No Evidence from FTIR Difference Spectroscopy That Aspartate-342 of the D1 Polypeptide Ligates a Mn Ion That Undergoes Oxidation during the S₀ to S₁, S₁ to S₂, or S₂ to S₃ Transitions in Photosystem II†

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Abstract: In the recent X-ray crystallographic structural models of photosystem II, Asp342 of the D1 polypeptide is assigned as a ligand of the oxygen-evolving Mn₄ cluster. To determine if D1-Asp342 ligates a Mn ion that undergoes oxidation during one or more of the S₀ → S₁, S₁ → S₂, and S₂ → S₃ transitions, the FTIR difference spectra of the individual S state transitions in D1-D342N mutant PSII particles from the cyanobacterium Synechocystis sp. PCC 6803 were compared with those in wild-type PSII particles. Remarkably, the data show that the mid-frequency (1800−1200 cm⁻¹) FTIR difference spectra of wild-type and D1-D342N PSII particles are essentially identical. Importantly, the mutation alters none of the carboxylate vibrational modes that are present in the wild-type spectra. The absence of significant mutation-induced spectral alterations in D1-D342N PSII particles shows that the oxidation of the Mn₄ cluster does not alter the frequencies of the carboxylate stretching modes of D1-Asp342 during the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions. One explanation of these data is that D1-Asp342 ligates a Mn ion that does not increase its charge or oxidation state during any of these S state transitions. However, because the same conclusion was reached previously for D1-Asp170, and because the recent X-ray crystallographic structural models assign D1-Asp170 and D1-Asp342 as ligating different Mn ions, this explanation requires that (1) the extra positive charge that develops on the Mn₄ cluster during the S₁ → S₂ transition be localized on the Mn ion that is ligated by the α-COO⁻ group of D1-Ala344 and (2) any increase in positive charge that develops on the Mn₄ cluster during the S₀ → S₁ and S₂ → S₃ transitions be localized on the one Mn ion that is not ligated by D1-Asp170, D1-Asp342, or D1-Ala344. In separate experiments that were conducted with L-[1-¹³C]alanine, we found no evidence that D1-Asp342 ligates the same Mn ion that is ligated by the α-COO⁻ group of D1-Ala344.

The catalytic site of water oxidation in photosystem II (PSII) contains a cluster of four Mn ions and one Ca ion. The Mn₄ cluster accumulates oxidizing equivalents in response to light-induced electron-transfer reactions within PSII, thereby serving as the interface between one-electron photochemistry and the four-electron process of water oxidation (for reviews, see refs 1–4). During each catalytic cycle, the Mn₄ cluster cycles through five oxidation states termed Sₙ, where “n” denotes the number of oxidizing equivalents that have been stored (n = 0–4). The S₁ state predominates in dark-adapted samples. Most interpretations of Mn-XANES data have concluded that the S₁ state consists of two Mn(III) and two Mn(IV) ions and that the S₂ state consists of one Mn(III) and three Mn(IV) ions (for review, see refs 5 and 6). Whether the additional oxidizing equivalent of the S₃ state is localized on a Mn ion (7, 8) or on a Mn ligand (9, 10) remains in dispute. The S₄ state is a transient intermediate that reverts to the S₀ state with the concomitant release of O₂.

The amino acid residues that ligate the Mn₄ cluster are provided mainly by the D1 polypeptide, one of the core subunits of PSII. One of these residues is D1-Asp342. This residue was originally proposed as a possible ligand of the Mn₄Ca cluster on the basis of early mutagenesis studies (11, 12) and is assigned as a ligand to Mn in the recent X-ray crystallographic structural models (13, 14). In the ~3.5 Å structural model (13), this residue is a unidentate ligand of...
a single Mn ion. In the ~3.0 Å structural model (14), this residue bridges two Mn ions, including the Mn ion that is ligated (in this model) by the α-COO − group of D1-Ala344. The differences between the two structural models are probably caused by differences in data quality, extent of radiation damage, and approach to interpreting the electron density. Recent XANES and EXAFS studies of PSII single crystals (15) and PSII membrane multilayers (16) have provided compelling evidence that the X-ray doses that were used in the crystallographic studies to irradiate the PSII crystals would have rapidly reduced the Mn4Ca cluster’s oxidized Mn(III/IV) ions to their fully reduced Mn(II) states and that this radiation-induced reduction would have significantly perturbed the structure of the Mn4Ca cluster, disrupting μ-oxo bridges and altering Mn–ligand interactions (15, 16). The extent that the ~3.5 and ~3.0 structural models of the Mn4Ca cluster and its ligation environment are distorted from the native structure remains to be determined.

