Flash-Induced FTIR Difference Spectroscopy Shows No Evidence for the Structural Coupling of Bicarbonate to the Oxygen-Evolving Mn Cluster in Photosystem II[†]

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ABSTRACT: Bicarbonate is known to be required for the maximum activity of photosystem II. Although it is well established that bicarbonate is bound to the nonheme iron to regulate the quinone reactions, the effect of bicarbonate on oxygen evolution is still controversial, and its binding site and exact physiological roles remain to be clarified. In this study, the structural coupling of bicarbonate to the oxygen-evolving center (OEC) was studied using Fourier transform infrared (FTIR) difference spectroscopy. Flash-induced FTIR difference spectra during the S-state cycle of OEC were recorded using the PSII core complexes from *Thermosynechococcus elongatus* in the presence of either unlabeled bicarbonate or ^{13}C-bicarbonate. The $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double difference spectra showed prominent bicarbonate bands at the first flash, whereas no appreciable bands were detected at the second to fourth flashes. The bicarbonate bands at the first flash were virtually identical to those from the nonheme iron, which was preoxidized by ferricyanide and photoreduced by a single flash, recorded using Mn-depleted PSII complexes. Using the bicarbonate bands of the nonheme iron as an internal standard, it was concluded that no bicarbonate band arising from OEC exists in the S-state FTIR spectra. This conclusion indicates that bicarbonate is not affected by the structural changes in OEC upon the four S-state transitions. It is thus strongly suggested that bicarbonate is neither a ligand to the Mn cluster nor a cofactor closely coupled to OEC, although the possibility cannot be fully excluded that nonexchangeable bicarbonate exists in OEC as a constituent of the Mn-cluster core. The data also provide strong evidence that bicarbonate does not function as a substrate or a catalytic intermediate. Bicarbonate may play major roles in the photoassembly process of the Mn cluster and in the stabilization of OEC by a rather indirect interaction.

Photosystem II (PSII[1]) in plants and cyanobacteria functions as light-driven water−quinone oxidoreductase, in which plastoquinone is reduced by electrons from water. Water oxidation that leads to the evolution of molecular oxygen is performed at the oxygen-evolving center (OEC) with a metal-cluster core, the so-called Mn cluster, which consists of four Mn ions and one Ca^{2+} ion (*1–3*). Although the X-ray crystallographic structures of PSII core complexes at 3.0–3.5 Å resolution (*4, 5*) in combination with the information by polarized EXAFS (*6*) provided the image of the Mn cluster, its detailed structure with a ligand environment has not been clarified yet.

Bicarbonate (hydrogencarbonate) has been proposed to be a cofactor necessary for the maximum activity of PSII reactions [reviewed in refs *7–12*]. The bicarbonate effect was first discovered by Warburg and Krippahl (*13*), who observed the acceleration of the Hill reaction by CO_2. Initially, the donor side of PSII was considered as a possible acting site of bicarbonate (*14*). Bicarbonate was proposed to be a substrate or a catalytic intermediate of oxygen formation from water possibly coupled with the carbonic anhydrase activity of PSII (*15, 16*). However, the real role of bicarbonate on the donor side remained unclear. However, evidence has been accumulated that bicarbonate functions on the electron acceptor side, and now it is well established that one bicarbonate molecule is bound to the nonheme iron to regulate the reactions of the quinone acceptors (*4, 5, 7, 8, 11, 12, 17–19*).

Since 1995, various data have been accumulated showing that bicarbonate is indeed functional in oxygen evolution (*9, 10, 12, 20–36*). In addition to the earlier proposal of a catalytic intermediate (*15, 16, 36*), it has been suggested that bicarbonate functions as a ligand to the Mn cluster or an integral cofactor of OEC (*20, 24, 26, 33*), a cofactor crucial in the photoassembly process of the Mn cluster as a transient ligand to Mn ions (*20, 21, 25, 27–31, 35*), a proton transfer mediator bound to a residue in the vicinity of the Mn cluster (*32*), and an element to indirectly stabilize OEC by binding to PSII proteins or extrinsic proteins (*20, 21, 34*). In fact, the X-ray structure by Ferreira et al. (*4*) at 3.5 Å resolution tentatively assigned a part of the nonprotein density to bicarbonate as a ligand bridging Mn and Ca ions. In contrast,

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[1] Abbreviations: DM, *n*-dodecyl-*β*-D-maltoside; FTIR, Fourier transform infrared; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving center; PSII, photosystem II.

