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Biochimica et Biophysica Acta 1232 (1995) 59–66

BBA Biochimica et Biophysica Acta

A carboxylate ligand interacting with water in the oxygen-evolving center of Photosystem II as revealed by Fourier transform infrared spectroscopy

Takumi Noguchi *, Taka-aki Ono, Yorinao Inoue

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

Received 17 April 1995; revised 11 July 1995; accepted 11 July 1995

Abstract

The effect of H/D exchange on a Fourier transform infrared (FTIR) difference spectrum between the S_1 and S_2 states of the oxygen-evolving center (OEC) in Photosystem II (PS II) has been investigated. Upon deuteration, a large upshift of a differential band by about 18 cm^{-1} was observed in the 1600–1500 cm^{-1} region, where asymmetric COO^- stretching and amide II modes mainly have intensities. This upshift was basically unaffected by ^{15}N substitution of the PS II membranes. FTIR measurements of three model carboxylate compounds showed that asymmetric COO^- stretching bands upshift upon deuteration when the carboxylate groups form hydrogen bonds with water; for free acetate and Mn-EDTA in which the carboxylate groups interact with bulk water, the upshift was 7 cm^{-1}, whereas $(CH_3COO)_2Ni \cdot 4H_2O$ whose carboxylate ligands form hydrogen bonds with water molecules coordinated to the metal ion, exhibits a much larger upshift of 20 cm^{-1}. The above observations indicate that the most plausible assignment of the upshifting band in the S_2/S_1 spectrum is the asymmetric COO^- mode of a carboxylate group interacting with water in OEC. The large upshift of 18 cm^{-1} found in PS II may suggest that this carboxylate group, which is possibly a ligand of a redox-active Mn ion, forms a hydrogen bond with a water molecule bound to the Mn-cluster, although the possibility that it forms a strong hydrogen bond with an exchangeable hydrogen atom of the neighboring amino acid residue cannot be excluded. In the amide I region of the S_2/S_1 spectrum, the bands around 1659 cm^{-1} exhibited large downshifts to around 1645 cm^{-1} upon deuteration. This frequency shift upon deuteration is typical of a random coil structure, and hence it is suggested that hydrophilic domains with random coil conformations are present in the close vicinity of the Mn-cluster.

Keywords: Photosystem II; Oxygen evolution; FTIR; H/D exchange; Carboxylate; Hydrogen bond

1. Introduction

Production of molecular oxygen on the earth entirely depends on photosynthetic water oxidation by plants and cyanobacteria. The oxygen-evolving reaction takes place in the oxygen-evolving center (OEC), which consists of four Mn ions called the Mn-cluster residing on the lumenal side of Photosystem II (PS II). It has been known that the reaction proceeds through the S-state cycle [1,2], in which one-electron oxidation of OEC advances the S_i ($i = 0$–3) state to the next S-state and molecular oxygen releases upon S_3-to-S_0 transition. The molecular mechanism of this reaction, however, still remains unclear (for recent reviews, see [3,4]).

The structure of the Mn-cluster has been studied mainly by means of X-ray absorption and electron paramagnetic resonance (EPR) spectroscopies, both of which detect signals directly from the Mn ions. The most probable structural model of the Mn-cluster at present is a tetranuclear cluster in which the Mn ions are connected by μ_2-oxo- and carboxylato-bridges (reviewed in [3,5]). As for ligands of the Mn-cluster, the ^{15}N ENDOR [6] and ^{14}N ESEEM [7,8] studies indicated that a few histidine residues serve as amino acid ligands. Also, the proton ENDOR study with spinach PS II by Kawamori et al. [9] showed the presence of H_2O ligands exchangeable with D_2O in the Mn-cluster, whereas Tang et al. [6] detected rather different ENDOR

Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; FTIR, Fourier transform infrared; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mn-EDTA, ethylenediaminetetraacetic acid manganese(II) disodium salt trihydrate; OEC, oxygen-evolving center; PS II, Photosystem II

* Corresponding author. Tel: +81 48 4621111 ex. 5543; fax: +81 48 4624685; e-mail: tnoguchi@postman.riken.go.jp.

SSDI 0005-2728(95)00111-5

spectra with PS II membranes from a cyanobacterium and suggested a 'dry' environment of the Mn-cluster.

Fourier transform infrared (FTIR) difference spectroscopy is a powerful method to investigate the structures of proteins and cofactors in the active site of a large protein complex (reviewed in [10]). Using this technique, we have been studying the structure of OEC including ligands of the Mn ions and protein conformations [11–14]. We successfully obtained a flash-induced FTIR difference spectrum that selectively represents the structural changes in OEC between the S_1 and S_2 states [11,12,14]. From the temperature dependence of the FTIR band appearance being identical with that of the S_2 formation studied by EPR [15], it was confirmed that the observed FTIR signals were in fact attributed to the S_1-to-S_2 changes of OEC [14]. The obtained S_2/S_1 difference spectrum was characterized by several COO^- stretching bands and complex band structures in the amide I region, suggesting that the ligands of the Mn-cluster are provided mostly by carboxylate groups and that conformational changes of the proteins around the Mn-cluster take place upon S_2 formation [11,12,14]. By comparing this spectrum with that of PS II membranes depleted of Ca^{2+}, which is an indispensable cofactor for oxygen evolution, it was found that a certain carboxylate group serves as a bridging ligand between the redox-active Mn and Ca^{2+} in the S_1 state, and the coordination of this carboxylate to Ca^{2+} is selectively broken upon transition to the S_2 state [14].

