A difference Fourier-transform infrared study of two redox-active tyrosine residues in photosystem II

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Communicated by Joseph J. Katz, August 23, 1993

ABSTRACT Photosystem II, the photosynthetic water-oxidizing complex, contains two redox-active tyrosine residues. Although current models suggest that these tyrosines are located in symmetric positions in the reaction center, there are functional differences between them. To elucidate those structural factors that give rise to this functional asymmetry, we have used difference Fourier-transform infrared spectroscopy to obtain the vibrational difference spectrum associated with the oxidation of each of these redox-active residues. Isotopic labeling was employed to definitively assign vibrational lines to the redox-active tyrosines. This work has shown that the vibrational spectra of the two redox-active species are significantly different from each other. This result suggests that the structure of the redox-active residue helps to determine its role in electron transfer in the reaction center.

Photosystem II (PSII) is the photosynthetic membrane complex that carries out the light-induced oxidation of water and reduction of plastoquinone in plants, green algae, and prokaryotic cyanobacteria. Isotopic labeling and electron paramagnetic resonance (EPR) spectroscopy have been used to show that PSII contains two redox-active tyrosine residues (1, 2). One of these tyrosines, Z, is involved in electron transfer between the primary chlorophyll donor, P680, and the manganese-containing catalytic site (for review, see ref. 3). The other tyrosine, D, is not directly involved in the electron-transfer events that lead to oxygen evolution; the function of D is not known (4). These tyrosines are believed to be symmetrically located in the reaction center (5–8).

The oxidized forms of D and Z, D+ and Z+, are neutral radicals that give rise to characteristic and similar EPR signals (1, 2). These EPR signals have been used to show that D and Z have different oxidation and reduction kinetics. Z is oxidized by P680+ (9), and Z+ is, in turn, reduced by the manganese cluster (10, 11). If the metal cluster is removed, the reduction of Z+ is slowed to the millisecond time regime (12–14). On the other hand, D is usually oxidized by the manganese cluster with a 1-sec rise time (15). In the absence of a functional manganese cluster, D can be oxidized by P680+ (16, 17). The decay time of D+ is on the order of several hours (18).

In addition to the kinetic differences described above, there are other functional differences between the Z and D tyrosines. For example, the redox potential of D has been measured to be 0.76 V (19), whereas the potential of Z has been estimated to be about 1 V (20). Further, the two radicals show different accessibility to exogenous reductants (21).

This functional asymmetry may be due to differences in the structure of the two redox-active residues. The EPR spectra of Z- and D- do not give detailed insight into this question. Since these radicals are immobilized, their EPR lineshapes are broadened by anisotropic interactions, and small structural alterations would not be readily observable. Recently, a small difference in EPR lineshape has been observed between specifically deuterated Z- and D-. This difference has been used to argue that the structures of the two radicals may be slightly different from each other (2). However, the nature of the structural alteration could not be conclusively identified by this technique.

To gain more detailed information about the structure of the D and Z tyrosines and their interaction with the environment, a high-resolution spectroscopic technique is necessary. To this end, we are using difference (light-minus-dark) Fourier-transform infrared (FTIR) spectroscopy. IR vibrational spectroscopy is sensitive to the geometry and protonation state of molecules. Light-minus-dark difference spectroscopy makes this technique specific for the geometric and protonation changes that accompany electron transfer, since a molecule must undergo a structural change in order to contribute to the difference spectrum. To definitively assign vibrational modes to tyrosine, it is essential to isotopically label tyrosine in these preparations. Isotopic labeling and difference FTIR have been used previously to obtain information about proton pumping mechanism in bacteriorhodopsin (reviewed in ref. 22). Difference IR spectroscopy has also been used to study light-driven electron transfer reactions in other photosynthetic proteins (reviewed in ref. 23).

In this study, we have used isotopic labeling and FTIR spectroscopy to obtain the vibrational difference spectrum associated with the oxidation of both the D and Z redox-active tyrosines.

MATERIALS AND METHODS

Cyanobacterial Culture Growth, Isotopic Labeling, and Protein Purification. Cultures of the cyanobacterium Synechocystis sp. PCC 6803 were grown on control tyrosine, l-[3H]tyrosine (1.4-hydroxyphenyl-d3-alanine, 99.6% labeled), or l-[13C]tyrosine (1.4-hydroxyphenol-13C6-alanine, 99% labeled) as described (1). Isotopically labeled tyrosine was from Cambridge Isotope Laboratories (Cambridge, MA). Samples were purified (2) and depleted of manganese (24).