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the structure by Loll et al. (*5*) at 3.0 Å did not show a bicarbonate ligand in the Mn cluster. More recently, the bicarbonate function as a substrate or a catalytic intermediate was excluded by UV detection of the OEC reaction under high CO_2 pressure and mass spectrometry detection of the CO_2 signal under $H_2{}^{18}O$ enrichment (*37*). A similar conclusion was obtained by quantifying the carbonic anhydrase activity of PSII and the flux of oxygen from bicarbonate into O_2 by rapid-mixing isotopic experiments using $^{18}O/^{13}C$-bicarbonate (*38*). Thus, the binding site of bicarbonate in OEC and exact physiological roles in the oxygen-evolving reaction remain to be elucidated.

Light-induced Fourier transform infrared (FTIR) difference spectroscopy is a powerful method to study the detailed structure and reactions of OEC (reviewed in refs *39–44*). Previously, Yruela et al. (*26*) studied the interaction of bicarbonate in OEC using this technique. They asserted that they found bicarbonate bands in the FTIR spectra of OEC and proposed that bicarbonate is a ligand to the Mn cluster. However, their measurement conditions, that is, continuous illumination at room temperature using a pellet sample with an exogenous electron acceptor, were not appropriate to properly control the S-state transitions and record FTIR difference spectra without artificial signals (*40, 41*).

In this study, we have re-examined the structural coupling of bicarbonate to OEC by means of FTIR difference spectroscopy. The measurement method of flash-induced FTIR difference spectra during the S-state cycle are now well established (*40–44*), and the spectra from several research groups are in agreement with each other (*45–50*). We have measured the FTIR difference spectra of the S-state cycle using the PSII core complexes from *Thermosynechococcus elongatus* in the presence of either unlabeled bicarbonate or ^{13}C-bicarbonate. If bicarbonate is coupled to the S-state transitions, the ^{13}C-induced shifts of bicarbonate bands should be detected in the difference spectra. We have used the signals of bicarbonate bound to the nonheme iron, which can be an endogenous electron acceptor in an oxidizing medium (*51, 52*), as an internal standard to estimate the signal intensity (or the absence of signals) as well as to confirm the replacement of bicarbonate in PSII proteins with ^{13}C species.

MATERIALS AND METHODS

Oxygen-evolving PSII core complexes from *T. elongatus* in which the carboxyl terminus of the CP43 subunit was genetically histidine-tagged were purified using Ni^{2+} affinity column chromatography as described previously (*53*). The PSII core complexes were suspended in a 10 mM Mes-NaOH buffer (pH 6.0) containing 5 mM $CaCl_2$, 5 mM NaCl, and 0.06% DM (buffer A), and concentrated to 4.5 mg of Chl/mL using Microcon-100 (Amicon). Mn-depleted PSII core complexes were prepared by NH_2OH (10 mM) treatment for 1 h at room temperature in the dark (*53*).

For measurements of FTIR spectra during the S-state cycle of OEC, the oxygen-evolving PSII core sample (4.5 mg Chl/mL; 4 μL) in buffer A was mixed with 1 μL of a potassium ferricyanide solution (100 mM) and then lightly dried under N_2 gas flow on a CaF_2 plate (25 mm in diameter). Subsequently, 4 μL of a 10 mM $NaHCO_3$ ($NaH^{12}CO_3$ or $NaH^{13}CO_3$ (Shoko Co. Ltd., 99 at. % ^{13}C)) solution was added to the sample and quickly mixed using a thin glass stick. The sample was dried under N_2 gas again to make a film in an oval shape (9 mm × 6 mm). The dry film was covered with another CaF_2 plate with a greased Teflon spacer (0.5 mm in thickness). In this sealed IR cell, 2 μL of a 20% (v/v) glycerol/water solution involving 50 mM $NaH^{12}CO_3$ or $NaH^{13}CO_3$ (for the sample with $NaH^{13}CO_3$) was placed without touching the sample to form a moderately hydrated film (*47*). The temperature of the sample was adjusted to 10 °C by circulating cooled water through a copper holder.