In this study, we have examined the effect of H/D exchange on the S_2/S_1 FTIR difference spectrum of OEC, in order to obtain structural information relevant to substrate water and exchangeable hydrogen atoms.

2. Materials and methods

BBY-type PS II membranes [16] capable of O_2 evolution were prepared from spinach according to Ono and Inoue [17]. Spinach whose nitrogen atoms were replaced by ^{15}N was cultured by hydroponics with a medium containing $K^{15}NO_3$ (99.8%, Shoko) and $Ca(^{15}NO_3)_2$ (99.5%, Shoko Co.) as nitrogen sources. The PS II membranes were suspended in Mes-NaOH buffer (400 mM sucrose, 20 mM NaCl, 20 mM $CaCl_2$, 40 mM MES, pH 5.5), and dark-adapted on ice overnight to make every OEC relax to the thermally stable S_1 state. For H/D exchange, PS II membranes were suspended in D_2O buffer (400 mM sucrose, 20 mM NaCl, 40 mM Mes-NaOD, p*D* 5.5) and incubated at 10°C for 1 h, followed by illumination with four saturating flashes from a frequency-doubled (532 nm) Nd:YAG laser (Quanta-Ray DCR-1) (pulse width: ≈ 7 ns) at intervals of 0.5 s. This illumination procedure was performed to accomplish H/D exchange of substrate water by completing a S-state cycle. The illuminated sample in the D_2O buffer was then incubated in the dark on ice overnight.

FTIR spectra were measured on a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101) with a spectral resolution of 4 cm^{-1}. Flash-induced FTIR difference spectra between the S_1 and S_2 states of OEC in PS II membranes were obtained as described previously [14]. The PS II suspension in the Mes buffer (pH(D) 5.5) was supplemented with 2 mM potassium ferricyanide and 18 mM potassium ferrocyanide, followed by centrifugation for 30 min at 150000 $\times g$. The resultant membrane pellet was then sandwiched between a pair of BaF_2 plates (13 mm ϕ). The absorbance of the peak at about 1655 cm^{-1} due to amide I and water (in the case of H_2O buffer) bands was less than 1.0 for all the samples. For D_2O buffer, in which a D_2O band does not overlap the amide I bands, a larger amount of sample could be mounted compared with H_2O buffer, and hence the spectrum with a higher S/N ratio could be obtained. The sample temperature was adjusted to 250 K in a cryostat (Oxford DN1704) with a temperature controller (Oxford ITC-4). In order to block the He-Ne laser beam partially leaking into the sample compartment, a Ge filter (OCLI LO2584-9) was placed in front of the sample. Light-induced difference spectra were obtained by subtraction between the two spectra measured after and before illumination (300 scans: 150 s accumulation). Light illumination was performed with a single pulse from a Nd:YAG laser (532 nm) with an energy of 10 mJ per pulse (≈ 10 mm ϕ) at the sample surface. For final data, five and two difference spectra were averaged for the PS II membranes in H_2O and D_2O buffers, respectively. No smoothing calculation was applied to the spectra. Each difference spectrum was measured with a newly-charged sample and the sample was illuminated only once.

FTIR spectra of model carboxylate compounds were measured at room temperature. Potassium acetate (CH_3COOK) (Wako Pure Chem. Ind.) and ethylenediaminetetraacetic acid manganese(II) disodium salt trihydrate (Mn-EDTA) (Dojindo) were dissolved in H_2O and D_2O as 10% and 20% solutions, respectively. FTIR spectra of these solutions were measured between a pair of ZnSe plates. For H_2O solutions, the spectra were corrected by subtracting the overlapping water band around 1645 cm^{-1}. Nickel(II) acetate tetrahydrates ($(CH_3COO)_2Ni \cdot 4H_2O$) (Wako Pure Chem.) and its deuterated compound ($(CH_3COO)_2Ni \cdot 4D_2O$) that was prepared by recrystallization from D_2O solution, were measured as a crystalline solid in KBr discs.

3. Results

3.1. Effect of H/D exchange on the S_2/S_1 difference spectrum of PS II membranes

Fig. 1 shows flash-induced FTIR difference spectra between the S_1 and S_2 states of OEC measured with PS II

membranes in H_2O (Fig. 1A) and D_2O (Fig. 1B) buffers. In these spectra, acceptor-side quinone signals are eliminated by abstracting an electron with ferricyanide, and redox reactions of the non-heme iron are also suppressed by controlling redox potential by ferricyanide/ferrocyanide couple (ferricyanide/ferrocyanide = 1:9) and pH(D) (pH(D) 5.5) [14]. In Fig. 1, prominent changes upon H/D exchange were seen in 1700–1500 cm^{-1}; A new negative band appeared at 1579 cm^{-1} with some intensity changes of bands around 1550 cm^{-1}, and rearrangement of the complex band structures in 1700–1600 cm^{-1} took place. In other regions, the two spectra were mostly identical. For example, there were practically no frequency changes in the small bands observed in 1750–1700 cm^{-1} (i.e., the bands at 1747/1737, 1730/1724, 1715/1708, and 1703/1698 cm^{-1}). The relatively large bands at 1404 and 1364 cm^{-1}, which we previously assigned to the symmetric COO^- modes of a carboxylate group connecting the Mn and Ca^{2+} ions [14], were not affected by H/D exchange. Also, no change was observed in the lower-frequency region of 1300–1000 cm^{-1}.

