Alterations in Carboxylate Ligation at the Active Site of Photosystem II*

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Photosystem II (PSII) is the photosynthetic enzyme catalyzing the oxidation of water and reduction of plastoquinone (Q). This reaction occurs at a catalytic site containing four manganese atoms and cycling among five oxidation states, the $S_n$ states, where $n$ refers to the number of oxidizing equivalents stored. Biochemical and spectroscopic techniques have been used previously to conclude that aspartate 170 in the D1 subunit influences the structure and function of the PSII active site (Boerner, R. J., Nguyen, A. P., Barry, B. A., and Debus, R. J. (1992) Biochemistry 31, 6660–6672). Substitution of glutamate for aspartate 170 resulted in an assembled manganese cluster, which was capable of enzymatic turnover, but at lower steady-state oxygen evolution rates. Here, we obtained the difference (light-minus-dark) Fourier transform IR spectrum associated with the $S_n Q_0$-minus-$S_1 Q_0$ transition by illumination of oxygen-evolving wild-type and DE170D1 PSII preparations at 200 K. These spectra are known to be dominated by contributions from carboxylate and carboxylic acid side chains that are close to or ligating the manganese cluster. Substitution of glutamate for aspartate 170 results in alterations in the $S_n Q_0$-minus-$S_1 Q_0$ spectrum; the alterations are consistent with a change in carboxylate coordination to manganese or calcium. In particular, the spectra are consistent with a shift from bridging/bidentate carboxylates in wild-type PSII to unidentate carboxylate ligation in DE170D1 PSII.

Photons in PSII are responsible for the light-driven oxidation of water and the reduction of quinone in plants, green algae, and cyanobacteria. The catalytic site contains a tetranuclear manganese cluster, and the catalytic cycle of water oxidation has been proposed to consist of five oxidation states ($S_n$) called the Joliot-Kok $S_0$ states. The subscript $n$ refers to the number of oxidizing equivalents stored at the active site. Absorption of four photons and four corresponding charge separations are necessary to complete one catalytic cycle. Each cycle results in the oxidation of two water molecules to form one molecule of molecular oxygen and four protons. Calcium is required for oxygen evolution and may be bound in proximity to the manganese cluster (reviewed in Refs. 1 and 2).

Many studies of PSII have focused on the $S_2$ state (for review, see Ref. 3). Dark adaptation at room temperature sets the catalytic center to the $S_1$ state because $S_0$ converts to $S_1$ by reduction of tyrosine radical, $D^−$ (4, 5). Two different EPR signals, a $g = 2$ multiline signal (6) and a $g = 4.1$ signal (7), are observed from the $S_0$ state. Illumination of plant or cyanobacterial PSII at 200 K results in the formation of the $g = 2$ multiline EPR signal (for examples, see Refs. 6 and 8) and an EPR signal from Fe$^{2+} Q_0^-$ (9). The $S_1$-to-$S_2$ transition is associated with oxidation of manganese, probably from Mn$^{3+}$ to Mn$^{4+}$ (reviewed in Ref. 10). Recently, we have obtained the difference FT-IR spectrum associated with the $S_1$-to-$S_2$ transition and have shown that this spectrum is dominated by contributions from carboxylate and carboxylic acid side chains that are close to or ligating the metal cluster (12–14).

A structural model for the manganese cluster in the $S_1$ state has been suggested; this model is based on x-ray absorption studies of the manganese cluster (reviewed in Ref. 10). According to this model, the manganese cluster has been proposed to contain four manganese atoms arranged in two bis-μ-oxo-bridged dimers. These dimers are linked by two carboxylato bridges and one bis-μ-oxo bridge to form a C-shaped tetranuclear metal center. Because each manganese atom must be five- or six-coordinate (15), amino acids must provide ligation to the metal atoms. Site-directed mutagenesis suggests that some of these ligands are provided by aspartate and glutamate residues in the luminal regions of the D1 and D2 polypeptides (reviewed in Ref. 16). Chemical modification experiments support this conclusion (17). Ligation by carboxylate groups would have the effect of stabilizing high oxidation states of the manganese ions (15).

Mutations have been made in carboxylate residues in the D1 polypeptide, with multiple substitutions generated at each site (for reviews, see Refs. 16, 18, and 19). The effect of mutations on oxygen evolution activity, photoautotrophic growth, and the variable fluorescence yield was assessed. From this work, likely metal ligands have been identified (reviewed in Refs. 16, 18, and 19).

The most thoroughly characterized mutations in this group were those introduced at aspartate 170 of the D1 polypeptide (20, 21). Eleven different amino acids were substituted for aspartate 170. All mutations, except glutamate and histidine, abolished photoautotrophic growth (21). Most mutations resulted in dramatic decreases in oxygen evolution activity (21), although residual low levels of oxygen evolution were detected in the DC170D1, DW170D1, DR170D1, DY170D1, and DM170D1 mutants (21). PSII particles containing the...
DN170D1 mutation had no detectable activity and showed electron transfer kinetics that were consistent with the absence of a functional manganese cluster (20). The manganese content of the DN170D1 mutant was reduced dramatically compared with manganese content in wild-type PSII (20).