In an experiment to ensure the replacement of bicarbonate, formate treatment was applied to the PSII sample. The PSII core suspension (4.5 mg Chl/mL; 4 μL) was mixed with 180 μL of a 50 mM Mes buffer (pH 6.0) containing 5 mM $CaCl_2$, 5 mM NaCl, 0.06% DM, and 250 mM sodium formate and incubated for 1 h on ice in the dark. The sample was then washed with the same buffer containing formate and subsequently washed four times with buffer A containing 5 mM $NaH^{13}CO_3$. The suspension was finally concentrated to 4 μL. It was dried under N_2 gas on a CaF_2 plate followed by the addition of 4 μL of a 5 mM $NaH^{13}CO_3$ solution (the total amount of $NaH^{13}CO_3$ is identical to that in the experiment without formate treatment), and a hydrated film was formed as mentioned above.

FTIR spectra were recorded on a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (InfraRed D316/8) at 4 cm^{-1} resolution. Flash-induced FTIR spectra of the S-state cycle of OEC were measured with a method similar to that described previously (*47*). Illumination was performed by a Q-switched Nd:YAG laser (Quanta-Ray GCR-130, 532 nm, ~7 ns fwhm, ~7 mJ $pulse^{-1}$ cm^{-2}). After two preflashes (1 Hz) followed by dark adaptation for 75 min, the sample was subjected to four consecutive flashes with 10-s intervals. Single-beam spectra (10-s scans for each) were recorded before, between, and after the flashes. Two spectra were recorded before the illumination. The sample was then dark-adapted for 75 min. This cycle from the preflash illumination to the final dark adaptation was repeated 6–8 times for one sample, and difference spectra upon the first−fourth flashes and a dark-minus-dark spectrum before illumination were calculated. Spectra recorded using two samples were averaged for final data.

The procedure of sample preparation for measurements of the nonheme iron spectra was identical to that for measurements of the S-state spectra except for using the Mn-depleted PSII core complexes instead of the oxygen-evolving ones. The dark-adapted sample was preilluminated by three flashes (1 Hz) to oxidize Y_D. After dark adaptation for 8 min, two single-beam spectra (50-s scans for each) before a single-flash illumination and one spectrum after illumination were recorded. The measurement was repeated 16 times every 8 min, and a dark-minus-dark spectrum and a flash-induced difference spectrum were calculated using averaged spectra.

RESULTS AND DISCUSSION

Figure 1 (black line) shows flash-induced FTIR difference spectra (1800–1050 cm^{-1}) of the S-state cycle of OEC measured using a hydrated film of the PSII core complexes from *T. elongatus* in the presence of unlabeled bicarbonate. The spectra at the first (a), second (b), third (c), and fourth