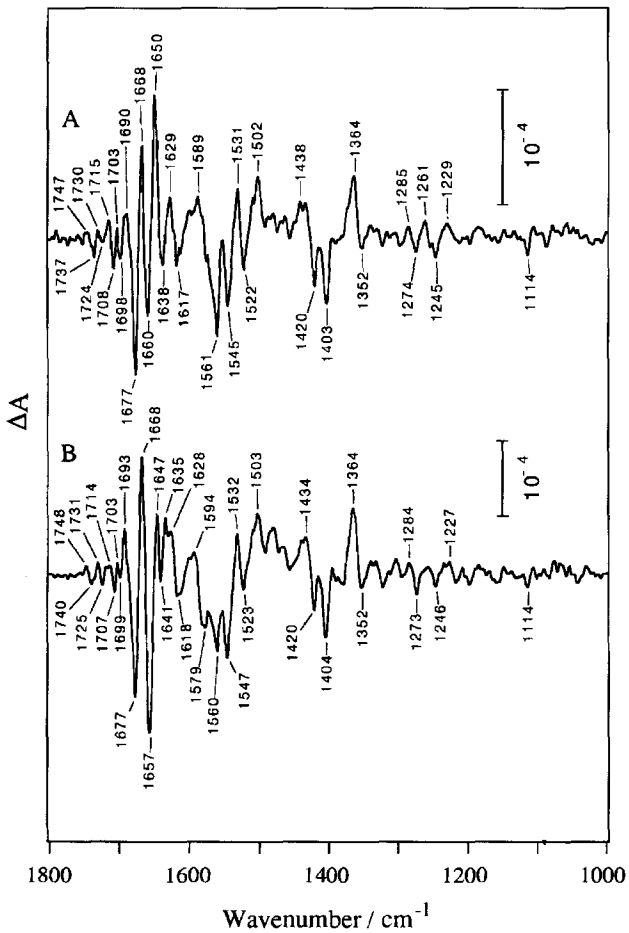

Fig. 1. Flash-induced FTIR difference spectra between S_1 and S_2 state of OEC in PS II membranes suspended in H_2O buffer (pH 5.5) (A) and in D_2O buffer (p*D* 5.5) (B). The spectra were measured after-minus-before single-pulse illumination from a Nd:YAG laser (532 nm) at 250 K.

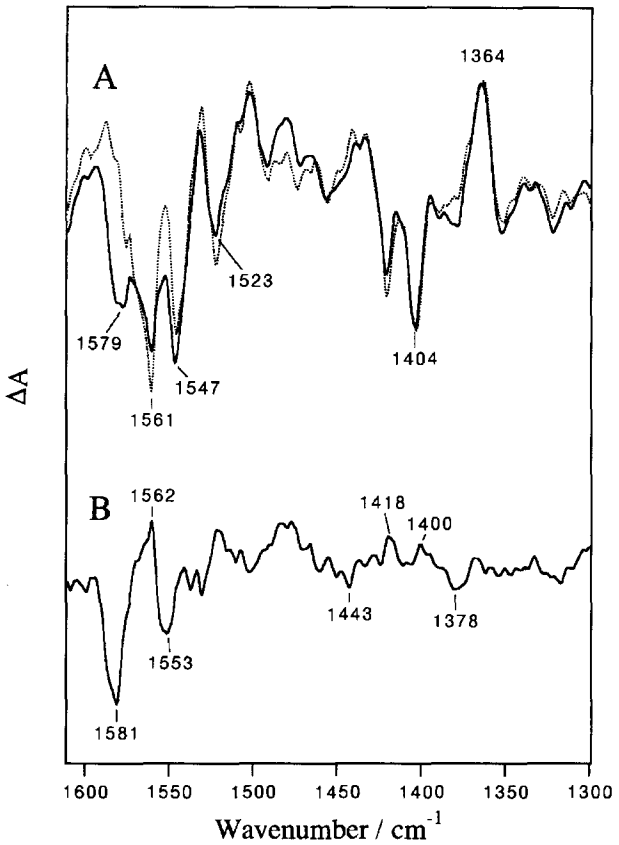

Fig. 2. (A) Expanded view (1610–1300 cm^{-1}) of the S_2/S_1 FTIR difference spectra of PS II membranes in H_2O buffer (dotted curve) and in D_2O buffer (solid curve). (B) Double difference spectrum by subtraction of the S_2/S_1 spectrum in H_2O buffer (A, dotted curve) from that in D_2O buffer (A, solid curve).

Fig. 2A shows an expanded view (in 1610–1300 cm^{-1}) of the above S_2/S_1 spectra in H_2O buffer (dotted curve) and D_2O buffer (solid curve). The two spectra were normalized on the unaffected 1404(−) and 1364(+) cm^{-1} bands (hereafter, positive and negative intensities are represented by + and −, respectively). In this superimposed view, it is clearly seen that the appearance of the 1579 cm^{-1} band upon deuteration is accompanied with an intensity decrease of the negative 1561 cm^{-1} band, in other words, the 1561 cm^{-1} band seems to upshift to 1579 cm^{-1} by 18 cm^{-1} upon H/D exchange.

