FEBS 15905

FEBS Letters 370 (1995) 241–244

Molecular interactions of the redox-active accessory chlorophyll on the electron-donor side of photosystem II as studied by Fourier transform infrared spectroscopy

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Received 12 June 1995

Abstract A Fourier transform infrared (FTIR) difference spectrum upon photooxidation of the accessory chlorophyll (Chl$_Z$) of photosystem II (PS II) was obtained at 210 K with Mn-depleted PS II membranes in the presence of fericyanide and silicomolybdate. The observed Chl$_Z^+$/Chl$_Z$ spectrum showed two differential bands at 1747/1736 and 1714/1684 cm^{-1}. The former was assigned to the free carbomethoxy C = 0 and the latter to the keto C = 0 that is hydrogen-bonded or in a highly polar environment. Also, the negative 1614 cm^{-1} band assignable to the macrocycle mode indicated 5-coordination of the central Mg. The negative 1660 cm^{-1} band, possibly due to the strongly hydrogen-bonded keto C = 0, may suggest oxidation of one more Chl$_Z$, although an alternative assignment, the amide I mode of proteins perturbed by Chl$_Z$ oxidation, is also possible.

Key words: Photosynthesis; Photosystem II; Fourier transform infrared spectroscopy; Chlorophyll; Radical cation

1. Introduction

On the electron-donor side of photosystem II (PS II), the primary chlorophyll donor, P680, oxidizes water via Y$_Z$ to evolve molecular oxygen (reviewed in [1]). Besides this main path, it has been known that there are various side paths of electron donation to P680 including Y$_D$ [2], cyt b_{559} [3,4], accessory chlorophyll (denoted as Chl$_Z$ [4]) [3–8], carotenoid [9–11], and the donor-side component of the A$_T$ thermoluminescence band [12]. Among them, photooxidation of Chl$_Z$ can be observed when the normal electron pathway to the oxygen-evolving center is blocked by Mn depletion or by lowering the temperature under the condition that cyt b_{559} has been preoxidized [3–8]. It has been proposed that oxidation of Chl$_Z$ on the electron-donor side is related to the photoinhibition of PS II [4]. The location of Chl$_Z$ in the PS II reaction center is under debate, because the His residues that coordinate the accessory bacteriochlorophylls in the bacterial reaction center are not conserved in PS II [13]. Recently, the distance between Chl$_Z$ and the non-heme Fe (II) in PS II was estimated by ESR to be much longer (~40 Å) [8] than that between the accessory bacteriochlorophylls and the Fe (II) in the bacterial reaction center (~25 Å) [14]. D1-His$_{118}$ or D2-His$_{118}$ has been proposed as a potential ligand of Chl$_Z$ in PS II [8,15].

In this study, we detected a Fourier transform infrared (FTIR) difference spectrum of Chl$_Z$ upon its photooxidation in Mn-depleted PS II membranes. The obtained FTIR spectrum provided information about molecular interactions of Chl$_Z$ in the reaction center protein.

2. Materials and methods

BBY-type PS II membranes [16] were prepared from spinach according to Ono and Inoue [17] and suspended in MES-NaOH buffer (400 mM sucrose, 20 mM NaCl, 40 mM MES, pH 6.5). ^{15}N-labelled spinach was cultured by hydroponics with a medium containing K^{15}NO$_3$ (99.8%, Shoko Co.) and Ca(^{15}NO$_3$)$_2$ (99.5%, Shoko Co.) as the nitrogen sources. Mn-depleted PS II membranes were prepared by NH$_2$OH treatment (0.5 mg chlorophyll/ml of PS II + 10 mM NH$_2$OH) followed by washes with the MES buffer.

FTIR spectra were measured on a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101) as described previously [11]. PS II suspension (0.5 mg chlorophyll/ml) supplemented with potassium ferricyanide (20 mM) and silicomolybdate (0.3 mM) was centrifuged for 30 min at 150,000 × *g*, and the resultant pellet was sandwiched between BaF$_2$ plates. The sample temperature was adjusted to 210 K in a cryostat (Oxford DN1704) with a controller (Oxford ITC-4). A light-induced difference spectrum was obtained as subtraction between the two single-beam spectra (300 scans: 150 s accumulation) measured before and after illumination (5 s). The light source for illumination was a halogen lamp (HOYA-SCHOTT HL150) equipped with heat-cut and red (>600 nm) filters (~30 mW/cm^2 at the sample surface).