EPR Spectroscopy. Samples were dried (24) and contained 105 μg of chlorophyll, 1.5 mM potassium ferricyanide, and 1.5 mM potassium ferrocyanide. Spectra were recorded at −9 ± 3°C as described (24). Spectral conditions were as follows: microwave frequency, 9.1 GHz; modulation amplitude, 3.2 G; microwave power, 0.67 mW; scan time, 4 min; time constant, 2 sec; gain, 1.6 × 104.

Spin quantitation was performed at −160°C and at room temperature by using Fremy’s salt as a spin standard (25). Samples for spin quantitation at −160°C were illuminated and dark adapted for 8 min at −9°C and then frozen in darkness to −160°C. Spin quantitation showed that there was one D-spin per 90 chlorophylls after 8 min of dark adaptation, in

Abbreviations: FTIR, Fourier-transform infrared; PSI, photosystem I; PSII, photosystem II.
reasonable agreement with room-temperature spin quantitation, which gave one D- spin per 70 chlorophylls. The difference in D- content under the two conditions was not due to D- decay during the 8-min dark adaptation, but to the conditions used for the dehydration of the sample (data not shown).

In addition to the experiment shown here (Fig. 1), EPR experiments were also performed on photosystem II samples labeled with [3H]- or [13C]tyrosine. Similar decay kinetics were obtained (data not shown). These data also show that the labeling was complete, in agreement with earlier isotope incorporation studies (1, 2). A distortion in the middle of the spectra in Fig. 1 was caused by a small amount of contamination from the oxidized primary donor, P700+, of photosystem I (PSI, see text). This distortion was not present in room-temperature EPR spectra of this preparation (2).

**FTIR Spectroscopy on PSII Samples.** Each sample contained 35 µg of chlorophyll, 1.5 mM potassium ferricyanide, and 1.5 mM potassium ferrocyanide and was dried (24). The potassium ferrocyanide and ferricyanide mixture has no vibrational modes in the spectral region of interest here (1900–1200 cm⁻¹) but does obscure the 2000-cm⁻¹ region (24). The absorbance in the amide I band at 1655 cm⁻¹ was always <0.9 absorbance units. Spectral conditions were as described (24). The spectral resolution was 4 cm⁻¹, data were recorded at −9 ± 1°C, the mirror velocity was 1.57 cm/sec, and 1000 scans were co-added for each interferogram. A dark spectrum was subtracted from a light spectrum to construct each difference spectrum. Seven to 11 such light-minus-dark difference spectra were then averaged. The spectra were reproducible in intensity and frequency from sample to sample.

**FTIR Spectroscopy on PSI Samples.** PSI was isolated as described (26). The amount of contaminating P700 in the PSI samples that were used in Figs. 2 and 4 was quantitated as described (16). To facilitate comparison, PSI samples for infrared spectroscopy contained approximately the same number of P700 molecules as the PSII samples. PSI samples contained 4 µg of chlorophyll, 1.5 mM potassium ferricyanide, and 1.5 mM potassium ferrocyanide and were dried as described (24). Six difference spectra were averaged for A and for B.

**RESULTS AND DISCUSSION**

Illumination of manganese-depleted PSI preparations results in stable charge separation between Z and the quinone acceptor QA, thus generating the state Z-QX. In the dark, relaxation occurs and generates the resting, neutral states of all electron-transfer intermediates. We can make use of the different kinetics of decay for the two redox-active tyrosines to obtain a vibrational difference (light-minus-dark) spectrum to which both redox-active tyrosines contribute or to which only Z contributes. For example, with a short, 8-min dark adaptation between the light and dark spectra, Z will make a positive contribution and Z will make a negative contribution to the difference spectrum. The difference spectrum of D- minus D will not be observed under these conditions, since D- does not decay significantly during the short dark adaptation. With a long, 1.5-hr dark adaptation that allows the decay of D-, the difference spectra associated with the oxidation of both D and Z will appear in the spectrum. If there are structural differences between the two tyrosine residues or radicals, the vibrational spectra of the two redox-active residues will reflect these differences.

The FTIR spectrum will also exhibit the vibrational difference spectrum of any other residue or prosthetic group that undergoes a light-induced structural change. For example, the spectrum will exhibit lines due to QA reduction. However, previous work has shown that plastoquinone is not labeled from [3H]tyrosine (1), and so isotopic labeling of tyrosine will have no effect on the quinone vibrations.