On the other hand, an aspartate-to-glutamate mutation did not abolish oxygen evolution, but the mutation resulted in a 2–3-fold decrease in the steady-state rate of oxygen evolution activity (20, 21). Spectroscopic characterization showed that a functional manganese cluster was assembled in this mutant (20, 21). The manganese content of the DE170D1 mutant was similar to the manganese content of wild-type PSII (20). Characterization of DE170D1 cells showed that oxygen evolution exhibited a period 4 oscillation with flash number; this oscillation was similar to the pattern observed in wild-type cells (21). The lifetime and oxidation kinetics of the S\textsubscript{2} state were perturbed (20, 21), giving a slightly more stable S\textsubscript{2} state in the mutant.

From the studies described above (20, 21), it was concluded that aspartate 170 influences the assembly and/or activity of the active site (see also Ref. 17). To investigate the mechanism by which aspartate 170 exerts its influence on the PSII catalytic site, we have employed vibrational spectroscopy. Use of oxygen-evolving PSII particles from the DE170D1 mutant provides an opportunity to obtain information about the catalytic site, we have employed vibrational spectroscopy. Use of oxygen-evolving PSII particles from the DE170D1 mutant provides an opportunity to obtain information about the catalytic site in a perturbed but active form. We report here the differences in carboxylate ligation at the active site of PSII.

RESULTS

Our previous work has shown that the difference FT-IR spectrum, S\textsubscript{2}QA-to-S\textsubscript{1}QA, can be acquired by 7.5 min of continuous illumination at 200 K (12–14). In Fig. 1, we present such S\textsubscript{2}QA-to-S\textsubscript{1}QA FT-IR spectra, and accompanying negative controls, obtained from spinach PSII complexes. To show that illumination for 7.5 min with red- and heat-filtered light has no deleterious effects on the sample, Fig. 1 presents an experiment conducted with shorter, 5-min illumination times. Fig. 1A presents the dark-minus-dark negative control experiment, which, as expected, gives a flat baseline. The first light-minus-dark difference spectrum (Fig. 1B) was acquired with 5 min of illumination. These data are similar to difference spectra obtained with 7.5 min of illumination from spinach PSII (Fig. 2A). Illumination for an additional 5 min (Fig. 1C) generates a difference spectrum indistinguishable from data obtained during the first 5 min of illumination (Fig. 1B).

Fig. 1. The light-minus-dark difference FT-IR spectra obtained at 200 K from spinach PSII complexes are shown in B and C. Data were obtained with 5 min of illumination, and the data acquisition scheme was as follows: dark (1), dark (2), light (1), light (2). Here, (1) and (2) refer to the order in which spectra were acquired. The dark (1)-minus-dark (2) (A) and light (1)-minus-light (2) (D) controls are also shown. The tick marks on the y axis represent \Delta A = 1 \times 10^{-3} absorbance unit. Other spectral conditions are given under “Experimental Procedures.” Figs. 2, 3, and 5. The absorbance of the amide I band at 1655 cm\textsuperscript{-1} was <0.9 absorbance units. Spectra were normalized to an amide II absorbance of 0.5 absorbance units. Such a normalization is equivalent to a correction for protein concentration and path length (12, 13, 27).

EPR spectra were obtained on a Bruker EMX spectrometer equipped with a temperature-controlled Wilmad flow-through Dewar inserts. A stream of cold nitrogen was used to maintain the EPR sample temperature at 80 K; the sample temperature was continuously monitored (22). PSII samples containing 1 eq of potassium ferricyanide were frozen in liquid nitrogen, and EPR spectra, corresponding to a dark scan, were recorded. PSII samples were then illuminated at 200 K with red- and heat-filtered light for 7.5 min and re-equilibrated in liquid nitrogen. EPR spectra, corresponding to the light scan, were then recorded. Spectra were obtained with the following instrument settings: microwave frequency, 9.383 GHz; microwave power, 1 milliwatt; modulation amplitude, 3.2 G; and time constant, 1.3 s. Twelve scans of 83 s were recorded and averaged. EPR spectra were integrated and analyzed through the use of the program IGOR (Wavemetrics, Lake Oswego, OR).

EXPERIMENTAL PROCEDURES

Chlorophyll and oxygen evolution assays were performed (22). Spinach PSII complexes were isolated (23). The steady oxygen evolution rates (22) of this preparation were \( \pm 1000 \mu\text{mol} \text{ of } O_2 \text{ (mg of Chl-h)}^{-1} \text{ h}^{-1} \). Construction of a wild-type kanamycin-resistant strain of Synechocystis sp. PCC 6803 and the DE170D1 mutant has been described (20). Cyanobacterial PSII particles were purified (20, 22, 24). Wild-type cells had average oxygen rates of 480 \( \mu\text{mol} \text{ of } O_2 \text{ (mg of Chl-h)}^{-1} \text{ h}^{-1} \). Wild-type PSII particles, employed for FT-IR experiments, exhibited rates of oxygen evolution of \( \sim 2000 \mu\text{mol} \text{ of } O_2 \text{ (mg of Chl-h)}^{-1} \), and DE170D1 PSII particles, employed for FT-IR studies, exhibited rates of \( \sim 500 \mu\text{mol} \text{ of } O_2 \text{ (mg of Chl-h)}^{-1} \). These steady-state oxygen evolution rates are in the range of values previously obtained (20). We have also shown that the chlorophyll antenna sizes of the wild-type and DE170D1 PSII preparations are similar (20). Hydroxylamine treatment of spinach and cyanobacterial PSII was performed (25).

