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Accelerated Publications

Detection of Structural Changes upon S_1-to-S_2 Transition in the Oxygen-Evolving Manganese Cluster in Photosystem II by Light-Induced Fourier Transform Infrared Difference Spectroscopy†

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ABSTRACT: The light-induced Fourier transform infrared (FT-IR) difference spectrum between the S_1 and S_2 states of the O_2-evolving photosystem II (PSII) was obtained for the first time. Detection of an S_2/S_1 difference spectrum virtually free from contributions by the acceptor-side signals was achieved by employing an exogenous electron acceptor, potassium ferricyanide, to trypsin-treated PSII membranes and using one-flash excitation at 250 K. A synthetic difference spectrum obtained by adding this S_2/S_1 spectrum to the Q_A^-/Q_A spectrum measured with Mn-depleted PSII was almost identical with the difference spectrum of the $S_2Q_A^-/S_1Q_A$ charge separation measured with untreated PSII. This successful simulation verifies the correctness of the S_2/S_1 spectrum thus obtained. The observed S_2/S_1 spectrum reflects the structural changes within the water-oxidizing Mn cluster upon the S_1-to-S_2 transition, most probably changes in vibrational modes of ligands coordinating to the Mn ion(s) that is (are) oxidized upon the S_2 formation and/or changes in protein conformation. The present results demonstrate that FT-IR difference spectroscopy is a promising method to investigate the structure of the intermediates of the Mn cluster involved in photosynthetic water oxidation.

Higher plants and cyanobacteria utilize water as the ultimate electron donor in successive photosynthetic redox reactions, resulting in cleavage of water to evolve molecular oxygen. Cleavage of water is accompanied by the release of protons, which generates a transmembrane potential gradient, the driving force for photophosphorylation. It is known that the so-called Mn cluster consisting of four Mn ions present on the donor side of photosystem II (PSII)[1] is the direct catalyst for water cleavage and that the reaction proceeds in a cyclic four-electron process involving five different oxidation intermediates designated as S_i states (i = 0–4) [for reviews see Babcock (1987), Brudvig et al. (1989) and Ghanotakis and Yocum (1990)].

Investigation of the structure of the Mn cluster has mostly relied on ESR (Dismukes & Siderer, 1981; de Paula et al., 1986; Hansson et al., 1987; Kim et al., 1990) and X-ray absorption spectroscopy, i.e., EXAFS and XANES (Kirby et al., 1981a,b; Yachandra et al., 1986, 1987; George et al., 1989; Guiles et al., 1990; Penner-Hahn et al., 1990; Kusunoki et al., 1990). These spectroscopic studies indicated the presence of an exchange-coupled multinuclear cluster and a possible involvement of a di-μ-oxo-bridged structure. Despite a variety of models so far proposed [reviewed by Thorp and Brudvig (1991)], the definite structure has not been determined yet. Also, many questions, such as ligand species, structural changes upon each S-state transition, and how and when the two water molecules are cleaved to yield an O–O bond, remain to be solved.

Vibrational spectroscopy is a method advantageous to investigation of molecular structures. In particular, recent de-

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[1] Abbreviations: Chl, chlorophyll; D, auxiliary electron donor of photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FT-IR, Fourier transform infrared; Mes, 4-morpholineethanesulfonic acid; Pheo, pheophytin; PSII, photosystem II; Q_A, primary quinone acceptor in photosystem II; Q_B, secondary quinone acceptor in photosystem II; XANES, X-ray absorption near-edge structure.

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velopment of Fourier transform infrared (FT-IR) spectroscopy, which is characterized by its high reproducibility and good signal-to-noise ratio, has enabled one to detect extremely small structural changes even in a large protein complex in aqueous solution. In the field of photosynthesis, light-induced FT-IR difference spectra of various photochemical intermediates have been obtained for bacterial reaction centers (Mäntele et al., 1985; Hayashi et al., 1986; Nabedryk et al., 1986, 1990b, 1991; Gerwert et al., 1988; Breton et al., 1991a,b; Morita et al., 1991) and for photosystems of higher plants (Tavitian et al., 1986; Nabedryk et al., 1990a,c; Berthomieu et al., 1990). As to PSII, difference spectra of Q_A^-/Q_A (Berthomieu et al., 1990) and of $Pheo^-/Pheo$ (Tavitian et al., 1986; Nabedryk et al., 1990a) have been recently reported.