Fig. 1 shows an EPR experiment that illustrates the decay kinetics of the two radicals in manganese-depleted PSI preparations from *Synechocystis* sp. PCC 6803. These data were recorded under conditions identical to those used in the FTIR studies. In the light, we obtain a spectrum (Fig. 1, solid line) that is a composite of the Z and D tyrosine radicals. After 8 min of dark adaptation, the Z-signal decays, leaving the signal of D- alone (Fig. 1, dot-dashed line). After 1.5 hr of dark adaptation, 24% of the D- signal has decayed (Fig. 1, dashed line). Spin quantification also shows that 54% of the centers generate Z- in the light (Fig. 1, compare solid and dot-dashed line).

Fig. 2 shows difference FTIR data on cyanobacterial PSI preparations. There was an 8-min dark adaptation between the light and dark scans. Z and Z- will contribute to this spectrum, but D and D- will not. 2H (Fig. 2, spectrum B) and 13C (spectrum C) labeling of tyrosine will change the intensities of the Z tyrosine residue (negative) and Z- tyrosine radical (positive) vibrational modes.

Comparison of Fig. 2 spectra A–C shows a negative mode at 1657 cm⁻¹ that decreases in intensity upon 2H or 13C labeling. We assign this mode to the tyrosine residue Z. Remaining intensity in this region could be due to a small amide I contribution. The amide I band arises from the C=O stretching vibration of the peptide backbone (27). New intensity at 1630–1620 cm⁻¹ in Fig. 2, spectrum C, is probably due to downshifted 13C modes. A negative mode at 1522 cm⁻¹ is reduced in intensity by 2H labeling (spectrum B); we assign this mode to Z, also. This is especially apparent in the overlay of the control (dashed line) and 2H (solid line) spectra A in Fig. 3 (arrow). It is difficult for us to use this region of the 13C spectrum for assignments (Fig. 2, spectrum C), since changes occur due to overlapping, downshifted 13C lines. The bands at 1657 and 1522 cm⁻¹ have reasonable frequencies to be ring modes of the tyrosine residue (28). In Fig. 2, spectrum C, the origin of the new negative line at ~1670 cm⁻¹ is unknown. A positive mode at 1480 cm⁻¹ decreases in intensity upon 2H labeling (Fig. 2, spectrum B, and Fig. 3, spectra A, arrow); we assign this mode to the oxidized tyrosine radical, Z-.

Resonance Raman studies of phenoxyl radical in *vitro* show two modes from the oxidized species in this spectral region, a ring stretching mode (υ8a) at 1552 cm⁻¹ and the C−O stretch (υ7a) at 1504 cm⁻¹ (29, 30). Comparable modes have

![Fig. 1. Low-temperature (-9°C) EPR of manganese-depleted *Synechocystis* PSI particles that were isolated from cells grown on unlabeled, control tyrosine. The solid line is the light-induced spectrum; the dot-dashed and dashed lines are the spectra obtained after 8 min and 1.5 hr of dark adaptation, respectively.](image-url)
In particular, Fig. 3, 1900 1800 effect of cm-'1450 perdeuteration, C-O stretching comparisons, we radical in A, arrow) has been observed after 8 min of dark adaptation.

C-O stretching mode of the Z- minus-ZD- spectrum (Fig. 2, spectrum A, arrow) or at the low-frequency side of the intense 1550 cm-'1 line.

Isotopic labeling should result in the appearance of new vibrational lines at different, probably lower, frequencies. Upon perdeuteration, the C-O stretching mode of the phenoxy radical in vitro downshifted 16 cm-'1 (29). There has been no comparable study of v8a for the phenoxy radical, but the v8a mode of the p-benzoquinone radical shifted 25 cm-'1 upon perdeuteration (32). There is little existing information about the effects of 13C labeling of phenoxy radicals in vitro. Also, to date, there has been no detailed study of the effect of isotopic substitution on tyrosine vibrational modes (33).

New positive and negative modes are observed in our spectra upon isotopic labeling (Fig. 2 and Fig. 3, spectra A). In particular, Fig. 3, spectra A, shows a new positive line at \( \approx 1450 \text{ cm}^{-1} \) in the \(^2\)H spectrum (arrow), and Fig. 2, spectrum C, shows a new positive line at \( \approx 1400 \text{ cm}^{-1} \). Since these are difference spectra, cancellations can occur. Therefore, it is premature for us to attempt assignments of these lines without more in vitro data. Note that there is still a vibrational mode at 1480 cm-'1 in Fig. 2, spectra B and C. A likely origin for this mode is the C-O bond of QX (32, 34).

We observe several negative lines in the region from 1264 to 1238 cm-'1 that are affected by isotopic labeling; one of these modes is likely to be the C-OH stretching mode of the Z tyrosine residue (29, 30, 35). The large change in frequency (from approximately 1260 to 1480 cm-'1) for this mode upon tyrosine oxidation is due to the delocalization of electron spin onto the phenol oxygen in the radical (29, 30).