To obtain independent evidence that D1-Asp342 ligates the Mn4Ca cluster, and to determine if D1-Asp342 ligates a Mn ion that undergoes oxidation during one or more of the individual S state transitions, we have compared the FTIR difference spectra of the S0 → S1, S1 → S2, S2 → S3, and S1 → S0 transitions in D1-D342N mutant PSII particles from the cyanobacterium Synechocystis sp. PCC 6803 with those in wild-type PSII particles. Both wild-type and mutant PSII preparations exhibit an extensive array of vibrational modes that undergo shifts in frequency during the individual S state transitions. Many of these shifts produce strikingly large features in specific Sn+1-minus-Sn FTIR difference spectra, and many are clearly attributable to frequency shifts of carboxylate residues (17–29). The identification of these modes will complement the X-ray crystallographic studies by providing information about the dynamic structural changes that accompany water oxidation. Few of these modes have yet to be identified (25, 27, 30, 31). We recently employed isotopic labeling to identify the symmetric carboxylate stretching [νsym(COO −)] mode of the α-COO − group of D1-Ala344 in the S2-minus-S1 FTIR difference spectrum of wild-type PSII particles containing Ca or Sr (30, 31) [also see refs 25 and 29]. Because the substitution of Sr for Ca significantly altered several νsym(COO −) modes, including some that may correspond to one or more metal ligands, but importantly did not alter the νsym(COO −) mode of the α-COO − group of D1-Ala344, we concluded that D1-Ala344 ligates Mn rather than Ca (31). The frequency of the νsym(COO −) mode of D1-Ala344 in the S1 state and its ~17 cm −1 or ~36 cm −1 downshift in the S2 state imply that this group is a unidentate ligand of a Mn ion whose charge increases during the S1 → S2 transition (25, 30, 31). A similar identification of one of the carboxylate stretching modes of D1-Asp342 in any of the Sn+1-minus-Sn difference spectra could provide unequivocal spectroscopic evidence for the ligation of the Mn4Ca cluster by D1-Asp342 and could provide information about the type of carboxylate ligation and the environment of the carboxylate group. If D1-Asp342 is ligand of a Mn ion whose charge increases during a specific Sn → Sn+1 transition, the increased charge should weaken the ligation

Mn–O bond(s), thereby decreasing the frequency of the D1-Asp342 symmetric carboxylate stretching mode and possibly shifting the frequency of the D1-Asp342 asymmetric carboxylate stretching mode. The shifted mode(s) should appear in the corresponding Sn+1-minus-Sn FTIR difference spectrum of wild-type PSII particles but not in the spectra of D1-D342N PSII particles because the mutation has eliminated the carboxylate group. The absence of the mode in the mutant would permit its identification in the wild-type control.

Our data show that the mid-frequency (1800–1200 cm −1 ) S1-minus-S0, S2-minus-S1, S3-minus-S2, and S0-minus-S3 FTIR difference spectra of D1-D342N PSII particles are remarkably similar to those of wild-type PSII particles. Importantly, the D1-D342N mutation eliminates no carboxylate modes from any of the difference spectra. The simplest explanation for these data is that the Mn ion that is ligated by D1-Asp342 does not change its charge or oxidation state during the S0 → S1, S1 → S2, or S2 → S3 transitions. In separate experiments that were conducted with L-[1-13C]-alanine, we found no evidence that D1-Asp342 ligates the same Mn ion that is ligated by the α-COO − group of D1-Ala344.

MATERIALS AND METHODS

Construction of Mutant and Propagation of Cultures. The D1-D342N mutation was constructed in the psbA-2 gene of Synechocystis sp. PCC 6803 (33) and transformed into a host strain of Synechocystis that lacks all three psbA genes and contains a hexahistidine-tag (His-tag) fused to the C-terminus of CP47 (34). Single colonies were selected for ability to grow on solid media containing 5 μg/mL kanamycin monosulfate. The control WT(D1) strain was constructed in an identical fashion except that the transforming plasmid carried no site-directed mutation. The designation “WT(D1)” differentiates this strain from the native wild-type strain that contains all three psbA genes, lacks a His-tag on the C-terminus of CP47, and is sensitive to antibiotics. Cells were propagated as described previously (30), but in the presence of 5 mM glucose and in three 7 L carboys instead of in multiple 1 L flasks. The light sensitivity of the D1-D342N strain that was noted previously for small scale cultures (12) was not a problem for cells propagated in carboys. For the isolation of isotopically labeled PSII particles, the liquid medium contained 0.5 mM L-[1-13C]alanine (99% 13C enrichment, Cambridge Isotope Laboratories, Andover, MA) (30, 31) (the presence of 5 mM glucose in the growth medium produced no noticeable difference in the FTIR difference spectra of isotopically labeled WT(D1) PSII particles). To verify the integrity of the mutant cultures that were harvested for the purification of PSII particles, an aliquot of each culture was set aside and the complete sequence of the psbA-2 gene was obtained after PCR amplification of genomic DNA (33). No trace of the wild-type codon was detected in any of the mutant cultures.