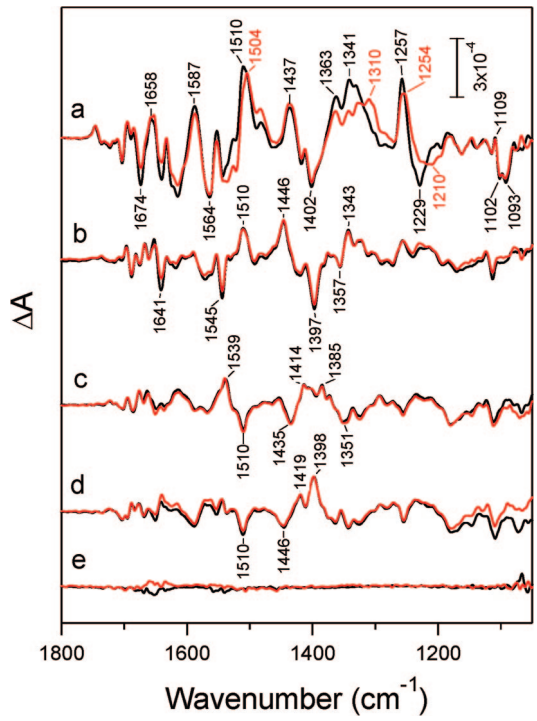

FIGURE 1: Flash-induced FTIR difference spectra (1800–1050 cm^{-1}) during the S-state cycle of OEC in the PSII core complexes from *T. elongatus* in the presence of $H^{12}CO_3^-$ (black line) and $H^{13}CO_3^-$ (red line). Difference spectra were recorded upon the first (a), second (b), third (c), and fourth (d) flash illumination at 10 °C. Dark-minus-dark spectra (e) recorded before illumination represent noise levels.

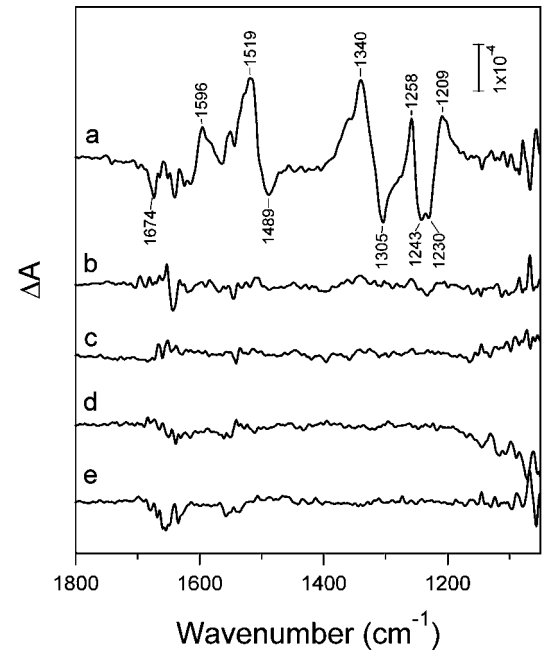

FIGURE 2: $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double-difference spectra of the S-state cycle for the first (a), second (b), third (c), and fourth (d) flashes. A double-difference spectrum of the dark-minus-dark spectra is shown in (e).

(c) flashes (spectrum d shows a noise level) virtually represent the structural changes upon $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$, and $S_0 \rightarrow S_1$ transitions, respectively (*47*). The overall spectral features were very similar to those previously reported (*45–50*); complex peaks at 1600–1500 cm^{-1} were assigned to the amide I vibrations (C═O stretch of backbone amides) due to conformational changes of proteins during the S-state cycle, and prominent bands at 1600–1500 and 1450–1300 cm^{-1} were mostly attributed to the asymmetric and symmetric stretching vibrations of carboxylate groups (*48, 49*). The amide II modes (NH bend + CN stretch of backbone amides) may also contribute to the 1600–1500 cm^{-1} region (*48, 49*).

Despite the similarities to the previous S-state spectra, additional features were observed in the first-flash spectrum at 1341, 1257, 1229, 1109, 1102, and 1093 cm^{-1} (Figure 1a). These peaks are typical of the nonheme iron signals (Fe^{3+}/Fe^{2+}) (*19, 54, 55*). The signals of the nonheme iron appear in the difference spectra because it is preoxidized by ferricyanide and reduced by the first-flash illumination (*51, 52*). In fact, the CN stretching peaks of ferricyanide/ferrocyanide at 2116/2039 cm^{-1} in the first-flash spectrum showed about half the intensity of those in the second- to fourth-flash spectra (data not shown), indicating that an electron was trapped by the nonheme iron at the first flash in half the centers. The intensities of the nonheme iron signals in the first-flash spectrum were clearly larger than those in the previous S-state spectra of the same *T. elongatus* core complexes (*45, 47, 48*). This may be because the present sample involves relatively high concentration of bicarbonate (approximately 40 mM in the core film), and hence most of the nonheme iron centers have a bicarbonate ligand; the nonheme iron with a bicarbonate ligand is readily oxidized by ferricyanide, whereas that without bicarbonate is not (*52*). It is also noted that signals from Y_D could slightly contaminate the first-flash spectrum judging from the small peaks at 1703/1696 cm^{-1} typical of $Y_D^{\bullet}/Y_D$ spectra (*56, 57*).

