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FTIR detection of the molecular reactions during the oxygen-evolving S-state cycle

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Introduction

Photosynthetic oxygen evolution is performed in photosystem II (PS II) of plants and cyanobacteria. The catalytic center of this reaction is the oxygen-evolving complex (OEC), which resides on the electron donor side of PS II. The OEC has a core structure of the tetra-nuclear Mn-cluster, which consists of four Mn and one Ca ions connected by μ-oxo and amino-acid ligands. In OEC, two water molecules are oxidized to cleave into one oxygen molecule and four protons. This reaction proceeds through a light-driven cycle called the S-state cycle (Joliot et al. 1969; Kok et al. 1970), which consists of five intermediates, S_0–S_4. Although this concept of the S-state cycle has been well established, the structure of OEC in the individual S-state intermediates, the molecular mechanism of oxygen evolution, and the involvement of proteins in the reactions mostly remain to be clarified. Fourier transform infrared (FTIR) spectroscopy is a powerful method to study the detailed structure of the active site and the reaction of a protein. In this study, we have used flash-induced FTIR difference spectroscopy to detect the reactions of substrate water and proteins in OEC during the S-state cycle.

Materials and methods

Oxygen-evolving PS II core complexes from *Synechococcus elongatus*, in which the carboxyl terminus of the CP43 subunit was genetically histidine-tagged, were purified using Ni^{2+}-affinity column chromatography as described by Sugiura and Inoue (1999). The PS II core complexes were suspended in 10 mM Mes-NaOH (pH 6.0) containing 5 mM NaCl, 5 mM $CaCl_2$, and 0.06% DM. An aliquot of the core suspension (4.5 mg of Chl/mL; 4 μL) mixed with 1.0 μL of 100 mM potassium ferricyanide was cast on a CaF_2 plate (25ϕ × 3 mm) about 10 mm in diameter. The sample was then dried under N_2 gas flow, and covered with another CaF_2 plate spaced with a greased Teflon spacer (0.5 mm in thickness). In this sealed cell, 1 μL of glycerol/water solution with various mixing ratios was placed to achieve desired humidity (Fig. 1).

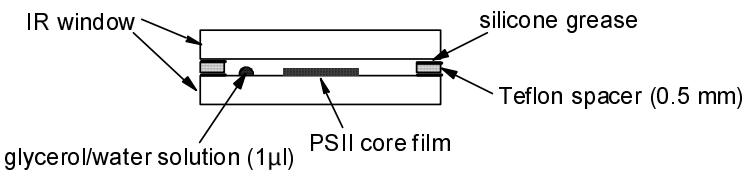

Fig. 1. Sample cell for FTIR measurements

FTIR spectra were measured on a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (InfraRed D316/8). The sample temperature was kept at 10 ˚C by circulating cool water through a copper sample holder. A Ge filter (OCLI, LO2584-9) was placed in front of the sample in the IR-beam path to block the red light of the He-Ne laser leaking from the interferometer. Flash illumination was performed by ns pulses from a frequency-doubled Q-switched Nd:YAG laser (Quanta-Ray GCR-130; 532 nm; ~7 ns FWHM). The pulse energy was ~10 mJ/pulse cm^2 at the sample point. Flash-induced FTIR measurement during the S-state cycle of OEC was performed by repeating the cycle of 6 successive flash illumination (10 s interval) and dark adaptation for 1 h. Seven single-beam spectra (10 s scan) were measured before the 1st flash, between the flashes and after the 6th flash. By the dark adaptation for 1 h, the S_2 and S_3 states relax to the S_1 state and also the S_0 state is oxidized to the S_1 state by ferricyanide in the sample. Difference spectra upon individual flashes were calculated as difference between the single-beam spectra measured before and after the flash. The spectral resolution was 4 cm^{-1}.

Results and discussion

Figure 2 presents the FTIR spectra (3800–2700 cm^{-1}) of the PS II core films showing the OH stretching region of water. All the spectra were normalised by intensities of the sharp bands at 3000–2800 cm^{-1} due to the CH stretching vibrations of methyl and methylene groups. In the dry film (no glycerol/water solution is placed in the cell), a band was observed around 3300 cm^{-1}, which is due to the NH stretching vibrations of polypeptide main chains with some contributions of the OH stretching vibrations of protein side chains, detergent (DM), and internal water in the protein. As the relative humidity (RH) in the IR cell increased by the presence of glycerol/water solution with a mixing ratio (v/v) of 80% (46% RH), 60% (73% RH), 40% (95% RH) and 20 % (> 98% RH), the OH bands of water centred at 3400 cm^{-1} became larger (Fig. 2). Thus, the extent of hydration of the PS II core film can be controlled using this method. This hydration control of the PS II sample makes it possible to measure the water region of FTIR difference spectra during the S-state cycle by avoiding absorption saturation.