Purification of PSII Particles. Isolated PSII particles were purified under dim green light at 4 °C with Ni-NTA superflow affinity resin (Qiagen, Valencia, CA) as described previously (31). For most samples, the purification buffer consisted of 1.2 M betaine, 10% (v/v) glycerol, 50 mM MES-NaOH (pH 6.0), 20 mM CaCl2, 5 mM MgCl2, 50 mM

2 The authors of ref 29 have recently arrived at the same conclusion. However, it should be noted that some authors continue to argue that the α-COO − group of D1-Ala344 ligates Ca rather than Mn (4, 32).
liquid nitrogen. The $S_2$ state was generated by illuminating with a frequency-doubled Q-switched Nd:YAG laser [Surelite I (Continuum, Santa Clara, CA)]. For experiments conducted at 250 K (30, 31), each sample was flash-illuminated only once. The single beam spectrum that was recorded after the flash was divided by the single-beam spectrum that was recorded before the flash, and the ratio was converted to units of absorption. Each single-beam spectrum consisted of 800 scans. For experiments conducted at 273 K (26, 28), after dark-adaptation, one preflash was applied followed by 5 min of additional dark-adaptation. This treatment was employed to oxidize $Y_D$ and to maximize the proportion of centers in the $S_1$ state. Six successive flashes then were applied with an interval of 12.2 s between each. Two single beam spectra were recorded before the first flash, and one single-beam spectrum was recorded starting 0.33 s after the first and subsequent flashes (each single-beam spectrum consisted of 100 scans). The 0.33 s delay was incorporated to allow for the oxidation of $Q_A^-$ by the ferricyanide. To obtain difference spectra corresponding to successive $S$ state transitions, the single-beam spectrum that was recorded after the $n$th flash was divided by the single-beam spectrum that was recorded immediately before the $n$th flash and the ratio was converted to units of absorption. To estimate the background noise level, the second preflash single-beam spectrum was divided by the first and the ratio was converted to units of absorption. The sample was dark-adapted for 30 min, then the entire cycle was repeated, including the preflash and the 5 min additional dark-adaptation period. The entire cycle was repeated 12 times for each sample, and the difference spectra recorded with several samples were averaged.

**Preparation of FTIR Samples.** All manipulations were conducted under dim green light at 4 °C. For experiments conducted at 250 K, samples were exchanged into FTIR analysis buffer [40 mM sucrose, 10 mM MES-NaOH (pH 6.0), 5 mM CaCl$_2$, 5 mM NaCl, 0.06% (w/v) n-dodecyl $\beta$-D-maltoside (22, 26)] by repeated concentration/dilution cycles. For experiments conducted at 273 K, most samples were exchanged into FTIR analysis buffer by passage through a centrifugal gel filtration column (28), although some of these samples [WT(D1b) in Figure 4] was exchanged into FTIR buffer by repeated concentration/dilution cycles. Concentrated samples (approximately 10 $\mu$L in volume) were mixed with 1/10 volume of fresh 100 mM potassium ferricyanide (dissolved in water), spread to a diameter of about 10 mm on a 15 mm diameter BaF$_2$ window, then dried lightly (until tacky) under a stream of dry nitrogen gas. For experiments conducted at 250 K, the lightly dried samples were placed in a humidifier at 95% RH for 10 min. For experiments conducted at 273 K, 1 $\mu$L of 20% (v/v) glycerol (in water) was spotted onto the window, adjacent to the lightly dried sample, but not touching it, to maintain the humidity of the sample in the FTIR cryostat at 99% RH (19). A second IR window with a Teflon spacer (0.5 mm thick) was placed over the first and sealed in place with silicon-free high-vacuum grease. The sample was immediately loaded into the FTIR cryostat and allowed to equilibrate in darkness to 250.0 K (for 2--4 h) or 273.0 K (for 2 h). Sample concentrations and thicknesses were adjusted so that the absolute absorbance of the amide I band at 1657 cm$^{-1}$ was 0.7--1.1.