Infrared spectra were recorded on a Nicolet 60-SX spectrometer equipped with a liquid nitrogen-cooled MCT-B detector (12, 13). The sample temperature was controlled to \( \pm 0.3 \text{ K} \) with a High-Tran liquid nitrogen cryostat (12, 13). The temperature was continuously monitored during data acquisition. The liquid nitrogen cryostat was equipped with TnSe (see Fig. 1) or CaF\textsubscript{2} windows (see Figs. 2, 3, and 5). Illumination was provided with a Dolan-Jenner fiber optic annular illuminator equipped with a red filter and a heat filter. Spectral resolution was \( 8 \text{ cm}^{-1} \) in Fig. 1 and \( 4 \text{ cm}^{-1} \) in Figs. 2, 3, and 5. A Happ-Genzel apodization function was used; two (Fig. 1) or three levels (Figs. 2, 3, and 5) of zero filling were employed; double-sided interferograms were collected; and the mirror velocity was \( 1.57 \text{ cm/s} \).

To construct a difference FT-IR spectrum, an interferogram recorded under illumination was ratioed directly to interferograms recorded in the dark before illumination. The resulting difference spectra were then averaged. The length of the scans was 5 min in Fig. 1 and 7.5 min in...
Our previous experiments, in which spectra were obtained as a function of illumination temperature, have led to the conclusion that the $S_2Q_A$--minus-$S_1Q_A$ spectra are dominated by contributions from the donor side (12–14). This conclusion was reached on the basis of alterations in the electron donor, changes in the temperature of illumination, and manganese removal (see Fig. 2, for example). We have recently identified $Q_A^-$ and $Q_A$ contributions to the spectrum through the use of isotopic labeling and confirmed that these vibrational lines are small in amplitude compared with the overall intensity of the spectrum.

**Difference FT-IR Studies of Oxygen-evolving Wild-type and DE170D1 PSII**—In Fig. 3, we present the results of 200 K illumination of oxygen-evolving PSII preparations isolated from the cyanobacterium *Synechocystis* sp. PCC 6803. The cyanobacterial $S_2Q_A$--minus-$S_1Q_A$ spectrum (Fig. 3A) is similar to data obtained from spinach PSII particles (Figs. 1B and 1A). Previous global $^{15}$N labeling experiments have led to the conclusion that the cyanobacterial spectrum is dominated by contributions from glutamic/aspartic acid and glutamate/aspartate residues that are ligating or close to the manganese cluster (12–14).²

In Fig. 3B (solid line), we present a difference FT-IR spectrum obtained by illumination at 200 K of oxygen-evolving PSII particles of the DE170D1 mutant. The spectrum exhibits an overall resemblance to the wild-type cyanobacterial $S_2Q_A$--minus-$S_1Q_A$ spectrum (Fig. 3A). Although we have not observed a $g = 2$ multiline signal from this mutant, our previous characterizations have shown that this mutant contains a functional manganese cluster and is capable of advancement from the $S_1$--to-$S_2$ state (20). Also, we have observed an $Fe^{2+}Q_A^-$ EPR signal upon 200 K illumination of DE170D1 PSII (data not shown); this result indicates that charge separation has occurred upon illumination.

Although there is an overall resemblance between $S_2Q_A$--minus-$S_1Q_A$ spectra obtained from wild-type and DE170D1 PSII, a detailed comparison of spectra obtained from mutant (Fig. 3B, solid line) and wild-type (dashed line) PSII revealed

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² M. R. Razeghifard, J. J. Steenhuis, S. Kim, J. Patzlaff, R. S. Hutchison, I. Ayala, and B. A. Barry, manuscript in preparation.
spectral changes in the carbonyl stretching region (1720 to 1670 cm$^{-1}$) and broad spectral changes between 1600 and 1400 cm$^{-1}$. These spectral alterations will be discussed in detail below.

**EPR Studies of Oxygen-evolving Wild-type and DE170D1 PSII**—When PSII preparations are illuminated at 200 K in glycerol-containing buffers, there are three possible donors to P$680^-$: the S$_1$ state, tyrosine D, and Chl (12–14). Cytochrome $b_{595}$ is low potential in these preparations and already oxidized (20, 31). Redox-active tyrosine D is preoxidized in both wild-type (12–14) and mutant PSII and does not significantly contribute to the light-minus-dark difference EPR spectrum (Fig. 4, A and B). In centers that lack a manganese cluster or are inhibited in oxygen evolution, 200 K illumination will oxidize Chl, and not the S$_1$ state of the manganese cluster (12–14, 28, 29).