ESR spectra were measured on a JEOL ES-FE1XG X-band ESR spectrometer. The sample temperature was adjusted to 210 K in a liquid N$_2$ cryostat (JEOL ES DVT-1). The PS II sample (5 mg chlorophyll/ml) with potassium ferricyanide (20 mM) and silicomolybdate (0.3 mM) was put into a capillary tube (1 mm diameter). Light illumination was performed through a cavity window for 30 s with the same light source as used for the FTIR measurements.

3. Results and Discussion

Fig. 1A shows an FTIR difference spectrum of Mn-depleted PS II membranes measured as after-minus-before illumination at 210 K in the presence of ferricyanide and silicomolybdate. Under this oxidizing condition, cyt b_{559} is kept oxidized and electron donation from cyt b_{559} to P680 is prevented [3,5,9,11]. In addition, on the electron-acceptor side, ferricyanide and silicomolybdate act as exogenous electron acceptors to abstract an electron out of the PS II complexes. In fact, the characteris-

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Abbreviations: Chl, chlorophyll *a* in vitro; Chl$_Z$, redox-active accessory chlorophyll of PS II; cyt b_{559}, cytochrome b_{559}; ESR, electron spin resonance; FTIR, Fourier transform infrared; MES, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor; PS II, photosystem II; Q$_A$, primary quinone acceptor; THF, tetrahydrofuran; Y$_D$, tyrosine electron donor in the D2 subunit; Y$_Z$, tyrosine electron donor in the D1 subunit.

SSDI 0014-5793(95)00833-0

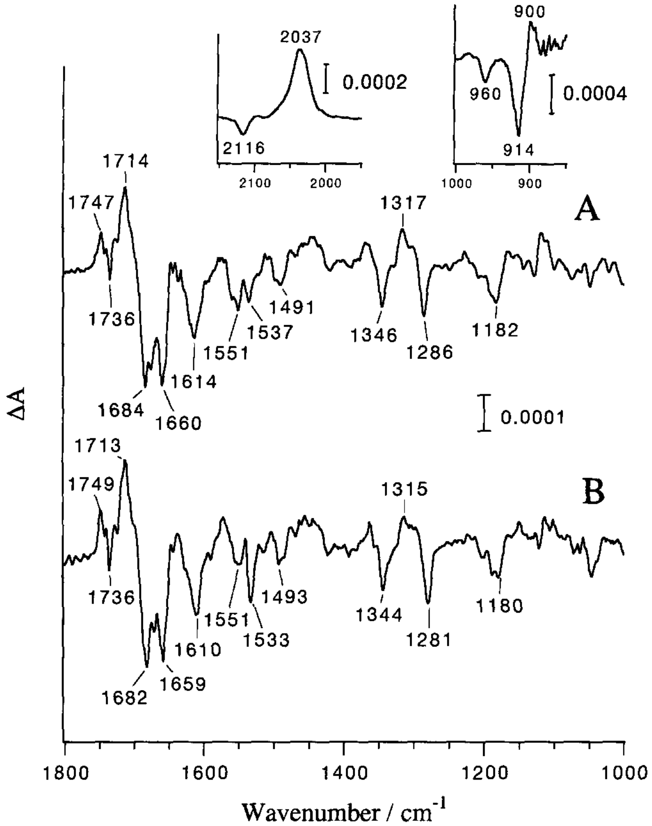

Fig. 1. (A) FTIR difference spectrum upon photooxidation of Chl_Z in Mn-depleted PS II membranes in the presence of ferricyanide and silicomolybdate measured at 210 K. The difference spectrum was obtained as after-minus-before illumination. Spectral resolution was 4 cm^{-1}. Insets: FTIR difference spectra in the regions of ferri- and ferrocyanide (2150–1950 cm^{-1}) and silicomolybdate (1000–850 cm^{-1}). (B) FTIR difference spectrum upon photooxidation of Chl_Z in ^{15}N-labeled, Mn-depleted PS II membranes. All other conditions were the same as (A).