**Measurement of FTIR Spectra.** Mid-frequency FTIR spectra were recorded with a Bruker Equinox 55 spectrometer (Bruker Optics, Billerica, MA) at a spectral resolution of 4 cm$^{-1}$ as described previously (26, 28, 30, 31). Flash-illumination (~20 mJ/flash, ~7 ns fwhm) was provided by a frequency-doubled Q-switched Nd:YAG laser [Surelite I (Continuum, Santa Clara, CA)]. For experiments conducted at 250 K (30, 31), each sample was flash-illuminated only once. The single beam spectrum that was recorded after the flash was divided by the single-beam spectrum that was recorded before the flash, and the ratio was converted to units of absorption. Each single-beam spectrum consisted of 800 scans. For experiments conducted at 273 K (26, 28), after dark-adaptation, one preflash was applied followed by 5 min of additional dark-adaptation. This treatment was employed to oxidize $Y_D$ and to maximize the proportion of centers in the $S_1$ state. Six successive flashes then were applied with an interval of 12.2 s between each. Two single beam spectra were recorded before the first flash, and one single-beam spectrum was recorded starting 0.33 s after the first and subsequent flashes (each single-beam spectrum consisted of 100 scans). The 0.33 s delay was incorporated to allow for the oxidation of $Q_A^-$ by the ferricyanide. To obtain difference spectra corresponding to successive $S$ state transitions, the single-beam spectrum that was recorded after the $n$th flash was divided by the single-beam spectrum that was recorded immediately before the $n$th flash and the ratio was converted to units of absorption. To estimate the background noise level, the second preflash single-beam spectrum was divided by the first and the ratio was converted to units of absorption. The sample was dark-adapted for 30 min, then the entire cycle was repeated, including the preflash and the 5 min additional dark-adaptation period. The entire cycle was repeated 12 times for each sample, and the difference spectra recorded with several samples were averaged.

**RESULTS AND DISCUSSION**

The $O_2$ evolving activity of the purified D1-D342N PSII particles was 1.4--1.8 mmol $O_2$ (mg of Chl) $^{-1}$ h $^{-1}$ compared to 5.1--5.5 mmol $O_2$ (mg of Chl) $^{-1}$ h $^{-1}$ for WT(D1) PSII particles. The lower $O_2$ evolving activity of the D1-D342N PSII particles (25--35% compared to wild-type) was expected because the $O_2$ evolving activity of D1-D342N cells has been reported to be only ~33% compared to wild-type (12).

A parallel polarization, integer spin, multil ine EPR signal was observed in dark adapted D1-D342N PSII particles (Figure 1A, lower trace). In terms of peak positions and spacings, this signal closely resembles the $S_1$ state multiline EPR signal that is observed in WT(D1) PSII particles (Figure 1A, upper trace), and we assign it to the $S_1$ state. A perpendicular polarization multiline EPR signal was observed in the same mutant PSI11 particles after illumination at 195 K (Figure 1B, lower trace). In terms of peak positions and spacings, this signal closely resembles the $S_2$ state multiline EPR signal that is observed in WT(D1) PSII particles after illumination (Figure 1B, upper trace), and we assign it to the $S_2$ state. Illumination produced no g $\approx$ 4.1 EPR signal nor any other recognizable Mn EPR signal in either sample (not shown). The similarity of the $S_1$ and $S_2$ state multiline
EPR signals in the mutant compared to wild-type shows that the magnetic interactions that give rise to these signals (35, 36) are not appreciably altered by the D1-D342N mutation. In contrast, both the D1-H332E mutation (34, 37) and the substitution of Sr for Ca (e.g., see refs 31 and 38) perturb these interactions and substantially alter the appearance of the S2 state multiline EPR signal.