When ^{13}C-bicarbonate was added to the sample instead of unlabeled bicarbonate, some spectral changes were observed in the first-flash spectrum, whereas basically no change was seen in the second- to fourth-flash spectra (Figure 1, red lines). The peaks at 1510, 1341, 1257, and 1229 cm^{-1} downshifted to 1504, 1310, 1254, and 1210 cm^{-1}, respectively. The effects of isotope substitution are better expressed in $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double difference spectra (Figure 2) calculated from the spectra in Figure 1. As expected, spectra at the second−fourth flashes did not show any meaningful peaks (Figure 2b−d), whereas at the first flash, prominent features were observed at 1674(−), 1596(+), 1519(+), 1489(−), 1340(+), 1305(−), 1258(+), 1243(−), 1230(−), and 1209(+) cm^{-1} (the + and − signs in parentheses indicate positive and negative peaks, respectively). A small negative peak at 1641 cm^{-1} could be a noise in the amide I region, which has a high absorption in the original infrared spectrum. Similar noises in this region were also seen in the spectra at the second−fourth flashes (Figure 2b−d) and in the dark-minus-dark spectrum (Figure 2e).

To identify whether the signals of HCO_3^- at the first flash arise from OEC or the nonheme iron, the FTIR spectrum of the nonheme iron was obtained using the Mn-depleted PSII core complexes in the presence of either unlabeled or ^{13}C-labeled bicarbonate (Figure 3a). The spectrum with unlabeled bicarbonate (Figure 3a, black line) was very similar to the previous Fe^{2+}/Fe^{3+} difference spectrum measured using PSII membranes of spinach (*19, 54, 55*). Note that a small amount of Y_D signals may contaminate the spectrum, as shown by the presence of the small peaks at 1703/1696 cm^{-1} due to the $Y_D^{\bullet}/Y_D$ difference (*56, 57*).

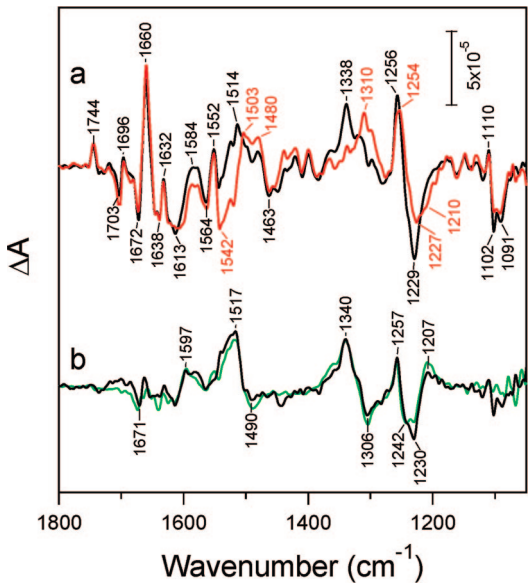

FIGURE 3: (a) FTIR difference spectra of the nonheme iron (Fe^{2+}-minus-Fe^{3+}) of the Mn-depleted PSII core complexes in the presence of $H^{12}CO_3^-$ (black line) and $H^{13}CO_3^-$ (red line). (b) A $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double difference spectrum of the nonheme iron (black line) in comparison with that of the S-state cycle at the first flash (green line; the same spectrum as that in Figure 2a).