tic marker signal of Q_A^- formation, a strong positive band at 1478 cm^{-1} [18], was not seen in the spectrum of Fig. 1A, indicating that no electron was trapped by Q_A. (Note that the electron flow from Q_A to Q_B is blocked at 210 K). Also, signals of the non-heme iron, another potential endogenous electron acceptor under the oxidizing condition, which are characterized by positive bands at 1335 and 1257 cm^{-1} and negative bands at 1227 and 1103 cm^{-1} [19,20], were absent in this spectrum. Abstraction of an electron by exogenous acceptors was further confirmed by the presence of bands at 2037/2116 cm^{-1} due to reduction of ferricyanide to ferrocyanide, and of negative bands at 960 and 914 cm^{-1} assignable to silicomolybdate (solid silicomolybdate in nujol showed bands at 970 and 912 cm^{-1}) and a positive band at 900 cm^{-1} probably due to its reduced form (Fig. 1A insets).

It has been known that when cyt b_{559} is preoxidized, Chl_Z acts as an electron donor to P680 at cryogenic temperatures [3–8]. To confirm that the photooxidized component in the above FTIR spectrum is Chl_Z, ESR measurements were performed under almost the same condition. Fig. 2 shows ESR spectra of Mn-depleted PS II membranes in the presence of ferricyanide and silicomolybdate measured before (dashed line) and after (solid line) illumination at 210 K. A 10 G-wide signal at $g = 2.0024$, which has been identified to the Chl_Z cation on the electron-donor side of PS II [3–8], was in fact observed after illumination. Before we draw a conclusion, however, we should check the possibility of contribution of a carotenoid cation, because it is not easily distinguished from a chlorophyll cation by means of ESR [3,6]. In fact, photooxidation of carotenoid has been observed in near-infrared absorption and FTIR spectra under similar oxidizing conditions at liquid-N_2 temperature [9,11]. However, this possibility is excluded, judging from the observation that in Fig. 1A, signals of a carotenoid cation, which are characterized by bands at 1465, 1441, 1148, 992 and 996 cm^{-1} [11], are absent. Probably, oxidation of carotenoid prefers lower temperatures compared with oxidation of Chl_Z. From the above considerations, it can be concluded that the FTIR difference spectrum in Fig. 1A represents the changes due to oxidation of Chl_Z on the donor side of PS II (denoted as Chl_Z^+/Chl_Z). Note that exogenous electron acceptors, ferricyanide and silicomolybdate, do not show any bands in the 1800–1000 cm^{-1} region.

The overall features of the Chl_Z^+/Chl_Z spectrum in Fig. 1A are very close to those of an electrochemically-induced FTIR difference spectrum between the cation and neutral species of chlorophyll *a* (Chl) in THF previously reported by Nabedryk et al. [21]; the differential signal of medium amplitude at 1747/1736 cm^{-1} and the neighboring largest one at 1714/1684 cm^{-1} of Chl_Z^+/Chl_Z (Fig. 1A) are compatible with the signals at 1751/1738 and 1718/1693 cm^{-1}, respectively, of Chl^+/Chl in THF [21], and the negative bands at 1614, 1551, 1537, 1491, 1346 and 1286 cm^{-1} (Fig. 1A) correspond to those of Chl at 1597, 1549, 1517, 1485, 1346 and 1289 cm^{-1} [21], respectively. This suggests that most of the bands in the Fig. 1A spectrum arise from vibrations of Chl_Z itself rather than from those of the protein moiety surrounding Chl_Z. The Chl_Z^+/Chl_Z spectrum (Fig. 1A), therefore, can be analyzed on the basis of extensive vibrational data of Chl in literatures (reviewed in [22–24]).