For the S1 state multiline EPR signal, the integrated area of the nine peaks indicated in the mutant spectrum (asterisks in Figure 1A) was approximately 23% of the integrated area of the corresponding peaks in the WT(D1) spectrum. For the S2 state multiline EPR signal, the integrated area of the nine peaks indicated in the mutant spectrum (asterisks in Figure 1B) was approximately 54% of the integrated area of the corresponding peaks in the WT(D1) spectrum. Because the two EPR signals were recorded on the same sample, with the dark-adapted S1 state signal recorded before illumination to produce the S2 state, we conclude that about 54% of the D1-D342N PSII particles contained Mn4Ca clusters. The presence of a significant fraction of mutant PSII reaction centers without Mn4Ca clusters is expected because we previously estimated that a significant fraction (30–40%) of D1-D342N PSII reaction centers lack Mn4Ca clusters in vivo on the basis of Chl fluorescence studies of intact cells (12). That significant fractions of D1-D342N PSII reaction centers lack Mn4Ca clusters in cells and PSII particles implies that the Mn4Ca cluster is assembled less efficiently or is less stable in D1-D342N than in wild-type, as was concluded previously (12). That the steady-state rates of O2 evolution in D1-D342N cells and PSII particles (relative to wild-type) are less than the fractions of PSII reaction centers estimated to contain Mn4Ca clusters implies that the Mn4Ca clusters in D1-D342N produce O2 less efficiently than those in wild-type. On the basis of the relative amplitudes of the S2 state multiline signals and the relative light-saturated rates of O2 evolution, we estimate that the Mn4Ca clusters in D1-D342N PSII particles evolve O2 at 50–65% the rate of the Mn4Ca clusters in wild-type PSII particles. The reasons for the different relative amplitudes of the S1 and S2 multline EPR signals in the mutant and wild-type PSII particles are unknown. The discrepancy presumably reflects our limited understanding of the factors that influence the S1 state multiline EPR signal.

To determine if the D1-D342N mutation alters the $\nu_{sym}(\text{COO}^-)$ mode of the $\alpha$-COO$^-$ group of D1-Ala344, the position of this mode was visualized by purifying PSII particles from cells that had been propagated in media containing $L$-[1-13C]alanine. A comparison of the mid-frequency S2-minus-S1 FTIR difference spectra of WT(D1) and D1-D342N PSII particles containing either unlabeled (13C) alanine (black traces) or $L$-[1-13C]alanine (red traces) is shown in Figures 2A and 2B. The spectrum of D1-D342N PSII particles (Figure 2B) resembles that of WT(D1) (Figure 2A). The most prominent differences are (1) the differential peaks at (−)1705/(+)1696 cm$^{-1}$ and (+)1552/(−)1543 cm$^{-1}$ appear with significantly larger amplitudes in the mutant, (2) the single positive peak at $\approx$1507 cm$^{-1}$ in WT(D1) is split into a doublet at $\approx$1511 and $\approx$1504 cm$^{-1}$ in the mutant, and (3) the positive peak at $\approx$1365 cm$^{-1}$ has a lower amplitude in the mutant. The larger amplitude differential peaks and the positive peak at $\approx$1504 cm$^{-1}$ are characteristic of the light-minus-dark FTIR difference spectrum of Y$_{65}$-
minus-Y_D that is observed in Mn-depleted PSII particles under the same experimental conditions (i.e., a single flash applied to dark-adapted samples at 250 K), as is the positive feature at 1599 cm\(^{-1}\) that appears in L-[1-\(^{13}\)C]alanine-labeled Mn-depleted PSII particles (30). Previously, we showed that the incorporation of L-[1-\(^{13}\)C]alanine produces no changes between 1450 and 1200 cm\(^{-1}\) in the light-minus-dark FTIR difference spectrum of Mn-depleted PSII particles (see Figure 4 of ref 30). Consequently, the [1-\(^{13}\)C]alanine-induced changes that are apparent in the \(\nu_{\text{sym}}(\text{COO}^-)\) region of the S\(_2\)-minus-S\(_1\) FTIR difference spectrum of Mn-depleted PSII particles (see Figure 2B) can be attributed to D1-D342N PSII particles that contain MnCa clusters, and not to PSII particles that lack MnCa clusters.

To isolate the 1-[\(^{13}\)C]alanine-shifted \(\nu_{\text{sym}}(\text{COO}^-)\) modes and to display them more clearly, the 13C-minus-12C double-difference spectra of the region between 1380 and 1270 cm\(^{-1}\) in the WT(D1) and D1-D342N samples are presented in Figure 2C. The data show that the positions of the 1-[\(^{13}\)C]alanine-shifted modes in the S\(_1\) and S\(_2\) states are nearly the same in D1-D342N mutant PSII particles as in WT(D1) PSII particles. In both samples, the data are consistent with a single \(S_1\) state mode at \(\sim 1356\) cm\(^{-1}\) in the unlabeled samples shifting to \(\sim 1337\) or \(\sim 1320\) cm\(^{-1}\) after the incorporation of L-[1-\(^{13}\)C]alanine, and with a single \(S_2\) state mode at \(\sim 1320\) cm\(^{-1}\) shifting to \(\sim 1302\) cm\(^{-1}\). These 1-[\(^{13}\)C]alanine-shifted modes were observed previously in wild-type PSII particles from \textit{Synechocystis} sp. PCC 6803 and were shown to originate from the \(\nu_{\text{sym}}(\text{COO}^-)\) mode of the \(\alpha\)-COO\(^-\) group of D1-Ala344 (25, 30, 31). It is not yet possible to distinguish between these two possibilities.