Since many bands overlap in this region, the spectral changes are more easily seen in a double difference spectrum (Fig. 2B) produced by subtracting the spectrum in H_2O buffer (Fig. 2A dotted curve) from that in D_2O buffer (Fig. 2A solid curve). As expected, a negative band at 1581 cm^{-1} and a positive band at 1562 cm^{-1} were observed in Fig. 2B, corresponding to the appearance of the negative 1579 cm^{-1} band and the intensity decrease of the 1561 cm^{-1} band upon H/D exchange in Fig. 2A. The double difference spectrum exhibits another negative band at 1553 cm^{-1} in the neighborhood of the 1581(−) and 1562(+) cm^{-1} bands. Generally, when a differential band

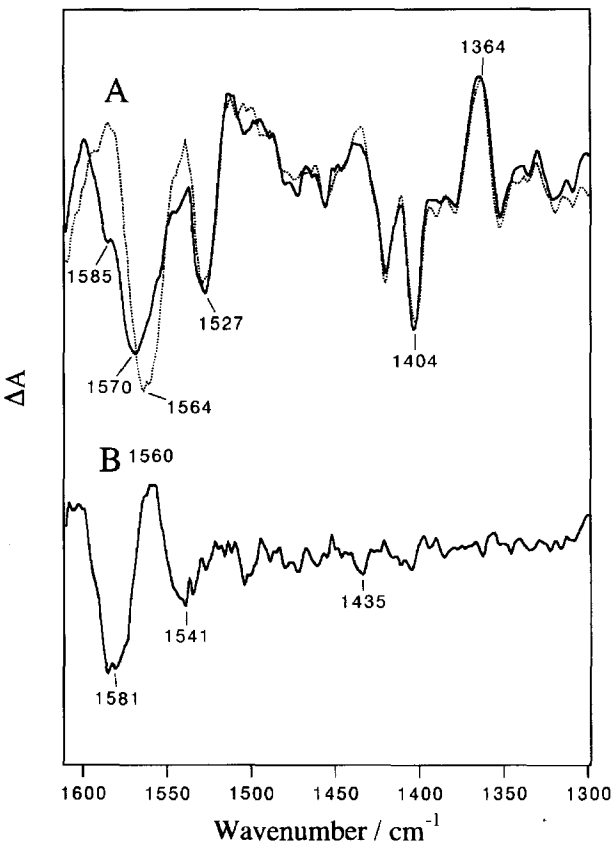

Fig. 3. (A) The S_2/S_1 FTIR difference spectra (1610–1300 cm^{-1}) of ^{15}N-labeled PS II membranes in H_2O buffer (dotted curve) and in D_2O buffer (solid curve). (B) Double difference spectrum by subtraction of the ^{15}N-substituted S_2/S_1 spectrum in H_2O buffer (A, dotted curve) from that in D_2O buffer (A, solid curve).

with a pair of negative and positive peaks undergoes a frequency shift that is comparative in its extent to the band width, subtraction between the two spectra gives rise to a spectrum with three peaks having alternative signs. Thus, the above three peaks at 1581(−)/1562(+)/1553(−) cm^{-1} in the double difference spectrum (Fig. 2B) can be interpreted as resulted from an upshift of the differential band with peaks at ≈ 1560(−)/≈ 1550(+) cm^{-1} upon H/D exchange.

The idea of characteristic group frequencies helps the assignments of IR bands of complex molecules (for standard textbooks, see [18,19]). From standard IR bands of amino acids and proteins with their extinction coefficients [20,21], we previously argued about the assignments of the bands near 1550 cm^{-1} in the S_2/S_1 spectrum [14]. The asymmetric COO^- stretching mode of Asp, Glu or C-terminus, the amide II mode (NH bending mode coupled with CN stretch of the backbone amide), and the symmetric NH_3^+ deformation mode of Lys or N-terminus were proposed as possible candidates.

Fig. 3 shows the results of the similar measurements using the ^{15}N-labeled PS II membranes. The features in the 1600–1500 cm^{-1} region of the S_2/S_1 spectra (Fig. 3A) are different compared with those of unlabeled PS II (Fig. 2A) probably because of some contribution of amide II bands. In the double difference spectrum (Fig. 3B), however, the three peaks at 1581(−)/1560(+)/1541(−) cm^{-1} were basically unchanged from unlabeled ones (Fig. 2A), except that the position of the third peak at 1541 cm^{-1} was a little different by about 10 cm^{-1}. This observation indicates that the contribution of the nitrogen-relating modes (most likely amide II modes) to the double difference spectrum is very small and restricted to the 1555–1540 cm^{-1} region.

The small contribution of amide II bands to the double difference spectrum (Fig. 2B) is also supported by the presence of only weak bands around 1450 cm^{-1} where is the region of amide II′ mode (N-D bending mode) due to deuterated backbone amide groups. Amide II′ bands appear in the double difference spectrum as the counterpart of amide II bands, and hence the weak amide II′ intensity implies that the amide II band is also weak. The small band at 1443 cm^{-1} in Fig. 2B seems to shift to 1435 cm^{-1} upon ^{15}N substitution (Fig. 3B) and is possibly assigned to the amide II′ band. Since the corresponding amide II band should be as small as the 1443 cm^{-1} amide II′ band, it is reasonable to think that the amide II band only partly contributes to the 1553 cm^{-1} peak (Fig. 2B). The downshift of this amide II contribution by 10–20 cm^{-1} upon ^{15}N-substitution may cause the apparent peak shift to 1541 cm^{-1} (Fig. 3B). Although it was somewhat unexpected that the effect of H/D exchange on the amide II bands in the S_2/S_1 spectrum was so small, the fact that only the part of peptides chains in OEC is deuterated by incubation in D_2O buffer, was demonstrated also in the amide I region (see below). Thus, it is considered that three bands at 1581(−)/1562(+)/1553(−) cm^{-1} in the double difference spectrum (Fig. 2B) are mostly due to vibrations unrelated to nitrogen atoms.