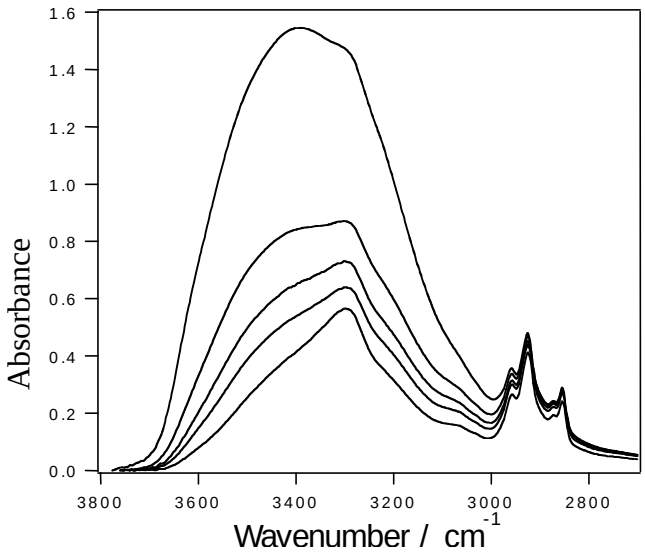

Fig. 2. OH stretching region of the FTIR spectra of moderately hydrated PSII films. The dry film (lowest trace) was hydrated with H_2O vapour of 80%, 60%, 40%, and 20% glycerol/water solution (from the second lowest to upper trances).

In Fig. 3 (solid line) are shown FTIR difference spectra (1800–1200 cm^{-1}) upon the 1st to 4th flashes measured using the PS II core film hydrated with water vapour of 20% glycerol/water solution. These spectra showed basically the same spectral features as those of a liquid PS II sample (Noguchi and Sugiura, 2001), indicating that the PS II sample was hydrated enough for a functional S-state cycle. The features of these four spectra significantly

differ from each other. The 1st-flash spectrum (Fig. 3a, solid line) was basically identical to the S_2/S_1 difference spectrum that has been measured at 250 K in the same core complexes of *S. elongatus* (Noguchi and Sugiura, 2000). The intensities at 1400 cm^{-1}, where prominent bands were present in all the spectra, were plotted in Fig. 4 (filled circle) as a function of the flash number. The intensities were negative at the 1st, 2nd, 5th and 6th flashes and positive at the 3rd and 4th flashes, showing a pattern of period-four oscillation. Other prominent peaks similarly showed a period-four oscillation pattern (not shown). Thus, the 1st, 2nd, 3rd and 4th-flash spectra of this hydrated PS II film virtually represent the S_2/S_1, S_3/S_2, S_0/S_3 and S_1/S_0 difference spectra, respectively. These difference spectra are similar to those measured using PS II membranes from spinach by Hillier and Babcock (2001).

In all the four spectra (Fig. 3, solid line), prominent bands are observed in the symmetric (1450–1300 cm^{-1}) and asymmetric (1600–1500 cm^{-1}) stretching regions of carboxylate groups (Noguchi et al., 1995), and the amide I region (1700–1600 cm^{-1}) of backbone amides, suggesting that drastic structural changes of the carboxylate ligands and protein conformations occur in every S-state transition. The band frequencies in the carboxylate regions are rather different between the S_2/S_1 and S_3/S_2 spectra (e.g., positive bands at 1508, 1437 and 1362 cm^{-1} vs 1514, 1446 and 1344 cm^{-1}; negative bands at 1541, 1417 and 1400 cm^{-1} vs 1545, 1396 and 1358 cm^{-1}). The band pattern in the amide I region is also different; the S_2/S_1 spectrum is characterised by a strong positive band at 1653 cm^{-1} and negative bands at 1676 and 1662 cm^{-1}, while in the S_3/S_2 spectrum, negative bands at 1687 and 1641 cm^{-1} are prominent. These observations indicate that the carboxylate groups and polypeptide main chains that react in the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions are different between the two transitions. It is further found that these structural changes in $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ are reversed in either $S_3 \rightarrow S_0$ or $S_0 \rightarrow S_1$ transition; In the S_0/S_3 and S_1/S_0 spectra (Fig. 3c, d, solid line), bands are observed at the frequencies close to the bands in the S_2/S_1 or S_3/S_2 spectra (Fig. 3a,b, solid line) but with opposite signs. For instance, in the carboxylate regions, the negative peaks at 1396 (S_3/S_2), 1400 (S_2/S_1), 1417 (S_2/S_1), and 1545 (S_3/S_2) cm^{-1} may correspond to the positive peaks at 1396 (S_1/S_0), 1398 (S_0/S_3), 1415 (S_0/S_3), and 1541 (S_0/S_3) cm^{-1}, respectively. Also, the positive peaks at 1344 (S_3/S_2), 1446 (S_3/S_2), 1508 (S_2/S_1) and 1587 (S_2/S_1) cm^{-1} may be the counterparts of the negative peaks at 1344 (S_0/S_3), 1450 (S_1/S_0), 1512 (S_0/S_3) and 1589 (S_1/S_0) cm^{-1}, respectively. In the amide I region, the prominent bands at 1653 and 1676 cm^{-1} in the S_2/S_1 spectrum (Fig. 3a solid line) have the counterparts at the same positions with opposite signs in the S_0/S_3 (Fig. 3c solid line) spectrum, and the counterparts of the bands at 1687 and 1641 cm^{-1} in the S_3/S_2 spectrum (Fig. 3b solid line) are found in the S_1/S_0 spectrum (Fig. 3d solid line).