3 Specific L-[1-\(^{13}\)C]alanine-labeling of PSII shifts the \(\nu_{\text{sym}}(\text{COO}^-)\) mode of the \(\alpha\)-COO\(^-\) group of D1-Ala344 by either 19 or 36 cm\(^{-1}\) (25, 30, 31). It is not yet possible to distinguish between these two possibilities.
features in response to the second, third, and fourth flashes. Clearly, an oxidized electron donor is photoaccumulated with high quantum yield in Mn-depleted WT(D1) PSII particles under these conditions. The most prominent spectral features of this oxidized electron donor, apparent in the first flash spectrum, include differential peaks at (−)1706/(+1698 cm⁻¹), positive peaks at 1677, 1651, 1550, 1531, and 1512 cm⁻¹, and negative peaks at 1624, 1453, and 1250 cm⁻¹. These spectral features identify this FTIR difference spectrum as that of $Y_Z^{-}$-minus-$Y_Z$ (39). Evidently, in lightly dried *Synechocystis* PSII particles at 99% RH in the presence of excess potassium ferricyanide, $Y_Z^{-}$ is stable for tens of seconds at 273 K, persisting for sufficient time to contribute to the first flash spectrum and to remain present when the subsequent flashes are applied. After normalizing the first flash spectra to the peak-to-peak amplitudes of the negative ferricyanide peak at 2115 cm⁻¹ and the positive ferrocyanide peak at 2038 cm⁻¹, the amplitude of the (−)1707 cm⁻¹ band in the D1-D342N PSII particles was found to be ~2.6-fold smaller than in the Mn-depleted WT(D1) PSII particles. If we assume that this band arises entirely from mutant PSII particles that lack Mn₄Ca clusters, then we estimate that ~38% of the mutant D1-D342N PSII particles lack Mn₄Ca clusters, an estimate that is in general agreement with our EPR data (described above) and with data that was presented earlier in ref 12.

On the basis of the data that is presented in Figure 3, the first flash spectrum of the D1-D342N PSII particles was corrected to eliminate the spectral contributions of mutant PSII particles lacking Mn₄Ca clusters. This correction was accomplished by subtracting ~38% of the spectrum of Mn-depleted WT(D1) from the first flash spectrum, thereby eliminating nearly all of the (−)1707 cm⁻¹ band. No correction was applied to the second, third, or fourth flash spectra.

The mid-frequency FTIR difference spectra of WT(D1) and D1-D342N PSII particles that were induced by four successive flashes applied at 273 K are compared in Figure 4 (panels A–D, traces a). (In Figure 4A, the spectrum of D1-D170H PSII particles was found in D1-D170H PSII particles (26, 40), despite evidence that ~50% of these mutant PSII particles lack Mn₄Ca clusters (26, 41). This difference between D1-D170H and D1-D342N PSII particles may be related to the substantial mutation-induced increase of the $Y_Z^{-}$/$Y_Z$ midpoint potential that was reported in D1-D342N cells (42), although it is unclear why the increased $Y_Z^{-}$/$Y_Z$ midpoint potential would increase the lifetime of $Y_Z^{-}$ in the FTIR samples at 273 K.

2C show that this is not the case. Therefore, we conclude that D1-Asp342 does not ligate the same Mn ion as the α-COO⁻ group of D1-Ala344.