To obtain an indirect measure of the amount of S$_2$ formation in the DE170D1 mutant, an EPR control experiment was performed to record the amount of Chl cation radical generated by 200 K illumination. These data are shown in Fig. 4. In wild-type cyanobacterial PSII (Fig. 4, A and a), EPR spin quantitation showed that the amount of Chl cation radical generated is 0.2 ± 0.1 spin/tyrosyl radical D$^-$. This result is similar to the amount of Chl$^+$ generated in spinach PSII complexes at 200 K, which has been reported as 0.33 ± 0.09 spin/D$^-$ (13). In DE170D1 PSII (Fig. 4, B and b), EPR spin quantitation showed that the amount of Chl cation radical generated at 200 K is ~2-fold the amount generated in wild-type PSII, giving again a result similar to the Chl$^+$ content determined in spinach PSII (13). Because this experiment was performed on DE170D1 samples with a somewhat lower oxygen evolution rate (300 μmol of O$_2$ (mg of Chl/h)$^{-1}$) compared with samples employed for FT-IR spectroscopy, this result provides an upper limit for the amount of Chl$^+$ that could be generated in an FT-IR experiment. Wild-type cyanobacterial and plant PSII give rise to an intense S$_2$ multiline signal upon illumination at 200 K (12, 13). Taken together, these results imply that PSII reaction centers in the DE170D1 sample can advance from the S$_1$ to the S$_2$ state.

This conclusion is consistent with previous characterization of these preparations that showed that the amount of variable fluorescence, produced after a single saturating flash to 3-hydroxyephyl-1, 1-dimethylurea containing, dark-adapted samples in the S$_1$ state, was indistinguishable in wild-type and DE170D1 PSII preparations (20). Because variable fluorescence is an indirect measure of the amount of Q$_A^-$ produced in the absence of fluorescence quenchers (20), this experiment suggests that the amount of charge separation is indistinguishable in oxygen-evolving wild-type and DE170D1 PSII. This deduction can explain the intensities of the difference FT-IR spectra obtained, which exhibit an overall similarity when wild-type and mutant PSII are compared (Fig. 3, A and B).

**Difference FT-IR Studies of Hydroxylamine-treated DE170D1 PSII**—Based on the reasoning described above, the light-minus-dark difference FT-IR spectrum obtained by illumination of DE170D1 PSII at 200 K (Fig. 3B) reflects oxidation of the S$_1$ state and not just the oxidation of Chl. To further test this idea, the difference FT-IR spectrum associated with illumination at 200 K was obtained from hydroxylamine-treated DE170D1 PSII (Fig. 5). If the spectrum shown in Fig. 3B reflects the production of the S$_1$ state in the DE170D1 mutant, then manganese removal is expected to change the spectrum (14). The contribution of Chl oxidation to the difference FT-IR spectrum will increase upon hydroxylamine treatment of DE170D1 PSII.

In Fig. 5, we present a comparison of difference FT-IR data obtained from oxygen-evolving DE170D1 PSII (Fig. 5A) and from hydroxylamine-treated, manganese-depleted DE170D1 PSII (Fig. 5B). Alterations in frequency and intensity are observed in the 1770 to 1700 cm$^{-1}$ and 1500 to 1200 cm$^{-1}$ regions when these spectra are compared. Note the apparent increase
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in intensity of a 1477 cm\(^{-1}\) spectral feature, which, under these conditions, can be assigned to Chl\(^+\) (32, 33). Spectra obtained from manganese-depleted DE170D1 PSII (Fig. 5B) are similar to Chl\(^+\)QA\(^-\)-minus-ChlQA data obtained from plant PSII (see Fig. 2 (B and C) and also Refs. 12–14, 32, and 33).

Because the difference spectra in Fig. 5 (A and B) are normalized on a protein and path length basis (27), the full amplitude of QA\(^-\) is expected under both conditions, and the amplitude of these quinone and semiquinone contributions is small (12–14), direct one-to-one subtraction (Fig. 5C) yields a DE170D1 double-difference spectrum, (S\(_2\)/S\(_1\))-(minus)-(Chl/Chl). This double-difference spectrum reflects a contribution from amino acid residues that are close to or ligating the manganese cluster in the DE170D1 mutant, as well as from the oxidation of Chl. This double-difference spectrum (Fig. 5C) exhibits sharp spectral features in the 1734 to 1659 cm\(^{-1}\) region as well as broad spectral features between 1560 and 1339 cm\(^{-1}\). The frequencies of these lines are 1719 cm\(^{-1}\) (positive), 1699 cm\(^{-1}\) (negative), 1684 cm\(^{-1}\) (positive), 1668 cm\(^{-1}\) (negative), 1560 cm\(^{-1}\) (negative), 1462 cm\(^{-1}\) (negative), and 1339 cm\(^{-1}\) (negative). Because the intensities of these lines decrease in amplitude upon removal of the manganese cluster, we assign these lines or a component of these lines to amino acid residues close to the manganese cluster. These natural assignments were confirmed from manganese-depleted DE170D1 mutants. These spectral features have the same sign in both double-difference spectra. Therefore, we assign these features to amino acid residues in the DE170D1 mutant that are perturbed by the S\(_1\)-to-S\(_2\) transition.