The straightforward assignment of the differential signal at 1747/1736 cm^{-1} of Chl_Z^+/Chl_Z (Fig. 1A) is the stretching mode of the carbomethoxy C = O group at the 10c-position, which was seen at 1751/1738 cm^{-1} in Chl^+/Chl [21]. The relative amplitude of the 1747/1736 cm^{-1} band with respect to other ones (e.g. the 1714 cm^{-1} band due to the keto C = O of Chl_Z^+, see below) in Fig. 1A, being close to that of the 1751/1738 cm^{-1} band to the corresponding ones in the Chl_Z^+/Chl spectrum [21], supports this assignment. The assignment of this band to the 7c-ester C = O, which is another possible mode of chlorophyll *a* in this region, is not likely, because this mode is essentially unaffected upon cation formation as proved by Nabedryk et al. [21] with pyrochlorophyll *a* in which the carbomethoxy C = O is replaced by a hydrogen atom. Some contributions of the COOH bands of protein side-chains (Glu or Asp) [25] to the 1747/1736 cm^{-1} band might be possible, for example as the shoulders seen at 1741 and 1728 cm^{-1} (Fig. 1A).

The carbomethoxy C = O frequency of 1736 cm^{-1} of neutral Chl_Z (Fig. 1A) is typical of that of Chl in non-protic solvents [26]. This indicates that the carbomethoxy C = O of Chl_Z in PS II is basically free from a hydrogen bond. It has been known that in non-protic solvents, downshifts in frequency of the carbomethoxy and keto C = O of neutral Chl correlate to the solvent polarity revealed by $(\varepsilon - 1)(n^2-1)/(2\varepsilon + 1)(2n^2 + 1)$, in which ε and n stand for the relative dielectric constant and refractive index of the solvent, respectively [26,27]. In hydrocar-

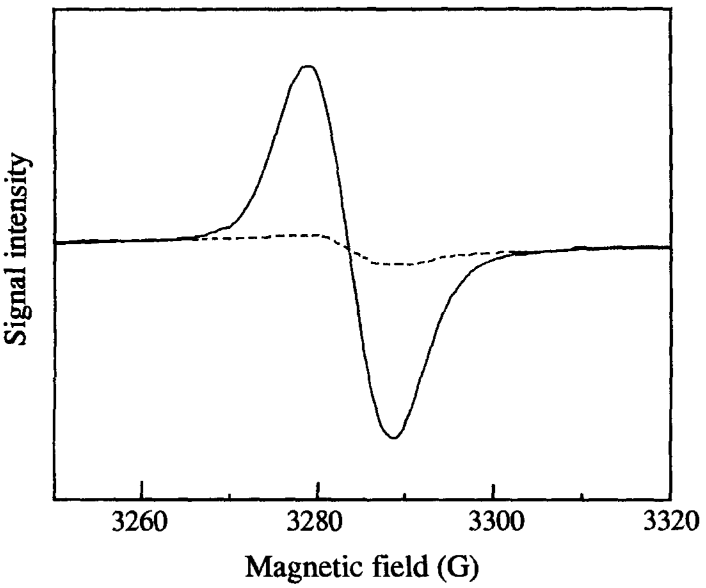

Fig. 2. ESR spectra of Mn-depleted PS II membranes in the presence of ferricyanide and silicomolybdate before (dashed line) and after (solid line) illumination at 210 K. Sample concentration, 5 mg chlorophyll/ml; temperature, 210 K; microwave frequency, 9.20 GHz; microwave power, 2 mW; field-modulation amplitude, 5 G; field-modulation frequency, 100 kHz.

bon solutions like *n*-hexane and cyclohexane, the carbomethoxy C = O band is observed at higher frequencies than 1740 cm^{-1} [26], and even in the photosynthetic protein one of the carbomethoxy C = O groups of the chlorophyll dimers (P700) of PS I shows a band at 1748 cm^{-1} [21]. Thus, we may assume that the carbomethoxy C = O of Chl$_Z$ is in a slightly polar environment, which may be produced by charged or aromatic amino acid residues. It is also possible that this carbomethoxy C = O is very weakly hydrogen-bonded with a relatively distant protic residue, the situation which does not occur in organic solutions.