In this study, we have investigated the structure of the Mn cluster in O_2-evolving PSII by means of light-induced FT-IR difference spectroscopy. Successful elimination of the contributions by acceptor-side signals has led us to observe the difference spectrum only due to the S_1-to-S_2 transition. The resultant spectrum reflects the changes in vibrational modes of ligands of the redox-active Mn ion(s) and/or the conformational changes of proteins. These results suggest that FT-IR difference spectroscopy is a promising method to investigate the structure of the Mn cluster and the O_2-evolving mechanism.

Materials and Methods

BBY-type PSII membranes (Berthold et al., 1981) capable of O_2 evolution were prepared from spinach according to Ono and Inoue (1986). Trypsination of PSII membranes was performed by addition of TPCK–trypsin (Cooper Biomedical) (50 μg/mL) to the PSII membranes (0.5 mg of Chl/mL) suspended in a Mes–NaOH buffer (400 mM sucrose, 20 mM NaCl, 40 mM Mes–NaOH, pH 6.5), followed by incubation at 22 °C for 20 min. Trypsination was stopped by addition of trypsin inhibitor (50 μg/mL) in the digestion mixture, and the trypsinized PSII membranes were washed twice with the same buffer. Mn-depleted PSII membranes were prepared by NH_2OH treatment (0.5 mg of Chl/mL of PSII + 10 mM NH_2OH) and subsequent washes with a Mes–NaOH buffer including 0.5 mM EDTA.

Untreated and trypsin-treated PSII membranes were incubated at 4 °C for several hours in order to relax every O_2-evolving center to the thermally stable S_1 state. Note that the relatively stable S_0 state is also converted to S_1 due to the oxidation by D^+ during this dark incubation (Styring & Rutherford, 1987). For measurement of the S_2/S_1 difference spectrum, the trypsinized PSII suspension (0.5 mg of Chl/mL) was supplemented with potassium ferricyanide (20 mM) and $CaCl_2$ (20 mM) and then centrifuged to form a pellet (see below). For measurement of the Q_A^-/Q_A spectrum, DCMU (0.1 mM) and NH_2OH (10 mM) were added to Mn-depleted PSII (0.5 mg of Chl/mL), and for the $S_2Q_A^-/S_1Q_A$ difference spectrum, DCMU (0.1 mM) was added to untreated PSII (0.5 mg of Chl/mL) before centrifugation.

FT-IR spectra were measured on a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101). The sample temperature was controlled at 250 K with a cryostat (Oxford DN1704) equipped with a temperature control unit (Oxford ITC-4). In order to block the He–Ne laser beam partially leaking into the sample room, a Ge filter (OCLI LO2584-9) was placed in front of the sample. The trypsin-treated PSII suspension (0.5 mg of Chl/mL in a Mes–NaOH buffer) was centrifuged for 25 min at 170000*g* (150000*g* for 15 min for untreated and Mn-depleted PSII), and the resulting pellet was pressed between a pair of BaF_2 plates. The absorbance at 1656 cm^{-1} which is due to the amide I band and some contribution by H_2O absorption was controlled between 0.5 and 1.0 for all the samples. Light-induced difference spectra were obtained by subtraction between the two single-beam spectra taken before and after illumination. Each single-beam spectrum was an average of 300 scans (150-s accumulation). The spectral resolution was 4 cm^{-1}.

Light illumination was performed at 250 K with a single flash for the S_2/S_1 spectrum or with continuous light for the Q_A^-/Q_A and the $S_2Q_A^-/S_1Q_A$ spectra (a few second illumination). The flash was white light supplied from a Xe flash lamp, 3 μs in pulse duration and 5 mJ in energy per one flash. Continuous light was red light supplied from a tungsten lamp through several layers of heat-cut filters and a red glass filter to cut shorter-wavelength light than 620 nm. The light intensity was about 20 mW/cm^2 at the sample surface.