Among the candidates of vibrational modes of proteins near 1550 cm^{-1} mentioned above, the vibration unrelated to nitrogen atoms is the asymmetric COO^- stretching mode. As will be shown below in the FTIR measurements of model carboxylate compounds, COO^- stretching frequencies generally upshift upon deuteration when the oxygen atom is hydrogen-bonded. Thus, the most plausible assignment of the band of the S_2/S_1 spectrum that underwent an upshift upon H/D exchange is the asymmetric stretching mode of a COO^- group hydrogen-bonded to an exchangeable hydrogen atom.

An asymmetric COO^- stretching mode is always coupled with a symmetric stretching mode, which exhibits a band in 1300–1450 cm^{-1}. In Fig. 2B, however, only small bands were observed in this region at 1418(+), 1400(+) and 1378(−) cm^{-1}. These weak intensities may be explained by the following two reasons. (1) The symmetric bands of this COO^- group may be originally small in the S_2/S_1 difference spectrum, that is, the band positions are very close between the S_1 and S_2 states. (2) The frequency

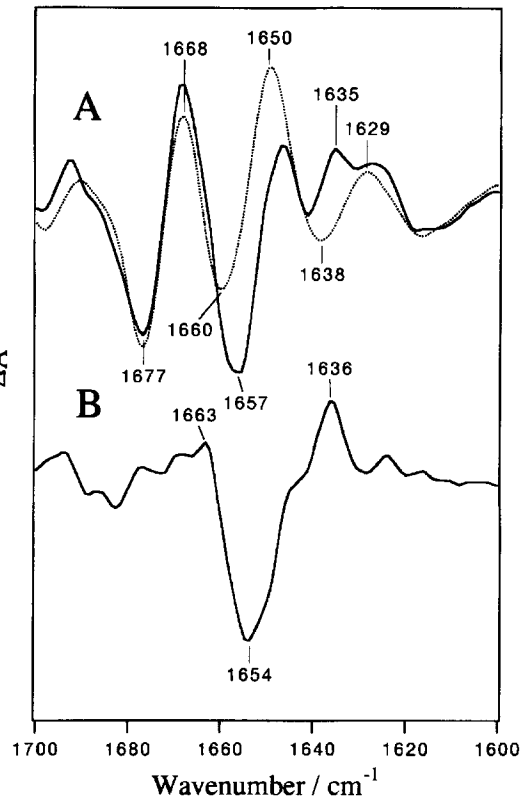

Fig. 4. (A) Expanded view (1700–1600 cm^{-1}) of the S_2/S_1 FTIR difference spectra of PS II membranes in H_2O buffer (dotted curve) and in D_2O buffer (solid curve). (B) Double difference spectrum by subtraction of the S_2/S_1 spectrum in H_2O buffer (A, dotted curve) from that in D_2O buffer (A, solid curve).

shifts of symmetric COO^- bands upon deuteration are small compared with the asymmetric ones. In fact, a small effect of H/D exchange on the symmetric COO^- frequency is demonstrated below in the measurements of model carboxylate compounds.

Fig. 4A shows an expanded view in 1700–1600 cm^{-1} of the S_2/S_1 spectra in H_2O (dotted curve) and in D_2O (solid curve) buffers. In our previous work [14], the peaks at 1677(−), 1668(+), 1660(−), 1650(+) and 1638(−) in H_2O buffer were absent altogether in the S_2/S_1 spectrum of Ca^{2+}-depleted PS II, and were ascribed to the changes of the amide I modes (C = O stretching modes of backbone amides) induced by Ca^{2+}-dependent perturbations on the protein backbones upon S_2 formation. Based on the general criteria of correlation between the amide I position and the protein secondary structure (reviewed in [22,23]), we previously assigned these amide I bands [14], as the bands around 1673 cm^{-1} (1677/1668 cm^{-1}) to turns, around 1664 cm^{-1} (1668/1660 cm^{-1}) to turns or random coil structures, around 1655 cm^{-1} (1660/1650 cm^{-1}) to α-helical or random coil structures, and around 1644 cm^{-1} (1650/1638 cm^{-1}) to β-strands. When PS II membranes were incubated in D_2O buffer (Fig. 4A solid curve), the negative band at 1660 cm^{-1} shifted to 1657 cm^{-1} with a stronger intensity accompanied by the appearance of a new positive band at 1635 cm^{-1}. Again, the double difference spectrum (Fig. 4B) between the two S_2/S_1 spectra exhibits these changes more clearly. Three peaks at 1663(+)/1654(−)/1636(+) cm^{-1} were observed with a typical band shape representing a parallel shift of a differential band. In this case, the spectrum may be interpreted that a differential band at about 1663(−)/1654(+) cm^{-1} downshifted by 10–20 cm^{-1} to about 1654(−)/1636(+) cm^{-1}.