To determine if D1-Asp342 ligates a Mn ion that undergoes oxidation during one or more of the S₀ → S₁, S₁ → S₂, and S₂ → S₃ transitions, we sought to compare the FTIR difference spectra of the individual S state transitions in WT(D1) and D1-D342N PSII particles. However, because our EPR and 250 K FTIR data (Figures 1 and 2) showed the absence of Mn₄Ca clusters in significant fractions of purified D1-D342N PSII particles, we first compared the FTIR difference spectra of D1-D342N and Mn-depleted WT(D1) PSII particles to ascertain the possible contribution to the mutant spectra of PSII particles lacking the Mn₄Ca cluster. In Figure 3, we compare the FTIR difference spectra of D1-D342N and Mn-depleted WT(D1) PSII particles that were induced by four successive flashes applied at 273 K under conditions that promote S-state turnover. The Mn-depleted WT(D1) PSII particles show substantial spectral features in response to the first flash, but show very small
The spectra labeled “WT(D1b)” correspond to WT(D1) samples that were exchanged into FTIR analysis buffer by repeated concentration/dilution cycles, as in ref 31. Both WT(D1a) and WT(D1b) spectra resemble those that have been reported previously for wild-type Synechocystis PSII particles (22, 25–27). The small differences that are apparent between the WT(D1a) and WT(D1b) spectra can be attributed to minor structural perturbations in the environment of the Mn$_4$Ca cluster that have little functional significance (28). Therefore, any spectral differences that are observed between D1-D342N and WT(D1) PSII particles would be significant only if they significantly exceed the spectral differences that are observed between the WT(D1a) and WT(D1b) PSII particles.

The data of Figure 4 show that the $S_{n+1}-S_n$ FTIR difference spectra of D1-D342N PSII particles are remark-
ably similar to the corresponding spectra of WT(D1a) and WT(D1b) PSII particles, with the possible exception of some of the features in the ν_{sym}(COO⁻) and ν_{asym}(COO⁻) regions of the S₂-minus-S₁ FTIR difference spectrum (Figure 4A, trace a). Several features in the corresponding regions of the WT(D1a)-minus-D342N and WT(D1b)-minus-D342N double-difference spectra (Figure 4A, traces b) are somewhat larger than the features that are present in these regions of the WT-(D1a)-minus-WT(D1b) double-difference spectra (Figure 4A, trace c), particularly the positive peaks near 1584, 1532, and 1502 cm⁻¹, the negative peaks near 1560 and 1521 cm⁻¹, and the differential peaks at (+)1365/(−)1352 cm⁻¹ in the two WT-minus-D342N double-difference spectra. If these small spectral differences correspond to a carboxylate group whose vibrational modes shift during the S₁ to S₂ transition, then these modes should be restored during the S₂ to S₀ and/or S₀ to S₁ transitions. Consequently, the differences between the mutant and wild-type S₂-minus-S₁ FTIR difference spectra should be mirrored in the S₀-minus-S₁, and/or S₁-minus-S₀ FTIR difference spectra (17-29). However, the D₁-D342N S₀-minus-S₁ and S₁-minus-S₀ FTIR difference spectra look remarkably like the WT(D1a) and WT(D1b) spectra (Figures 1C and D, traces a). Notably, for all Sₙ-minus-S₁ FTIR difference spectra with the exception of S₂-minus-S₁, the features that are present in the WT(D1a)-minus-D342N and WT(D1b)-minus-D342N double-difference spectra (Figures 4B-4D, traces b) are similar in amplitude to the features that are present in the WT(D1a)-minus-WT-(D1b) double-difference spectra (Figures 4B-4D, traces c). Therefore, we conclude that the small differences between the S₂-minus-S₁ FTIR difference spectra of D₁-D342N and WT(D1) PSII particles probably reflect imperfect subtraction of features arising from mutant PSII particles that lack Mn₄-Ca clusters.

On the basis of the comparisons shown in Figure 4, we conclude that our data provide no indication that the D₁-D342N mutation eliminates a specific carboxylate vibrational mode from any of the Sₙ-minus-S₁ FTIR difference spectra. If D₁-Asp342 ligated a Mn ion whose charge or oxidation state increased during one or more of the S state transitions, then replacing Asp with Asn should eliminate 1-2 specific carboxylate modes from one or more of the Sₙ-minus-S₁ FTIR difference spectra, replacing them with a combination of ν(C=O) and δ(NH₂) modes. The elimination of the carboxylate modes should be as obvious as the [1-¹³C]-alanine-induced shift of the ν_{sym}(COO⁻) mode of the α-COO⁻ group of D₁-Ala344 that was observed in Figure 2 of this study and in earlier studies (25, 30, 31). Yet, no such elimination is observed (Figures 4A-4D, traces b). Therefore, our data show that neither the symmetric nor the asymmetric carboxylate stretching mode of D₁-Asp342 is altered significantly when the Mn₄-Ca cluster is oxidized during the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions.