Results and Discussion

In order to selectively detect the signals due to structural changes restricted within the O_2-evolving system as free as possible from acceptor-side signals, we employed a combination of proteolytic digestion and an exogenous electron acceptor. It has been well-known that mild trypsin treatment below pH 7.25 selectively modifies the Q_B site while keeping the Mn cluster intact (Völker et al., 1985). The modification of the Q_B site is characterized by a markedly enhanced efficiency of electron flow from Q_A^- to ferricyanide accompanied by a loss in DCMU sensitivity (Renger et al., 1981). We thus expect that, with trypsin-treated PSII in the presence of ferricyanide, one-flash illumination should lead to the S_2 formation from the thermally stable S_1 state without involving any change on the acceptor side. By illumination at a moderately low temperature, e.g., at 250 K, the S_2 state is fully generated and stays rather stable. The redox reaction of D, the auxiliary electron donor of PSII, is another matter of concern, because neutral D generated during dark incubation is known to react with the S_2 state at room temperature to form the S_1 state and D^+ in 25% of the centers (Styring & Rutherford, 1987). However, our EPR measurements have shown that such oxidation of D does not take place at 250 K (unpublished data).

Figure 1A shows a one-flash-induced FT-IR difference spectrum of trypsin-treated PSII measured at 250 K in the presence of ferricyanide. There appear a negative band at 2117 cm^{-1} and a positive band at 2036 cm^{-1} (Figure 1A inset) assignable to the CN stretching modes of ferricyanide and ferrocyanide, respectively (Jones, 1963), indicating that ferricyanide accepts an electron to be reduced to ferrocyanide. [Note that the adsorption coefficients of the CN stretching bands are different between ferricyanide and ferrocyanide (Jones, 1963), which gives rise to the apparent imbalance in intensities of the two bands.] In the wavenumber region between 1200 and 1800 cm^{-1}, 10–20 bands are observed with similar numbers of positive and negative peaks. Since neither ferricyanide nor ferrocyanide has absorption in this region, these bands are considered to arise from the structural changes induced by the S_1-to-S_2 transition effected by the flash illumination. The absence of a band at 1478 cm^{-1}, where a typical band of Q_A^- exists (see below), confirms that this difference spectrum involves no contribution by the acceptor-side signals.

In order to further confirm the correct measurement of the S_2/S_1 difference spectrum, we have tried to synthesize a difference spectrum corresponding to the $S_2Q_A^-/S_1Q_A$ charge separation by adding the two difference spectra, the above S_2/S_1 spectrum and a Q_A^-/Q_A spectrum. The Q_A^-/Q_A