It has been known that the amide I bands of α-helical and random coil conformations are located in a similar frequency region (1660–1650 cm^{-1}) in a H_2O medium, and hence are difficult to be distinguished from each other (see a review by Surewicz and Mantsch [23]). Upon deuteration of the amide groups, however, the amide I band of random coil structure largely downshifts to around 1644 cm^{-1}, while that of α-helix shows only a small shift ($< \approx 5$ cm^{-1}) [23,24]. This frequency difference in a D_2O medium has been used to discriminate the two conforma-

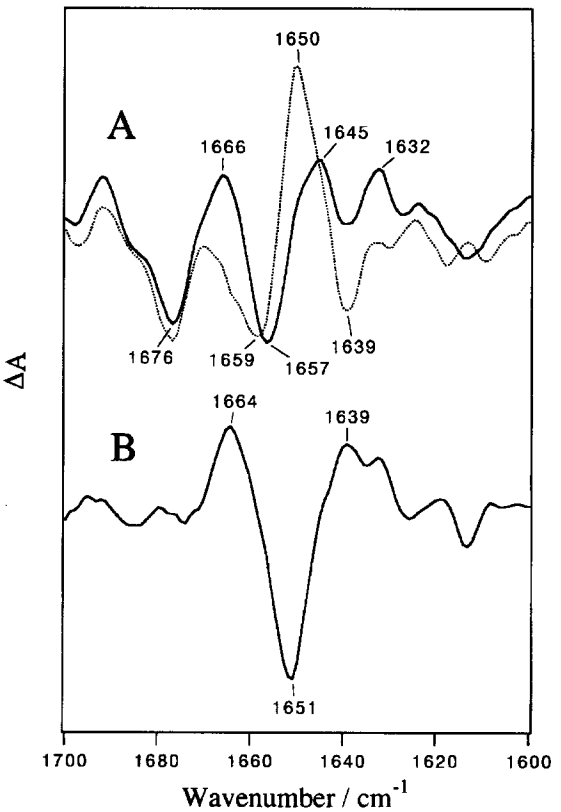

Fig. 5. (A) The S_2/S_1 FTIR difference spectra (1700–1600 cm^{-1}) of ^{15}N-labeled PS II membranes in H_2O buffer (dotted curve) and in D_2O buffer (solid curve). (B) Double difference spectrum by subtraction of the ^{15}N-substituted S_2/S_1 spectrum in H_2O buffer (A, dotted curve) from that in D_2O buffer (A, solid curve).

tions [23]. The observed band shift upon deuteration from around 1659 cm^{-1} (1663/1654 cm^{-1}) to around 1645 cm^{-1} (1654/1636 cm^{-1}) found in PS II (Fig. 4) is typical of the random coil conformation, suggesting that these bands are ascribed to the amide I modes with random coil structures.

The assignment of the bands around 1650 cm^{-1} influenced by deuteration to the amide I modes was supported by the similar measurements with ^{15}N-labeled PS II membranes (Fig. 5). The double difference spectrum (Fig. 5B) showed three peaks at 1664(+)/1651(−)/1639(+) cm^{-1}. These peak positions are only slightly different from those of unlabeled spectrum (Fig. 4B) and hence exclude the possibility of assignment to the stretching modes of the guanidine group ($-CN_3H_5^+$) in Arg and the symmetric NH_3^+ deformation mode in Lys and N-terminus, which show bands in this region. The slight shift by a few wavenumbers upon ^{15}N substitution is consistent with the fact that the amide I mode is weakly coupled with C-N stretching vibration.

Table 1
Effects of H/D exchange on vibrational frequencies of the COO^- stretching modes of model carboxylate compounds and the O_2-evolving center of PS II

	$\nu_{as}(cm^{-1})$ [a]	$\nu_s(cm^{-1})$ [b]
CH_3COO^- in H_2O [c]	1557	1417
CH_3COO^- in D_2O [c]	1564 (+7) [f]	1418 (+1)
Mn-EDTA in H_2O	1585	1416
Mn-EDTA in D_2O	1592 (+7)	1416 (0)
$(CH_3COO)_2Ni \cdot 4H_2O$ [d]	1534	1421
$(CH_3COO)_2Ni \cdot 4D_2O$ [d]	1554 (+20)	1428 (+7)
OEC (S_1) in H_2O [e]	1561	≈ 1400
OEC (S_1) in D_2O [e]	1579 (+18)	≈ 1400

[a] Vibrational frequency of the asymmetric COO^- stretching mode.
[b] Vibrational frequency of the symmetric COO^- stretching mode.
[c] Potassium acetate was dissolved in H_2O or D_2O.
[d] Measured as a crystalline solid in a KBr disc.
[e] Measured as flash-induced S_2/S_1 difference spectra.
[f] Figures in parentheses indicate the frequency shift upon H/D exchange.