The Fe^{2+}/Fe^{3+} spectrum with ^{13}C-bicarbonate (Figure 3a, red line) showed clear shifts of bands in the 1600–1470 and 1340–1200 cm^{-1} regions. The $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double difference spectrum of the Fe^{2+}/Fe^{3+} spectra (Figure 3b, black line) showed peaks at 1671(−), 1597(+), 1517(+), 1490(−), 1340(+), 1306(−), 1257(+), 1242(−), 1230(−), and 1207(+) cm^{-1}. This double difference spectrum is very similar to the corresponding spectrum of the Mn-depleted PSII membranes of spinach measured by Hienerwadel et al. (*19*), which showed peaks at 1658(−), 1600(+), 1530(+), 1495(−), 1339(+), 1305(−), 1258(+), ∼1240(−), 1230(−), and 1205(+). They assigned the 1658/1530 and 1230/1339 cm^{-1} peaks to the asymmetric and symmetric CO stretching vibrations, respectively, of the bicarbonate ligating the Fe^{3+}/Fe^{2+} ions (*19*). The close similarity of the bicarbonate signals in the present study to those of spinach indicates that the structure and interaction of the bicarbonate bound to the nonheme iron are basically identical between cyanobacteria and plants.

In Figure 3b, the $H^{12}CO_3^-$-minus-$H^{13}CO_3^-$ double difference spectrum of the nonheme iron (black line) was compared with that of the S-state cycle at the first flash (green line; identical spectrum to Figure 2a). It is shown that the two spectra are virtually identical to each other. This observation indicates that all of the bicarbonate peaks in the first flash spectrum of the S-state cycle (Figure 2a) arise from the nonheme iron on the electron acceptor side and that there is no contribution of the signals from OEC.

The bicarbonate bands of the nonheme iron at the first flash (Figure 2a) can be an ideal internal standard to judge the presence or absence of bicarbonate signals of OEC. Because photoreduction of the preoxidized nonheme iron took place in about half the centers of the PSII sample (see above), if OEC includes a strongly coupled bicarbonate in all the centers, its signals should appear in the S-state spectra with intensities (and at different positions) two times larger than the bicarbonate bands of the nonheme iron. Thus, the observation that bicarbonate signals from OEC could not be detected above the noise level (∼5% of the bicarbonate signal of the nonheme iron; Figure 2) indicates that such a coupled bicarbonate molecule does not exist in OEC.

The presence of ^{13}C-bicarbonate signals of the nonheme iron in the first flash spectrum (Figures 1a and 2a) also indicates that the experimental conditions used in the present study were appropriate to exchange bicarbonate molecules attached to PSII (at least the bicarbonate bound to the nonheme iron) and that the added ^{13}C-bicarbonate was not replaced with an unlabeled one by CO_2 in atmosphere during the procedure. $NaH^{13}CO_3$ was added to the sample just before closing an infrared cell, and the glycerol/water solution placed in the cell to hydrate the sample also contained 50 mM $NaH^{13}CO_3$ to exchange CO_2 in the cell for $^{13}CO_2$. Because the typical bicarbonate band at 1338 cm^{-1} in the Fe^{2+}/Fe^{3+} spectrum (Figure 3a, black line) was fully shifted to 1310 cm^{-1} by ^{13}C-bicarbonate addition (Figure 3a, red line), it can be considered that the bicarbonate attached to the nonheme iron was fully replaced with the ^{13}C species.