Assignment of the Double-difference Spectrum DE170D1-minus-Wild-type—Our aim is the interpretation of the spectral alterations observed in the DE170D1 S\(_2\)-QA\(^-\)-minus-S\(_1\)-QA spectrum (Fig. 3B). This interpretation will yield structural information about the catalytic site of DE170D1. The S\(_2\)-QA\(^-\)-minus-S\(_1\)-QA spectra obtained from wild-type (Fig. 3A) and DE170D1 (Fig. 3B) PSII are complex and are made up of many overlapping positive and negative lines. Therefore, a quantitative comparison of Fig. 3 (A and B) is necessary. These spectra are corrected for any differences in protein concentration and path length (27). A double-difference spectrum, DE170D1 (Fig. 3B)-minus-wild-type (Fig. 3A), will reveal the structural changes induced by the DE170D1 mutation and associated with the S\(_1\)-to-S\(_2\) transition (Fig. 3C). Observed spectral alterations will be due to the substitution of a glutamate for aspartate 170.

As shown in Fig. 3C (striped areas), the double-difference spectrum DE170D1-minus-wild-type exhibits positive lines at 1719 and 1683 cm\(^{-1}\) and negative lines at 1696 and 1669 cm\(^{-1}\). Broad positive spectral features, with center frequencies at 1570 and 1496 cm\(^{-1}\), and a negative spectral feature, with a center frequency at 1543 cm\(^{-1}\), are also observed (Fig. 3C, dotted areas). The narrow line width of the negative 1543 cm\(^{-1}\) may be the result of cancellation of intensity in the double-difference spectrum. An additional broad, less intense negative line may be observed at 1415 cm\(^{-1}\) (Fig. 3C, dotted areas). These spectral features are above the level of noise and base line variations observed in Fig. 3D, which shows the averaged results of wild-type-minus-wild-type and mutant-minus-mutant subtraction.

In the double-difference spectrum constructed from wild-type and DE170D1 data (Fig. 3C), positive features are due to the wild-type S\(_1\) state or the mutant S\(_2\) state, whereas negative features are due to the wild-type S\(_2\) state or the mutant S\(_1\) state. Amino acid residues that are perturbed by oxidation of manganese and by the DE170D1 mutation will contribute to this double-difference spectrum. Thus, contributions from both aspartate/aspartic acid 170 (wild-type) and glutamate/glutamic acid 170 (mutant) would be expected in Fig. 3C, if these residues are ligating or close to the manganese cluster. Also, contributions from other amino acid residues close to or ligating the metal cluster and perturbed by the DE170D1 substitution are expected. Vibrational lines may originate from amino acid residues in the DE170D1 mutant or in wild-type PSII.

These alternatives can be evaluated by comparison with the spectrum shown in Fig. 5C, which compared data obtained from oxygen-evolving and manganese-depleted DE170D1 PSII. Lines assignable to the S\(_1\)-to-S\(_2\) transition in the mutant will be in common and have the same sign when these two double differences (Figs. 3C and 5C) are compared. Another possible contributor to Fig. 3C is vibrational modes of Chl\(^+\)-minus-Chl. In such a case, ester and keto contributions to the spectrum, arising from Chl, should be observed in the 1750 to 1650 cm\(^{-1}\) region (see, for example, Figs. 2 and 5). The EPR control experiments shown in Fig. 4 suggest an increase in Chl\(^+\) content in the mutant when compared with wild-type PSII. However, the spectrum obtained from oxygen-evolving preparations of the DE170D1 mutant exhibits spectral differences when compared with such a Chl\(^+\)QA\(^-\)-minus-ChlQA spectrum (Fig. 5). Lines assignable to Chl\(^+\) will increase in intensity upon manganese depletion.

Assignment of the 1750 to 1670 cm\(^{-1}\) Spectral Region—Spectral features with frequencies of 1719 cm\(^{-1}\) (positive), 1696 cm\(^{-1}\) (negative), 1683 cm\(^{-1}\) (positive), and 1669 cm\(^{-1}\) (negative) are observed in Fig. 3C (striped areas), derived from a comparison of wild-type and DE170D1 PSII. Furthermore, lines with similar frequencies (1719, 1699, 1684, and 1668 cm\(^{-1}\)) and line widths are observed in Fig. 5C (striped areas), derived from a comparison of oxygen-evolving and manganese-depleted DE170D1 mutants. These spectral features have the same sign in both double-difference spectra. Therefore, we assign these spectral features to amino acid residues in the DE170D1 mutant that are perturbed by the S\(_1\)-to-S\(_2\) transition.

Interpretation of the Lines in the 1750 to 1670 cm\(^{-1}\) Spectral Region Assigned to the DE170D1 Mutant—The only amino acid residues with fundamental vibrational transitions in the 1750 to 1670 cm\(^{-1}\) region are glutamic and aspartic acid (34–37). The observation of two different, derivative-shaped lines (1719 (positive)/1696 (negative) cm\(^{-1}\) and 1683 (positive)/1669 (negative) cm\(^{-1}\)) suggests that at least two, and possibly more, glutamic or aspartic acid residues are perturbed in the DE170D1 mutant during the S\(_1\)-to-S\(_2\) transition. One of these perturbed residues may be glutamic acid 170. Because these amino acid residues are perturbed by the S\(_1\)-to-S\(_2\) transition, these amino acid residues must be close to or ligating the metal cluster. There are three types of structural changes that would result in a carboxylic acid contribution to the spectrum. The first is a frequency shift of the C=O stretching vibration, perhaps due to a perturbation in the pKa or hydrogen bonding of these groups (12). This first type of structural change would give rise to derivative-shaped spectral features in the 1750 to 1720 cm\(^{-1}\) region (35). The second type of structural alteration that could give rise to such a spectral contribution is a protonation change (35–37). The C=O and C=O stretches of the carboxylate are intermediate in frequency between the C=O and C=O stretching vibrations of the carboxylic acid (35, 38). The third explanation is that these groups are ligating manganese and that the oxidation of metal causes a frequency shift of vibrational modes, assignable to the ligand (38, 39). On the basis of the pattern of observed frequencies, we favor the third possible explanation of these spectra, as described below.