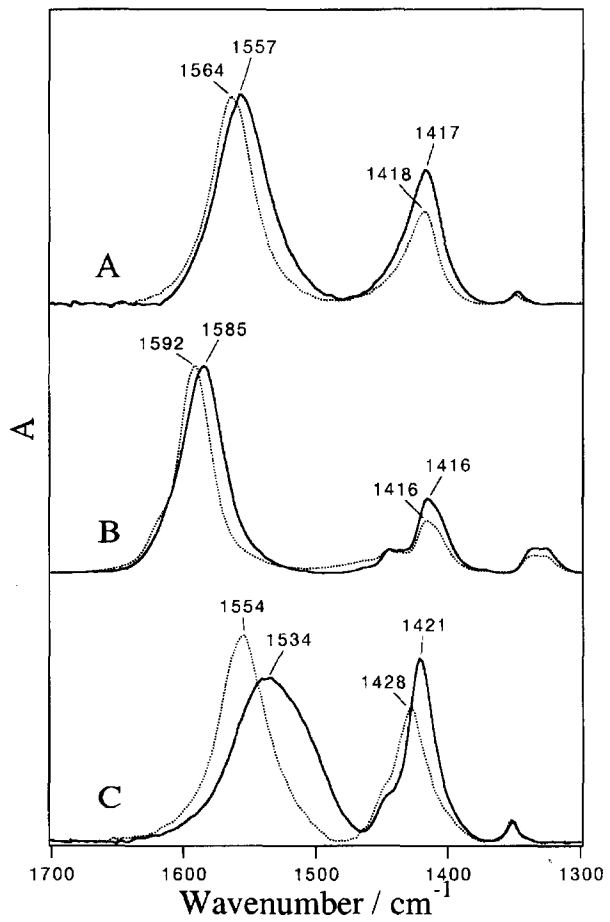

Fig. 6. FTIR spectra of model carboxylate compounds interacting with H_2O or D_2O. (A) Free acetate ion in H_2O (solid curve) and D_2O (dotted curve). (B) Mn-EDTA in H_2O (solid curve) and D_2O (dotted curve). (C) $(CH_3COO)_2Ni \cdot 4H_2O$ (solid curve) and $(CH_3COO)_2Ni \cdot 4D_2O$ (dotted curve). For free acetate and Mn-EDTA in H_2O, the H_2O band around 1645 cm^{-1} was subtracted.

It should be noted that the H_2O bending mode overlaps the amide I region at around 1645 cm^{-1}. Since water molecules are thought to be bound to the Mn-cluster as a substrate and will be replaced by D_2O upon incubation in D_2O buffer and subsequent illumination with four flashes, one might think that the spectrum of Fig. 4B includes the changes in H_2O bands. However, in the D_2O bending region at ≈ 1210 cm^{-1}, no new band was observed in the S_2/S_1 difference spectrum measured in D_2O buffer (Fig. 1B). This indicates that there is also no detectable contribution of H_2O bands in the amide I region of the S_2/S_1 difference spectrum. This view is rationalized by the fact that the extinction coefficient of the H_2O bending mode is much smaller than that of the C = O stretching mode. (For example, our measurement of an equimolar mixture of water and acetone showed that the band intensity (peak height) of the H_2O bending mode was less than 10% of that of the C = O stretch of acetone.)

3.2. COO^- stretching frequencies of model carboxylate compounds

Fig. 6 shows FTIR spectra of three model carboxylate compounds interacting with H_2O (or D_2O), and Table 1 lists the observed band positions of the COO^- stretching modes. In a free acetate ion prepared by dissolving potassium acetate in water, both of the two oxygen atoms will form hydrogen bonds with bulk water. The asymmetric (ν_{as}) and symmetric (ν_s) COO^- stretching frequencies were higher by 7 and 1 cm^{-1}, respectively, in D_2O solution as compared with those in H_2O solution. In Mn-EDTA dissolved in water, one of the oxygen atoms of COO^- coordinates to a Mn ion whereas the other is hydrogen-bonded to bulk water. ν_{as} upshifted by 7 cm^{-1}

upon deuteration while ν_s is left at the same value. The similar upshifts found between free acetate ion and Mn-EDTA suggest that the shift extent depends neither on coordination to the metal nor the structural symmetry. In $(CH_3COO)_2Ni \cdot 4H_2O$ crystal, one of the oxygen atoms of acetate coordinates to the Ni ion whereas the other is hydrogen-bonded to the water molecule that also coordinates to Ni [25]. In this case, the effect of deuteration was much more drastic than the former two cases: ν_{as} and ν_s upshifted by 20 cm^{-1} and 7 cm^{-1}, respectively. For all the three compounds, the shift was always larger for ν_{as} than for ν_s.

4. Discussion

Upon incubation of PS II membranes in D_2O buffer, exchangeable hydrogen in the backbone amides and side chains in proteins are replaced by deuterium. In general, the degree of H/D exchange depends on the nature of the protein, e.g., hydrophobicity, secondary structures, and hydrogen-bond networks. Since the PS II proteins possess large hydrophobic regions, only a part of the hydrogen atoms may be exchanged. As for the substrate H_2O and its possible intermediates (e.g., hydroxide) bound to the Mn-cluster, the H/D exchange should be accomplished by illumination with four saturating flashes in D_2O buffer.

The most drastic change upon H/D exchange in the S_2/S_1 difference spectrum was observed in 1600–1500 cm^{-1}. The negative band at 1561 cm^{-1} seemed to upshift by 18 cm^{-1} to 1579 cm^{-1} upon deuteration (Fig. 2A) and the band shape of the double difference spectrum (Fig. 2B) could by explained by an upshift of a differential band at $\approx 1560(-)/\approx 1550(+)$ cm^{-1}. Since ^{15}N substitution little affected the band changes upon deuteration (Fig. 3B) and the model carboxylate compounds showed upshifts of the asymmetric COO^- stretching bands by 7–20 cm^{-1} upon deuteration (Fig. 6, Table 1), the band that upshifts in the S_2/S_1 spectrum could be reasonably assigned to the asymmetric COO^- stretch of a carboxylate group interacting with exchangeable hydrogen. Although the final definitive assignment should be given by ^{18}O and ^{13}C substitution of Glu, Asp or C-terminus, the above assignment is most probable when we take into consideration the group frequencies of amino acids and proteins [18–21]. Also, it is noted that although the band shape with alternative three peaks in the double difference spectrum (Fig. 2B) might be explained by a downshift of a differential band at $\approx 1580(+)/\approx 1560(-)$ cm^{-1}, the non-nitrogen vibration that shows such a behavior upon deuteration cannot be found among the modes near 1550 cm^{-1} of amino acids and proteins [20,21].