Fig. 4 shows difference FTIR data recorded on the same sample as in Fig. 2, but with a 1.5-hr dark incubation between the dark and light scans. This lengthy dark adaptation allows both D and Z to contribute to the difference spectrum. The vibrational difference spectrum of Z will be in common between Fig. 2, spectrum A, and Fig. 4, spectrum A; an overlay of the two spectra is shown in Fig. 3, spectra B. The D- minus-D spectrum will account for unique vibrational lines. As we will discuss below, at our present signal/noise ratio, we can identify one unique mode from D and one from D- (Fig. 3, spectra B, arrows). Note that the intensity of the D and D- contributions will be smaller than the Z and Z- contributions, since D- decays by only 24% in 1.5 hr (see discussion of Fig. 1).

Upon \(^2\)H (Fig. 4, spectrum B) or \(^1\)H (spectrum C) incorporation into tyrosine, negative lines at 1657 and 1524 cm-'1 decrease in intensity. Similar lines were also found in Fig. 2, where we assigned them to Z. However, the 1524 cm-'1 mode has a slightly different frequency when Figs. 2 and 4 are compared. This might be due to an alteration of the Z vibrational spectrum, caused by oxidation of D.
Therefore, reappears.

Subtraction of the data in Figs. 2-4 to make D and Z assignments. We see little intensity in the regions that we have assigned to tyrosine modes, and we observe only small changes in the PSII spectrum with length of dark adaptation (Fig. 5, spectrum C). For example, the small PSII line at 1534 cm\(^{-1}\) is not changed in intensity by long dark adaptation (Fig. 5, spectrum C), while the PSII line that we have assigned to D is changed in intensity. Also, IR spectra of PSII particles, which were isolated from cyanobacteria labeled with \([\text{H}]\) or \([^{13}\text{C}]\)tyrosine, show no isotopic shifts in the regions of interest (data not shown). Mass spectral analysis shows that chlorophyll is not labeled from \([\text{H}]\)tyrosine (data not shown).

Our experiments show that the vibrational modes of the two tyrosines, D and Z, and the two tyrosine radicals, D- and Z-, are not identical. These results suggest that there are significant structural differences between D and Z in both the oxidized and the unoxidized form. To pinpoint and describe these structural alterations, we need an assignment of per-

Fig. 5. Light-minus-dark difference FTIR spectra of purified PSI. The tick marks on the y axis correspond to \(4 \times 10^{-4}\) absorbance unit. Spectrum A: light spectrum minus a dark spectrum recorded after 8 min of dark adaptation. Spectrum B: light spectrum minus a dark spectrum recorded after 1.5 hr of dark adaptation. Spectrum C: spectrum B minus spectrum A.
turbed vibrational modes to particular atomic displacements in the molecules. As yet there has been no detailed normal coordinate analysis for tyrosine, and there has been no infrared study of tyrosine oxidation in vitro. Therefore, definitive assignments of our spectra are not possible without further studies.

However, we can speculate on the cause of the different vibrational frequencies that we observe for the D and Z tyrosine radicals. One possibility is that perturbation of the ring modes (38) or the C—O stretching vibrations (29, 31) of the radicals could be caused by a difference in the strength of a hydrogen bond to the phenol oxygen of Z and D. While D is known to be a neutral, but hydrogen-bonded radical from a variety of studies (1, 39–42), there is very little experimental information about the hydrogen-bonding characteristics of the neutral radical Z. Modeling studies of the donor side of PSII have suggested a difference in the strength of a hydrogen bond to D and Z (7, 8). A change in the strength of such an interaction could affect the pKa of the phenol oxygen and, thus, could explain the lower midpoint potential of D as compared with Z (24). It should be noted that Raman and resonance Raman studies of the effect of hydrogen bonding on the vibrational spectrum of tyrosine and tyrosine model compounds have been reported (43, 44), but there has been no comparable study of the vibrational spectrum of the oxidized species.

In summary, we have used isotopic labeling to definitively assign vibrational modes in the difference infrared spectrum to the redox-active tyrosines, D and Z. We have obtained evidence for a structural difference between these two tyrosines that may be consistent with a difference in the strength of a hydrogen bond to each phenol oxygen.

We thank Dr. Renee Boerner for advice concerning protein purification and cyanobacterial culture growth. This work was supported by the National Institutes of Health Grant GM43273, by a University of Minnesota-McKnight award to B.A.B., and by a National Institutes of Health training grant award to G.M.M. (GM08277). Acknowledgment is also made of support from the donors of the Petroleum Research Fund, which is administered by the American Chemical Society.