Frequencies—The frequencies of carboxylic acid C=O stretches reflect changes in double-bond character in the C=O bond as well as changes in the basicity of the oxygen (35). A typical range of frequencies is from 1750 to 1720 cm\(^{-1}\) (35).
Therefore, a frequency of 1719 cm$^{-1}$, as observed in the DE170D1 mutant (Figs. 3C and 5C), can be regarded as typical of the C=O vibration of uncoordinated carboxylic acid groups. Free carboxylic acids give rise to C–O frequencies in the 1300 to 1200 cm$^{-1}$ range; high absorption makes it difficult to interpret this spectral region.

However, the other frequencies, 1696 cm$^{-1}$ (negative), 1683 cm$^{-1}$ (positive), and 1669 cm$^{-1}$ (negative), observed in the DE170D1 mutant (Figs. 3C and 5C) are out of the range expected for the C=O vibration of free carboxylic acid residues in proteins. Because of their low frequency, we attribute these lines to carboxylate ligands to manganese or calcium. Carboxylate groups may ligate metals with unidentate, bidentate, or bridging geometries. An asymmetric CO frequency in the range from 1629 to 1604 cm$^{-1}$ is consistent with unidentate ligation to metals in mononuclear clusters (38, 40). However, the nuclearity and the oxidation state of the metal are expected to influence these frequencies (38–40). The energy gap between the asymmetric and symmetric stretching vibrations of carboxylates is also influenced by metal binding (38, 40). The $\Delta\nu$ for free aspartate in aqueous solution is 183 cm$^{-1}$, and that for free glutamate is 236 cm$^{-1}$ (8). Unidentate ligands are expected to have energy gaps larger than these values (38, 40).

On the basis of these considerations, spectral features at 1696, 1683, and 1669 cm$^{-1}$ (Fig. 3C, striped areas) are assigned to the asymmetric CO vibration of unidentate carboxylate ligands to the DE170D1 manganese cluster (Fig. 6A). Unidentate ligands to mononuclear clusters are expected to give rise to symmetric CO stretching frequencies in the 1300 to 1200 cm$^{-1}$ range (38, 40).

The direction of the shift provides additional information about the origin of vibrational lines. The four lines discussed above are components of two derivative-shaped lines, 1719/1696 and 1683/1669 cm$^{-1}$. The fact that derivative-shaped lines are observed is consistent with a perturbation of frequency upon manganese oxidation and the assignment to unidentate manganese ligands. In addition, the direction of the frequency shift observed upon oxidation of manganese during the $S_1$-to-$S_2$ transition supports the assignment of these vibrational features to manganese ligands (Fig. 3A). These lines are assigned to the DE170D1 mutant; therefore, the double-difference spectrum (Fig. 3C) is associated with $S_2$-minus-$S_1$. For a unidentate carboxylate ligand, oxidation of bound manganese should result in a shortening of the O–Mn bond and a corresponding increase in the frequency of the asymmetric stretching vibration (Fig. 6A). The pairing of 1683 cm$^{-1}$ (positive, $S_2$ state) and 1669 cm$^{-1}$ (negative, $S_1$ state) lines is consistent with such an upshift in CO frequency upon manganese oxidation (Fig. 3C, striped areas). The pairing of the 1719 cm$^{-1}$ (positive, $S_2$ state) and 1696 cm$^{-1}$ (negative, $S_1$ state) lines is also consistent with such an upshift (Fig. 3C, striped areas). However, as discussed above, the $S_1$-to-$S_2$ transition-induced change from 1696 to 1719 cm$^{-1}$ is also consistent with an oxidation-induced change from unidentate ligation to free carboxylic acid residue (Fig. 6A).

**Assignment of the 1600 to 1400 cm$^{-1}$ Spectral Region—Spectral features with center frequencies of 1570 cm$^{-1}$ (positive), 1543 cm$^{-1}$ (negative), 1496 cm$^{-1}$ (positive), and possibly, 1415 cm$^{-1}$ (negative) are observed in Fig. 3C (dotted areas), derived from a comparison of wild-type and DE170D1 PSII. Furthermore, the same pattern of frequencies and intensities is not observed in Fig. 5C, derived from a comparison of oxygen-evolving and manganese-depleted DE170D1 mutants. Therefore, the simplest interpretation of the spectrum is that these spectral features arise from amino acid residues in wild-type PSII that are perturbed by the $S_1$-to-$S_2$ transition. Note the unusually large line width of these lines, which can be attributed to homogeneous or inhomogeneous broadening mechanisms (see Refs. 12–14 and “Discussion”).**