The inference from our results is that the carboxylate group of D₁-Asp342 is not sensitive to the oxidation of the Mn₄-Ca cluster during the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions. One explanation of these data is that D₁-Asp342 ligates a Mn ion that does not increase its charge or formal oxidation state during any of the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions. As previously discussed with respect to similar data obtained with mutants of D1-Glu189 (28), this explanation presents some challenges. First, in both of the recent X-ray crystallographic structural models, D₁-Asp342 ligates one of the Mn ions that are located in the Mn₄-Ca cluster (13, 14). If D₁-Asp342 ligates one of these Mn ions, then its carboxylate group is insensitive to the Mn oxidations that must occur elsewhere in this portion of the Mn₄-Ca cluster during the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions. Second, the same explanation was offered previously for D₁-Asp170 to explain similar FTIR data that were obtained with D₁-D170H mutant PSII particles (26). The recent X-ray crystallographic structural models assign D₁-Asp170 and D₁-Asp342 as ligands of different Mn ions (13, 14). Consequently, if neither of these Mn ions increases its charge or oxidation state during the S₁ → S₀ transition, then the extra positive charge that develops on the Mn₄-Ca cluster during this transition must necessarily be localized, probably to a single Mn ion. However, this conclusion conflicts with a resonant inelastic X-ray scattering (RIXS) study, whose authors concluded that this extra charge is strongly delocalized over the Mn₄ cluster (42). Third, this explanation constrains the identity of the Mn ion(s) whose charge or formal oxidation state increases during the S₀ → S₁ and S₂ → S₃ transitions. Because there is evidence that the α-COO⁻ group of D₁-Ala344 is ligated to a Mn ion whose charge increases during the S₁ → S₂ transition (25, 30, 31), decreases during the S₁ → S₀ transition (25), and remains unchanged during the S₀ → S₁ and S₁ → S₂ transitions (25), any increase in positive charge that develops on the Mn₄-Ca cluster during the S₀ → S₁ and S₁ → S₂ transitions must necessarily be localized on the one Mn ion that is not ligated by D₁-Asp170, D₁-Asp342, or D₁-Ala344 [Mn₃ in the ~3.0 Å structural model (14)]. We are currently testing this possibility by analyzing mutations of the residue CP43-Glu354, identified as a Mn ligand in both the ~3.0 Å (14) and ~3.5 Å (13) structural models.

An alternate explanation for our data is that D₁-Asp342 does not ligate a Mn ion. We consider this explanation to be less likely because it would conflict with both recent X-ray crystallographic structural models (13, 14), and because D₁-Asp342 was proposed to be a possible ligand to the Mn₄-Ca cluster on the basis of earlier mutagenesis studies (11, 12). These studies showed that O₂ evolving activity is retained when D₁-Asp342 is replaced with a possible metal ligand (e.g., Glu, Asn, or His) but not when D₁-Asp342 is replaced by Ala or Val.

Recently, it has been suggested that the vibrational modes of the carboxylate ligands of the Mn ions in the Mn₄-Ca cluster may be insensitive to changes in the formal oxidation states of the Mn ions during any of the S state transitions (4, 32, 43). This suggestion could explain the observed insensitivity of D₁-Asp170 (26, 40), D₁-Glu189 (28), and D₁-Asp342 (this work) to oxidations of the Mn₄-Ca cluster during the S₀ → S₁, S₁ → S₂, or S₂ → S₃ transitions. However, this suggestion does not account for the strikingly large number of features that are present in the individual Sₙ-minus-S₁ FTIR difference spectra, all of which correspond to vibrational modes that change frequency in response to the oxidation of the Mn₄-Ca cluster, and many of which correspond to the symmetric and asymmetric modes of carboxylate groups. If these features do not correspond to metal-ligating carboxylate residues, then to what carboxylate residues do they correspond? They do not correspond to changes in the protonation states of free carboxylates because
the intensities of the features that would correspond to such changes [those in the ν(C=O) regions of the Sν+1-minus-Sν FTIR difference spectra (44)] are very weak in comparison to the strong intensities of the features in the νsym(CO2−) and νasym(CO2−) regions (Figures 4A–4D, traces a).

CONCLUDING REMARKS

The mid-frequency Sν+1-minus-Sν FTIR difference spectra of D1-D342N mutant particles are remarkably similar to those of wild-type PSII particles. In particular, there is no indication that the D1-D342N mutation eliminates any carboxylate modes. Therefore, either D1-Asp342 ligates a Mn ion that does not increase its charge or oxidation state during any of the S0 → S1, S1 → S2, or S2 → S3 transitions or D1-Asp342 does not ligate the Mn4 cluster. If D1-Asp342 does ligate the Mn4 cluster (as is expected), then the FTIR data provides a constraint on the identity of the Mn ion(s) whose charge or oxidation state increases during the S0 → S1 and S2 → S3 transitions.

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