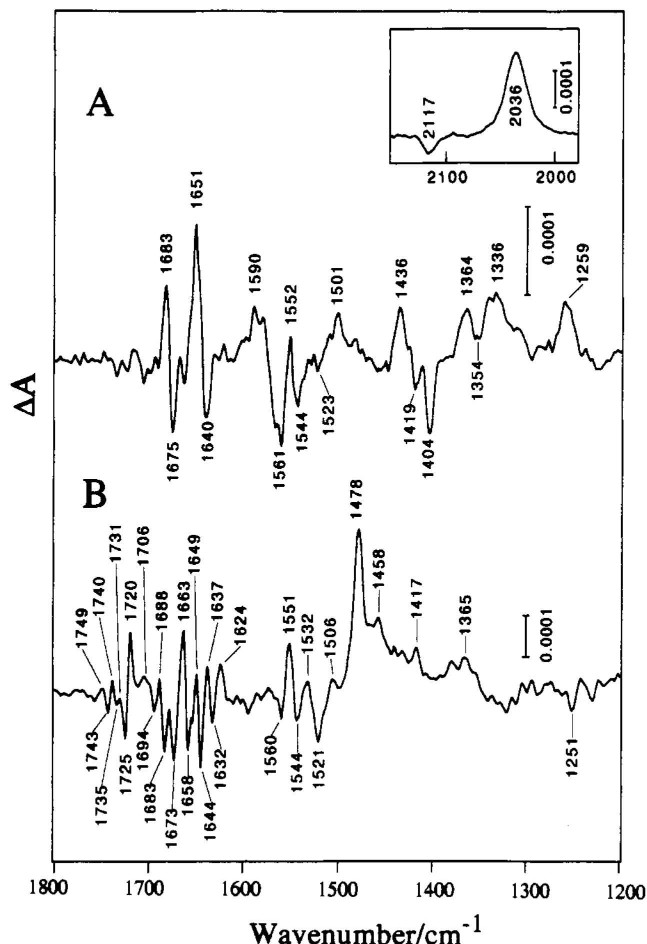

FIGURE 1: Light-minus-dark FT-IR difference spectra of PSII membranes measured under two different conditions. (A) S_2/S_1 difference spectrum. Trypsin-treated PSII was excited by one-flash illumination at 250 K in the presence of potassium ferricyanide. The inset indicates the CN stretching region of ferricyanide and ferrocyanide. (B) Q_A^-/Q_A difference spectrum. Mn-depleted PSII was illuminated with continuous light at 250 K in the presence of DCMU and NH_2OH. Mn depletion was performed by 10 mM NH_2OH treatment. (A) and (B) are averages of four and two sets of measurements, respectively, where one set stands for two single-beam measurements of 300 scans. The spectral resolution is 4 cm^{-1}.

spectrum was separately measured by a method similar to that of Berthomieu et al. (1990): Mn-depleted PSII membranes were illuminated with continuous light in the presence of NH_2OH and DCMU as an exogenous electron donor and as an inhibitor of the electron transfer from Q_A to Q_B, respectively. Figure 1B shows a light-induced FT-IR difference spectrum of Mn-depleted PSII measured at 250 K. This spectrum is basically identical with the spectrum reported by Berthomieu et al. (1990) measured at room temperature. The typical strong band at 1478 cm^{-1} was assigned by them to the CO stretching mode of the semiquinone anion (Q_A^-). They also assigned the negative bands at 1644 and 1632 cm^{-1} to the CO stretches of neutral Q_A (Berthomieu et al., 1990).

Figure 2A shows an FT-IR difference spectrum of untreated PSII membranes induced by continuous-light illumination at 250 K. DCMU was included in the sample to inhibit the Q_A-to-Q_B electron transfer. Under this condition, only a single turnover occurs, and consequently, the continuous-light illumination yields an $S_2Q_A^-$ charge-separated state. Since the recombination temperature between the S_2 state and Q_A^- is as high as 275 K (Rutherford et al., 1982), we may expect that at 250 K this charge-separated state is stably preserved during the measurement period (150 s). Thus, the difference

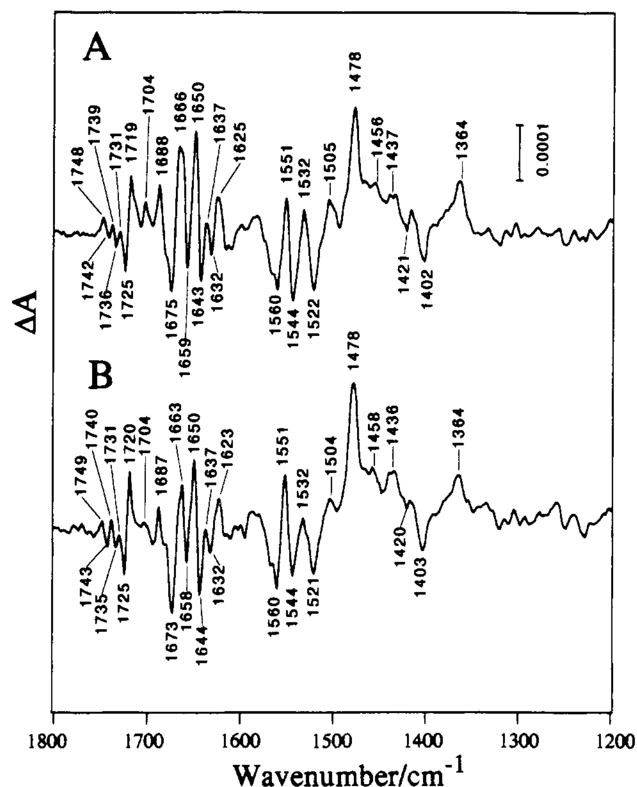

FIGURE 2: Comparison of the two $S_2Q_A^-/S_1Q_A$ difference spectra, directly measured one (A) vs synthetic one (B). (A) FT-IR difference spectrum of $S_2Q_A^-/S_1Q_A$. Untreated PSII was illuminated with continuous light at 250 K in the presence of DCMU. The spectrum is an average of two sets of measurements. Other conditions are the same as in Figure 1. (B) Synthesized FT-IR spectrum (S_2/S_1 + Q_A^-/Q_A) calculated by addition of the S_2/S_1 (Figure 1A) and the Q_A^-/Q_A (Figure 1B) difference spectra with an appropriate factor.

spectrum in Figure 2A exhibits the structural difference between S_1Q_A and $S_2Q_A^-$ ($S_2Q_A^-/S_1Q_A$).