In the Chl$^+$/Chl spectrum in THF, the most prominent bands are the large differential signal at 1718/1693 cm^{-1} that is ascribed to the keto C = O group [21]. In the Chl$_Z^+$/Chl$_Z$ spectrum of PS II (Fig. 1A), a large positive band at 1714 cm^{-1} and two large negative bands at 1684 and 1660 cm^{-1} were observed. The 1714 cm^{-1} band can be unambiguously assigned to the keto C = O band of Chl$_Z^+$, based on its frequency and strong amplitude. Upon ^{15}N-substitution, the two negative bands at 1684 and 1660 cm^{-1} were basically unaffected or only slightly shifted to lower frequencies (less than 2 cm^{-1}) (Fig. 1B), indicating that these bands are uncoupled or very weakly coupled to nitrogen vibrations. This excludes the involvement of Arg and Lys vibrations that are potential modes in this region [25], and supports the assignment of these bands to the C = O mode. The 1684 cm^{-1} band is most probably attributed to the keto C = O band of neutral Chl$_Z$, by taking into consideration the frequency difference of 30 cm^{-1} from the cation band at 1714 cm^{-1}, which is in good agreement with the reported values of 25–37 cm^{-1} for Chl in organic solvents [21]. The 1660 cm^{-1} band is also possibly assigned to the keto C = O of neutral Chl$_Z$. In this case, the frequency difference from the 1714 cm^{-1} band amounts to 54 cm^{-1}, which seems a little too large to be readily accepted. Since the keto C = O band of the cation has been reported to be much less sensitive to the solvent polarity than that of the neutral species [21], however, it may be possible to assume that both the bands at 1684 and 1660 cm^{-1} upshift to the same position of 1714 cm^{-1} upon cation formation. According to the analysis based on the criteria of solvent effect on the keto C = O frequency [26,27], the keto C = O at 1684 cm^{-1} is either hydrogen-bonded or in a highly polar environment, while the C = O at 1660 cm^{-1} is strongly hydrogen-bonded. These considerations lead us to assume that there are two different Chl$_Z$ molecules that can be oxidized by P680.

Another, but completely different assignment of the 1660 cm^{-1} band is the amide I mode (C = O stretching mode) of the protein backbone that was perturbed upon Chl$_Z$ oxidation. The fact that PS II proteins show the amide I peak at 1656–1659 cm^{-1} [28,29], which is indicative of the α-helical conformation [30], is in support of this assignment. At the present stage, however, we cannot determine which assignment is correct for the 1660 cm^{-1} band.

The negative bands at 1614, 1551 and 1537 cm^{-1} can be attributed to the macrocycle C = C stretching modes of neutral Chl$_Z$ [24,31]. The only small downshift (by 4 cm^{-1}) of the 1614 cm^{-1} band to 1610 cm^{-1} upon ^{15}N-substitution (Fig. 1B), implying weak coupling with nitrogen vibrations, supports the above assignment as to this band. Appreciable changes in the relative amplitudes and frequencies of the 1551 and 1537 cm^{-1} bands upon ^{15}N-substitution (Fig. 1B), suggest that the amide II band (NH bending mode of backbone amide) may have some contribution in this region. It has been known that some of the macrocycle bands are sensitive to the coordination number of the central Mg, and can be used as markers for determining it [24,31]. The most explicit marker among them is the band at ~1610 cm^{-1} in 5-coordination that downshifts to ~1600 cm^{-1} in 6-coordination [24,31]. The band frequency at 1614 cm^{-1} in the Chl$_Z^+$/ Chl$_Z$ spectrum (Fig. 1A) indicates that Chl$_Z$ has a 5-coordinate structure, in other words, only one amino acid residue coordinates the central Mg.

In conclusion, the light-induced FTIR difference spectrum of Chl$_Z$ in PS II showed that Chl$_Z$ has a 5-coordinate structure and its carbomethoxy C = O group is basically free from a hydrogen bond whereas the keto C = O group is hydrogen-bonded or present in a highly polar environment. It was also suggested that another Chl$_Z$, whose keto C = O is strongly hydrogen- bonded, may be oxidized in PS II. Such FTIR detection of the molecular interactions, in combination with site-directed mutagenesis, will be of great use to study the location and the role of Chl$_Z$ in the PS II reaction center.

Acknowledgements: We would like to thank Dr. T. Ono for the helpful discussion. This work was supported by grants for Photosynthetic Sciences and Biodesign Research Program, and by Special Grant for Promotion of Research at The Institute of Physical and Chemical Research (RIKEN) given by the Science and Technology Agency of Japan.

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