Interpretation of the Lines in the 1600 to 1400 cm$^{-1}$ Spectral Region Assigned to Wild-type PSII—Positive spectral features, observed at 1570 and 1496 cm$^{-1}$, and negative spectral features, observed at 1543 cm$^{-1}$ and, possibly, 1415 cm$^{-1}$, have been assigned to amino acid residues in wild-type PSII (Fig. 3C, dotted areas). The frequencies of these lines provide information concerning their origin. Frequencies of lines assigned to wild-type PSII (double-difference spectrum; Fig. 3C, dotted areas), 1570 cm$^{-1}$ (positive), 1543 cm$^{-1}$ (negative), 1496 cm$^{-1}$ (positive), and 1415 cm$^{-1}$ (negative), are in the range of frequencies expected for carboxylate anions that are ligating to high valence manganese. For example, bridging carboxylate ligands to Mn$^{3+}$ dinuclear complexes gave rise to bands in the 1570 to 1415 cm$^{-1}$ region, and Mn$^{3+}$-Mn$^{4+}$ dinuclear complexes gave rise to bands in the 1540 to 1440 cm$^{-1}$ region (39, 41). By contrast, free aspartate has asymmetric and symmetric stretching vibrations at 1574 and 1391 cm$^{-1}$, respectively, and free glutamate has asymmetric and symmetric stretching vibrations at 1555 and 1399 cm$^{-1}$, respectively. On the basis of this comparison and consideration of $^{15}$N labeling experiments (13), we favor the assignment of these lines to carboxylate ligands. It is reasonable to expect that a component of or all of these broad spectral features in the double-difference spectrum (Fig. 3C, dotted areas) may arise from aspartate 170 (see “Discussion”).

The energy gap between the asymmetric and symmetric stretching vibrations of carboxylates is influenced by metal binding (38, 40) and provides information about the origin of spectral features. The $\Delta\nu$ for free aspartate in aqueous solution is 183 cm$^{-1}$, and that for free glutamate is 236 cm$^{-1}$. As discussed above, unidentate ligands are expected to have energy gaps larger than these values (38). When carboxylates bind to metals with bridging ligation, the energy gap between the asymmetric and symmetric stretching vibrations may be similar to that of the free carboxylate (38, 40). In recent studies,
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Δν was also found to be dependent on the oxidation state of the manganese, (39, 40). When carboxylates bind to metals with bidentate ligation, the characteristic spectral change is a decrease in Δν between the asymmetric and symmetric modes of the carboxylate (38, 40).

Consideration of the spectral features assigned to wild-type PSII (Fig. 3C, dotted areas) gives Δν equal to 74 cm⁻¹ for the positive lines and 128 cm⁻¹ for the negative lines. Although these values can be influenced by the oxidation state of the metal and by cancellation of intensity in the difference spectra (13, 39, 40), these numbers are far smaller than the expected energy gap between asymmetric and symmetric stretching vibrations of free carboxylates that are not ligating metals. These numbers are smaller or on the same order of magnitude as the expected energy gap for bridging carboxylate ligands. For example, in a study of trimeric and dimeric manganese compounds, bridging carboxylates gave rise to energy splitting of 220 to 100 cm⁻¹ with the smaller values of Δν arising from Mn₃⁺⁻Mn₄⁺ dinuclear complexes (39, 41). We conclude that the broad lines in Fig. 3C (dotted areas) may arise from bidentate or bridging carboxylate ligands to the manganese cluster (Fig. 6B).

The direction of the shift provides additional information about the origin of vibrational lines. These broad lines are assigned to wild-type PSII; therefore, the double-difference spectrum is an S₂-minus-S₁ spectrum. When manganese is oxidized, both asymmetric and symmetric vibrations of bridging carboxylate ligands downshift (39). For example, the asymmetric stretching vibration of bridging carboxylates downshifts from 1636 to 1540 cm⁻¹ in comparison with Mn²⁺ and Mn⁴⁺ dinuclear complexes (39, 41). We expect similar behavior for bidentate ligands. The pairing of 1570 cm⁻¹ (positive, S₁ state) and 1543 cm⁻¹ (negative, S₂ state) lines is consistent with such a downshift in the C–O frequency upon manganese oxidation (Fig. 3C, dotted areas). The pairing of the 1496 cm⁻¹ (positive, S₁ state) and 1415 cm⁻¹ (negative, S₂ state) lines is also consistent with such a downshift (Fig. 3C, dotted areas). Thus, both the direction of the shift and the frequency of the lines are consistent with assignment to bridging or bidentate carboxylate ligands of the manganese cluster (Fig. 6B).