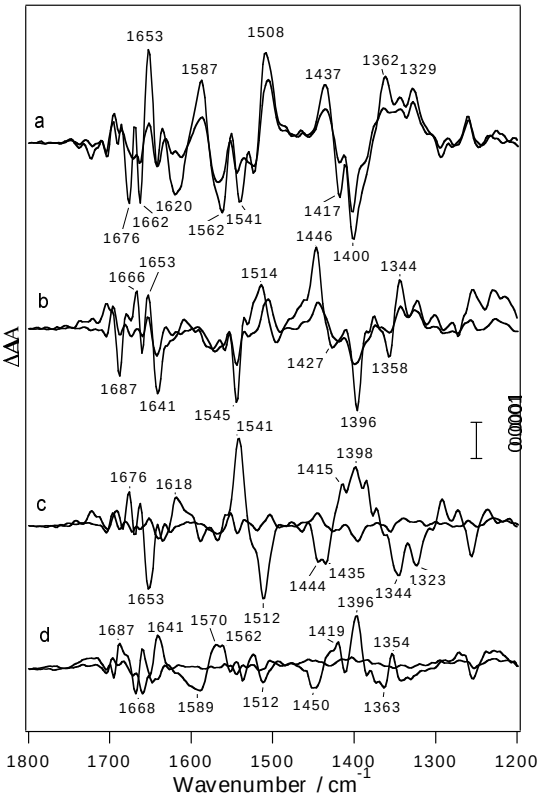

Fig. 3. Flash-induced FTIR difference spectra of the PS II core films hydrated by water vapour of 20% (solid line) and 80% (dotted line) glycerol/water solution. Spectra show differences upon the 1st (a), 2nd (b), 3rd (c) and 4th (d) flashes.

When the PSII core film is less hydrated, the S-state cycle is inhibited. As an example, the flash-induced difference spectra of the PS II sample hydrated using 80% glycerol/water (46% RH) are shown in Fig. 3 (dotted line). The 1st- and 2nd-flash spectra (Fig. 3a, b dotted line) were similar to the S_2/S_1 and S_3/S_2 spectra, respectively, of the PS II film hydrated using 20% glycerol/water (Fig. 3a, b solid line), although the spectral intensities were a little smaller. However, upon the 3rd and later flashes, the spectrum similar to the S_3/S_0 spectrum was not observed.

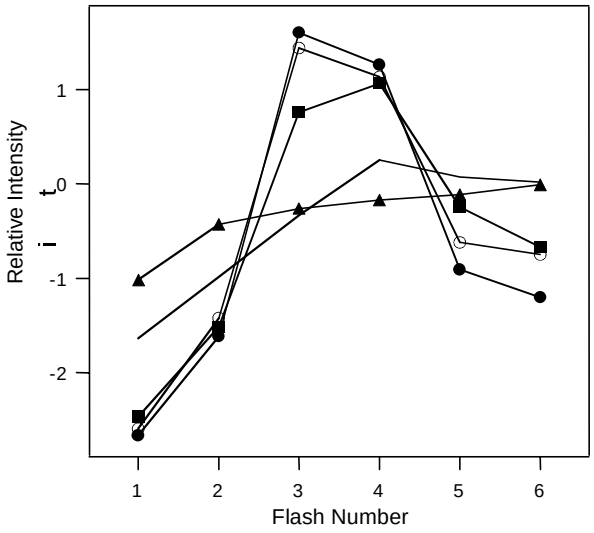

Fig. 4. Relative intensities at 1400 cm^{-1} of the FTIR difference spectra as a function of the flash number. Dry PS II film (filled triangle) was hydrated by water vapour of 20% (filled circle), 40% (open circle), 60% (filled square), 80% (open square) glycerol/water solution.

