decaying on a time scale much faster than the 1.3-s data acquisition time would not be detected by this infrared method.

This assignment of the 1478 cm^{-1} line is in agreement with previous characterization of site-directed mutants and isotopically labeled PSII (MacDonald et al., 1993; Bernard et al., 1995; Kim et al., 1998; Kim and Barry, 1998a). The Z·-minus-Z difference spectra presented in those previous studies were recorded under continuous illumination. Z· contributions to the spectrum were distinguished from D· contributions by varying the intervals between dark and light scans. The 1478 cm^{-1} line was assigned to the C-O stretching vibration of Z· through isotopic labeling of tyrosine with three different isotopomers (MacDonald et al., 1993; Kim et al., 1998; Kim and Barry, 1998a).

An alternative difference FTIR spectrum has been assigned to Z·-minus-Z (Berthomieu et al., 1998). This spectrum lacks a positive 1478 cm^{-1} line. These data were obtained on PSII in a buffer containing 50 mM MES, pH 6.0, 10 mM NaCl, 5 mM $MgCl_2$, and approximately 10 mM sorbitol and 100 mM potassium ferricyanide. We have obtained EPR data under these conditions and found that the photo-induced yield of Z· is low when compared to the conditions we employ (data not shown). This is expected, based on studies of buffer effects on PSII structure (Kim and Barry, 1998b). Significantly, the spectrum reported (Berthomieu et al., 1998) was obtained by data acquisition in 3.6-s intervals before and 1 s after illumination at a repetition rate of 0.02 Hz. The amount of Z remaining oxidized during this time regime was estimated to be only 15–20%.

In our PSII samples, a 15–20% level of Z· oxidation is obtained in EPR kinetic transients recorded more than 6 s after illumination (Fig. 2 *B*). The difference FTIR spectra, D4-minus-D6 and D5-minus-D6, were constructed from data obtained after 6 s (Fig. 5). These spectra lack an intense 1478 cm^{-1} spectral feature (Fig. 4, *D* and *E*), because the decay of Z· is essentially complete. This observation may explain the failure of Berthomieu et al. (1998) to observe a 1478 cm^{-1} line.

A 1512 cm^{-1} line in the data of Berthomieu et al. (1998) was assigned to the C-O vibration of Z· (Berthomieu et al., 1998). This feature may correspond to the 1508 cm^{-1} line observed in Fig. 4 *A-D*. This positive 1512 cm^{-1} line was downshifted upon $^{13}C_1$ (at position 4) tyrosine labeling (Berthomieu et al., 1998). When $^{13}C_6$-labeled tyrosine was used, this line did not shift, and no change of amplitude was observed at 1512 cm^{-1}. This result is inconsistent with the assignment of this line to the C-O stretch of Z· (discussed in Kim et al., 1998), but may be consistent with the assignment of the 1512 cm^{-1} line to a ring stretching vibration of the tyrosine ring (Dollinger et al., 1986). This tyrosine may be located in the environment of Z.

The identities of the Q_A^- and Q_A contributions to the difference FTIR spectrum are important. By isotopic labeling of plastoquinone and comparison to model compound data, ring and CO vibrations of Q_A^- have recently been assigned to lines at 1482 and 1469 cm^{-1} (Razeyhifard et al., 1999). Any overlapping Q_A^- contributions at 1478 cm^{-1} would decay too rapidly to be detected in our FTIR experiments.

The data presented in Fig. 4 resemble previous difference FTIR data recorded with time resolution in the millisecond time regime (Zhang et al., 1997). This work was the first reported FTIR study of PSII with this time resolution and, thus, represents an important breakthrough in kinetic studies of this enzyme. In this previous work, a small difference in the decay kinetics of the 1478 cm^{-1} line, in the presence and absence of DCMU, was observed. This observation was used to conclude that the entire 1478 cm^{-1} feature arises from Q_A^- (Zhang et al., 1997). However, with an alternative explanation, based on recent results in the literature (Kim and Barry, 1998a), these previous results are consistent with our findings. The FTIR data, obtained in the presence and absence of DCMU, were obtained with different repetition rates (Zhang et al., 1997). It has recently been shown that both the Z· and D· radicals contribute intensity to the 1478 cm^{-1} spectral feature (Kim and Barry, 1998a). Therefore, a difference in repetition rate could cause a small change in the D· contribution to the spectrum and could account for an apparent difference in the decay rate of the 1478 cm^{-1} line (Zhang et al., 1997). Alternatively, overlapping Q_A^- and Z· contributions at 1478 cm^{-1} (see discussion of Q_A^- assignments, above) may have been the foundation of the kinetic differences observed on this faster time scale.

Difference infrared spectroscopy provides a new technique to use in the study of proton and electron transfer events in PSII. Our experiments have confirmed the assignment of a component of the positive 1478 cm^{-1} line to the C-O stretching vibration of Z· (MacDonald and Barry, 1992; MacDonald et al., 1993; Bernard et al., 1995; Kim et al., 1997; Kim et al., 1998; Kim and Barry, 1998a). The identification of the vibrational spectrum of Z· will allow the use of FTIR spectroscopy to study proton and electron transfer events in PSII. This work will give new information concerning the mechanism of photosynthetic water oxidation. In addition, an eventual normal coordinate analysis will yield a complete structural model of Z· and Z.

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