Figure 2B presents the synthetic spectrum obtained by calculation, that is, the addition of the S_2/S_1 (Figure 1A) and the Q_A^-/Q_A (Figure 1B) spectra with an appropriate factor, in comparison with the measured $S_2Q_A^-/S_1Q_A$ spectrum (Figure 2A). It is seen that the spectral features are mostly identical between the synthetic and the measured ones, and the frequencies of all the prominent bands are in agreement with each other within the spectral resolution (4 cm^{-1}). This indicates that the S_2/S_1 difference spectrum in Figure 1A has been properly measured in spite of the artificial conditions employed, i.e., trypsin treatment and the presence of an exogenous electron acceptor, ferricyanide. The result also suggests that the structural changes on both the donar and the acceptor sides are restricted within individual domains of PSII and do not influence the other side.

It is now evident that the FT-IR difference spectrum of S_2/S_1 (Figure 1A) selectively reflects the structural changes induced by the transition from S_1 to S_2. It has been known that the S_1-to-S_2 transition is not accompanied by proton release (Saphon & Crofts, 1977; Fowler, 1977). Also, it has been observed that the Mn K-edge energy of the XANES spectrum upshifts upon this transition with no detectable change in EXAFS, indicative of an increase in the oxidation state of the Mn atom (Yachandra et al., 1987). Hence, this reaction is likely to be one-electron oxidation of the Mn ion(s) with no breakage or formation of chemical bonds. Thus, the spectrum in Figure 1A involves the changes in force constants of the vibrational modes of ligands, which are brought about by oxidation of the coordinated Mn. In other words, assign-

ments of the bands in this spectrum can clarify the ligand species of the redox-active Mn ion(s) in the Mn cluster.

Tentative assignments of some of the major bands in the S_2/S_1 spectrum (Figure 1A) can be given as follows: Between 1600 and 1700 cm^{-1}, there appear two large bands of a differential form at 1683/1675 cm^{-1} and at 1651/1640 cm^{-1}. One possible assignment of these bands is the CO stretch of the $-CONH_2$ group in an Asn or Gln that coordinates to Mn. Another possibility is the CO stretch of backbone amides (the so-called amide I band). In the latter case, the 1683/1675-cm^{-1} band could be attributed to the change in a β-turn structure while the 1651/1640-cm^{-1} band to the change in a β-sheet structure or an unordered structure (Dong et al.). As to the cause for the appearance of the amide I bands, three cases are considered: (1) a backbone CO is directly coordinated to Mn, (2) the secondary structure around the Mn cluster is indirectly influenced by the change in bond lengths between Mn and ligands or within ligands, and (3) the apoprotein undergoes other changes in its secondary structure upon the S_2 formation, perhaps to facilitate the subsequent S_2-to-S_3 transition.

An ionized carboxyl (COO^-) has two CO stretching bands around 1550 cm^{-1} (antisymmetric mode) and around 1400 cm^{-1} (symmetric mode). Hence, the bands observed between 1600 and 1500 cm^{-1} (positive bands at 1590, 1552, and 1501 cm^{-1} and negative bands at 1561, 1544, and 1523 cm^{-1}) can be assigned to the antisymmetric COO^- vibration, while the bands between 1450 and 1350 cm^{-1} (positive bands at 1436 and 1364 cm^{-1} and negative bands at 1419 and 1404 cm^{-1}) are assignable to the symmetric COO^- vibration. Thus, a few Glu or Asp residues may be involved in ligating the redox-active Mn ion(s) in the Mn cluster. Alternative assignments of the bands around 1550 cm^{-1} are the amide II bands from the protein backbone, which may also arise from conformational changes of the apoprotein as discussed for the appearance of the amide I bands.

The above tentative assignments must be carefully scrutinized by means of isotope substitution, site-directed mutagenesis, etc. before being used for a more definite analysis of the structure of the Mn cluster including ligand species. However, the present study has shown that FT-IR difference spectroscopy is a promising method for investigating the mechanism of photosynthetic water oxidation.

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