The concentration of $NaHCO_3$ in the PSII hydrated film was approximately 40 mM (4 μL of 10 mM $NaHCO_3$ is concentrated to approximately 1 μL in a hydrated film). The pH of the buffer after mixing the $NaHCO_3$ solution was about 6.6, and the concentration of HCO_3^- in the medium was estimated to be ∼26 mM. This concentration should be enough to replace bicarbonate in OEC, because the K_d value of bicarbonate required for OEC activity has been estimated to be 20–34 μM in PSII membrane fragments from spinach (*25*). Also, the exchange rate of bicarbonate should not be more than a few minutes because inhibition of O_2 activity and the formation of the S_2-multiline signal in a bicarbonate-depleted (flushed by CO_2-depleted gas) medium have been reported without specification of the incubation time (*25, 27*). However, one may still be concerned about the possibility that bicarbonate in OEC is very strongly bound and cannot be exchanged spontaneously with ^{13}C-bicarbonate. To ensure bicarbonate exchange, the PSII core complexes were treated with 250 mM formate for 1 h to forcefully remove bicarbonate from the PSII protein and then washed with a buffer involving 5 mM $NaH^{13}CO_3$. This concentration of formate was reported to fully inhibit the O_2 evolution and S_2-multiline formation in PSII membranes of spinach (*58*). We verified that the 250 mM formate treatment was also effective to the core complexes of *T. elongatus* and inhibited the O_2-evolving activity by 88% (from 3420 to 420 μmol O_2 mg Chl^{-1} h^{-1}). The S-state spectra measured using this sample are shown in Figure S1 (Supporting Information) (red lines) in comparison with those of the untreated PSII sample in the presence of ^{13}C-bicarbonate (black lines; identical to red lines in Figure 1). The spectra with and without formate treatment were virtually identical. This observation excludes the possibility that bicarbonate was not exchanged because of its strong binding to OEC.

From these results, it is concluded that there are no detectable bicarbonate signals in the FTIR difference spectra of the four flash-induced S-state transitions ($S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$, and $S_0 \rightarrow S_1$). This conclusion is at odds with the previous assertion by Yruela et al. (*26*) that they found bicarbonate signals in the FTIR difference spectra of OEC. However, their FTIR measurements of OEC were signifi-

cantly problematic, and the resultant spectra mostly arose from the mixture of various artifacts. The details of the problems in their FTIR spectra are discussed in Supporting Information.

The absence of bicarbonate bands in the FTIR difference spectra of the S-state cycle indicates that the structure and interaction of bicarbonate are not affected by the structural changes of the Mn cluster and the proteins upon any of the four S-state transitions. This conclusion strongly suggests that bicarbonate is neither a ligand to the Mn cluster nor a cofactor strongly coupled to the Mn cluster. This is consistent with the recent results that neither the miss parameter of the S-state cycling nor the life-times of the S states were affected by washing with bicarbonate-free medium (*35*). The possibility cannot be excluded, however, that bicarbonate is silent in FTIR spectra because it is a ligand to a Mn ion that is not affected by any S-state transitions or is located in the vicinity of the Mn cluster but not affected by any structural changes of proteins upon the S-state transitions. Even in this case, bicarbonate is probably not directly related to the water oxidizing reaction by regulating the redox potential of the Mn cluster and working as an immediate proton abstractor through a hydrogen-bond with substrate water. Another possibility cannot be fully excluded that bicarbonate is incorporated into the Mn cluster during the photoassembly process to be a constituent of the core structure, and hence, it cannot be released even by high-concentration formate. Further experiments of exchanging $^{12}C/^{13}C$-bicarbonate during photoassembly of the Mn cluster may be necessary to clarify this point.

The present results also provide strong evidence that bicarbonate is neither a substrate nor a catalytic intermediate, in agreement with recent studies using UV detection under high CO_2 pressure and ^{18}O mass spectrometry (*37, 38*); if bicarbonate is directly involved in the substrate reaction, its structural changes should be detected in FTIR difference spectra. However, bicarbonate can be indirectly involved in water reactions as a proton transfer mediator (*32*). In this case, the structure of bicarbonate does not necessarily change before and after proton release. Probably, bicarbonate plays major roles in the photoassembly process of the Mn cluster as a ligand to its precursor (*20, 21, 25, 27–31, 35*) and stabilization of the Mn cluster through an indirect interaction by binding at PsbO or some site in PSII proteins (*20, 21, 34*).

SUPPORTING INFORMATION AVAILABLE

FTIR difference spectra of the S-state cycle recorded using the PSII core complexes in the presence of ^{13}C-bicarbonate after 250 mM formate treatment in comparison with those without formate treatment and the details of the problems of the previous FTIR measurements and the spectra by Yruela et al. (*26*). This material is available free of charge via the Internet at http://pubs.acs.org.

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