The effect of dehydration on the S-state transitions is more clearly expressed in the flash-dependent intensity at 1400 cm^{-1} (Fig. 4). The intensities of the PS II film hydrated using 40% glycerol/water (open circle) was basically the same as those of the film hydrated by 20% glycerol/water (filled circle). In the sample hydrated using 60% glycerol/water, the intensities follow those of the more hydrated ones up to the 2nd flash, but the oscillation is slightly

dumped after the 3rd flash. In the film with 80% glycerol/water (open square), only little intensities were observed after the 3rd flash. In the dry PS II film (filled triangle), the intensity of the 1st-flash spectrum was less than half of that of the fully hydrated film, and the intensity never became positive by further flashes. These observations suggest that the $S_3 \rightarrow S_0$ transition is more inhibited upon dehydration than the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions. This means that the chemical reaction of the $S_3 \rightarrow S_0$ transition is more coupled to water. This is consistent with the view that the substrate water is incorporated into the catalytic site in the $S_4 \rightarrow S_0$ transition after release of molecular oxygen.

Figure 5 (solid lines) shows the OH stretching region of the 1st–4th-flash spectra of the PS II film hydrated with H_2O vapour of 40% glycerol/H_2O. The 1st-flash spectrum (Fig. 5a, solid line) exhibited a differential band at 3620/3587 cm^{-1}, which was previously observed with a frozen sample at 250 K (Noguchi and Sugiura, 2000). In the 2nd and 3rd-flash spectra (Fig. 3b,c), negative bands at 3635 and 3620 cm^{-1}, respectively, were observed. The 4th-flash spectrum (Fig. 3d) showed a differential signal at 3616/3591 cm^{-1}, which seems to be the counterpart of the signal of the 1st-flash spectrum with opposite signs. All of these peaks downshifted by about 10 cm^{-1} upon $H_2{}^{18}O$ substitution (Fig. 5 dotted lines), indicating that these are due to the OH stretching bands of water. The different positions and shapes of the water bands among the 1st–4th-flash spectra mean that the observed bands reflect the reactions of water molecules coupled to individual S-state transitions. It is highly likely that the bands arise from substrate water molecules. Alternatively, other internal water coupled to the Mn-cluster can be responsible for the observed bands.

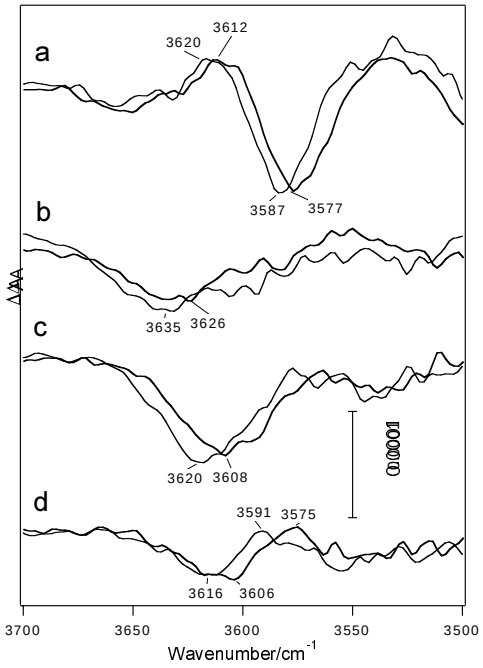

Fig.5. The OH stretching region of the FTIR difference spectra of the PS II core films upon the 1st (a), 2nd (b), 3rd (c), and 4th (d) flashes. The PS II films were moderately hydrated by water vapour of 40% glycerol/H_2O (solid line) and glycerol/$H_2{}^{18}O$ (dotted line).

The above results show that flash-induced FTIR difference spectroscopy is a fruitful method to monitor the reactions of amino acid ligands and substrate water during the oxygen-evolving S-state cycle. Further FTIR studies using isotope substitution in combination with site-directed mutagenesis are expected to clarify the molecular mechanism of photosynthetic oxygen evolution.

Acknowledgments

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