DISCUSSION

Upon illumination of PSII at 200 K, the S₁-to-S₂ transition can be studied independently of other S state transitions (12–14, 42), and the S₃Qₓ⁻minus-S₄Qₓ⁻ FT-IR spectrum has been obtained under these conditions (12–14). In these studies, we have used the S₃Qₓ⁻minus-S₄Qₓ⁻ FT-IR spectrum to detect a structural change, involving carboxylate coordination of manganese or calcium, in DE170D1 PSII. The frequencies, direction of the frequency shift, and observed splittings of observed spectral features support the interpretation that the mode of manganese/calcium ligation is altered from bridging/bidentate to unidentate when aspartate 170 is changed to a glutamate. The exact number of ligands altered is not known, but because two different, new derivative-shaped lines (1719/1696 and 1683/1669 cm⁻¹) are observed in the DE170D1 spectrum, we deduce that at least two different coordinating carboxylates are affected. The ~1.5-Å increase in side chain length is presumed to be the cause of these alterations in metal coordination. The frequencies of the observed vibrational lines and the direction of the observed shifts are consistent with the assignment of spectral lines to high valence manganese ligands. Significantly, the altered active site is still active in oxygen evolution.

Carboxylate rearrangements involving conversion between bridging and unidentate coordination are known to occur on metal oxidation in methane monooxygenase (43, 44) and ribonucleotide reductase (45). Our work has now documented carboxylate shifts at the catalytic site for photosynthetic water oxidation. Therefore, the large line width of the carboxylate ligand vibrations in wild-type PSII (Fig. 3C) may arise from inhomogeneous broadening due to carboxylate rearrangements on the time scale of the measurement (13, 14). Alternatively, the line width may arise from inhomogeneous broadening due to a contribution to the spectrum from multiple carboxylate groups in slightly different protein environments (13).

A change in inner-sphere coordination of a metal is also observed when an aspartate-to-glutamate mutation is introduced at a Ca²⁺ ligand in staphylococcal nuclease (46). In this case, the substitution of glutamate for aspartate 21 results in a change from unidentate ligation by aspartate 21 to bidentate ligation by glutamate 21. Other substantial changes in coordination of calcium occur, including a change in the number of coordinating water molecules and new ligation by glutamate 43, which is not coordinating in the wild-type protein. In addition, more long-range alterations in protein structure are also observed (46). This work provides a precedent for the interpretation of our vibrational spectra.

The question of whether aspartate 170 and/or glutamate 170 is directly ligating manganese is of importance. The simplest interpretation of our results is that aspartate 170 is a bridging/bidentate ligand in the S₁ and S₂ states and that glutamate 170 is a unidentate ligand in the S₂ and, possibly, S₃ states (Fig. 6). If correct, other changes in coordination must also occur to explain our data. For example, in the S₁ state, another bridging/bidentate ligand must be replaced with a unidentate ligand (Fig. 6). These additional changes in coordination may be rearrangements of bound water, which may not contribute to the spectral region investigated here (38), or additional carboxylate rearrangements, contributing to the spectra presented here. Although there is precedent for this interpretation (see discussion of staphylococcal nuclease above (46)), the possibility of long-range structural changes induced by the mutation must be considered. In this scenario, substitution of glutamate for aspartate 170 causes a structural change that results in the substitution of unidentate for bridging carboxylate ligands.

The catalytic site of the DE170D1 mutant is functional, but perturbed. This is evident in the decreased steady-state rate of oxygen evolution, in the longer lifetime of the S₁ state, and in observed changes in the rate of oxidation of the catalytic site (20, 21). Also, we have not yet observed a g = 2 multil ine signal from this mutant (data not shown), indicating that alterations in magnetic interactions have occurred. We attribute these functional alterations to the change in ligation described above (Fig. 6). For example, ab initio calculations have associated a 10 kcal/mol stabilization of energy with the exchange of a bidentate ligand in the first solvation shell of Mg²⁺ with a unidentate ligand (30). A decrease in energy upon substitution of unidentate for bidentate coordination rationalizes our results with previous studies showing a stabilization of the DE170D1 S₂ state (20, 21).

Additional indirect evidence for a perturbation of manganese binding is observed in data obtained after hydroxylamine treatment of DE170D1. In spinach and wild-type cyanobacterial PSII, treatment with hydroxylamine and EDTA is required to remove manganese and to generate a Chl¹⁻Qₓ⁻minus-ChlQₓ⁻ spectrum upon illumination at 200 K (14). This work is consistent with a high affinity binding site for manganese on the donor side of wild-type PSII. On the other hand, hydroxylamine treatment of DE170D1 results in a PSII preparation that gives rise to a characteristic Chl¹⁻Qₓ⁻minus-ChlQₓ⁻ spectrum upon 200 K illumination without treatment with EDTA (Fig. 5). This result is consistent with a change in binding affinity for manganese in this mutant. A change in the Kₘ for manganese...
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We have shown that substitution of glutamate for aspartate 170 gives rise to a change in coordination of manganese or calcium in PSII. The spectral changes observed are consistent with an alteration from bridging/bidentate carboxylate ligands to unidentate carboxylate ligation in the mutant. The cluster, which exhibits vibrational spectra characteristic of unidentate carboxylate ligation, is still active in water oxidation (20, 21). Our work is the first characterization of a mutation that alters the structure and function of the photosynthetic water-oxidizing complex through the use of vibrational spectroscopy. These studies have established that carboxylate shifts are possible at the metal center, which carries out photosynthetic water oxidation. This result indicates that PSII is a member of the same class of enzymes as ribonucleotide reductase and methane monoxygenase, which undergo carboxylate shifts at their metal centers (43–45).

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14616 oxidation has been observed previously in non-oxygen-evolving core preparations from the DE170D1 mutant (21).