The similar type of upshift of C = O stretching band has been already demonstrated for acetic acid [26]. When acetic acid forms a hydrogen-bonded dimer in carbon tetrachloride, the C = O band shifted to a higher frequency upon deuteration [26]. The larger C = O upshifts of $(CH_3COO)_2Ni \cdot 4H_2O$ observed in the present study can be explained by a stronger hydrogen bond formed with a ligand H_2O molecule compared with bulk water: When a H_2O molecule is coordinated to a metal ion, the hydrogen atom becomes more acidic than that of bulk water and capable of forming a stronger hydrogen bond. This stronger hydrogen bond was in fact expressed by the OH stretching frequencies of $(CH_3COO)_2Ni \cdot 4H_2O$ as observed around 3150 cm^{-1} which was much lower than those of bulk water at about 3400 cm^{-1}.

Using the above criteria, the change in the COO^- band of the S_2/S_1 spectrum upon deuteration can be characterized. The upshift by about 18 cm^{-1} for the asymmetric COO^- band in PS II is much larger than 7 cm^{-1} of the carboxylate compounds interacting with bulk water, but is comparable to 20 cm^{-1} of $(CH_3COO)_2Ni \cdot 4H_2O$ whose COO^- is hydrogen-bonded with ligand H_2O. It is therefore suggested that this COO^- group in PS II is hydrogen-bonded with a H_2O molecule that ligates to the Mn-cluster, although an alternative interpretation that the COO^- group is strongly hydrogen-bonded with an exchangeable hydrogen atom of the neighboring amino-acid residue cannot be excluded at the present stage.

We previously argued about the presence the COO^- bridge between Mn and Ca^{2+} in OEC [14]. From a double difference spectrum between normal and Ca^{2+}-depleted S_2/S_1 spectra, it was proposed that this bridging COO^- has bands at 1560 and 1403 cm^{-1} in the S_1 state and at 1587 and 1364 cm^{-1} in the S_2 state [14]. The COO^- group interacting with H_2O found in the present study is considered to be a different one from the bridging COO^-, because (1) the symmetric stretching bands of bridging COO^- at 1404/1364 cm^{-1} were not affected by H/D exchange at all (Fig. 2A, Fig. 3A), and (2) the band shape of the double difference spectrum (Fig. 2B) is explained by an upshift of the $\approx 1550(+)/\approx 1560(-)$ cm^{-1} band but not the $1587(+)/1560(-)$ cm^{-1} band of the bridging COO^-.

Since in the 1750–1700 cm^{-1} region, where only C = O stretching modes of COOH groups are present in protein IR spectra [20,21], there appear only weak bands with a pair of negative and positive peaks and basically no effect of H/D exchange was observed on these bands (Fig. 1), it is considered that neither protonation nor deprotonation reaction of COO(H) groups takes place upon S_1-to-S_2 transition. This, taken together with the fact that the COO^- bands discussed above have a differential shape in the S_2/S_1 difference spectrum, indicates that the appearance of these COO^- bands in the light-induced spectrum is not due to the protonation of a COO^- group but due to the change in circumstances that substantially affect the bond order. Thus, this COO^- group is probably a ligand of the redox-active Mn ion that is oxidized upon S_2 formation. The structure of hydrogen-bonding COO^- ligand may hold not only for the S_1 state but also for the S_2 state,

since the band shape in the double difference spectrum (Fig. 2B) having three peaks with alternative signs can be explained by a parallel shift of a differential band. If the COO^- ligand were not hydrogen-bonded in the S_2 state, there would be no effect of H/D exchange on the positive COO^- band in the S_2/S_1 spectrum and the double difference spectrum would show only two peaks.

It is likely that the H_2O molecule interacting with the COO^- ligand is a substrate for oxygen evolution. If this is the case, the role of the hydrogen bond between the COO^- group and the substrate H_2O will be speculated as follows. This hydrogen bond will facilitate proton release from the H_2O molecule in the S_2-to-S_3 or S_3-to-S_0 transition. The COO^- ligand may work as the first proton acceptor and transfer the proton to another amino acid residue.

In the amide I region of the S_2/S_1 difference spectra, the H/D exchange induced a downshift of a differential band around 1659 cm^{-1} (1663/1654 cm^{-1}) to around 1645 cm^{-1} (1654/1636 cm^{-1}) (Fig. 4). This relatively large downshift upon deuteration led us to assign these bands to random coil conformations. This assignment implies in turn that a protein domain with random coil structure is present around the Mn-cluster and its structure is perturbed upon S_2 formation. The domains with other conformations, i.e., α-helices, β-strands and turns, which are also influenced upon S_2 formation, do not seem to be deuterated by the H/D-exchange procedure used in this study. This result may be consistent with the general tendency that amide groups in random coil conformations are more easily deuterated than those in other conformations that form more rigid hydrogen bonds. Alternatively, this observation may indicate that only a limited part of the protein regions in OEC is water accessible. If this is the case, the arrangement of hydrophilic and hydrophobic domains in OEC may control the accessibility of substrate water to the Mn-cluster.

Acknowledgements

This work was supported by a grant for Photosynthetic Sciences and a Special Grant for Promotion of Research at The Institute of Physical and Chemical Research (RIKEN) given by the Science and Technology Agency (STA) of Japan, and partially by a Grant-in-Aid for Cooperative Research (No.05304006) (to T.N. and T.O.) from the Ministry of Education, Science and Culture of Japan.

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