Light-induced Fourier transform infrared (FTIR) spectroscopic investigations of primary reactions in photosystem I and photosystem II

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Light-induced Fourier transform infrared (FTIR) difference spectroscopy has been applied for the first time to primary reactions in green plant photosynthesis. Photooxidation of the primary electron donor (P700) in photosystem I-enriched particles as well as in thylakoids, and photoreduction of the pheophytin (Pheo) intermediary electron acceptor in photosystem II-enriched particles, have led to reproducible difference spectra. In the spectral range investigated (between 1800 and 1000 cm^{-1}) several bands are tentatively attributed to changes in intensity and position of the keto and ester carbonyl vibrations of the chlorophyll or Pheo molecule(s) involved. For some of these groups, possible interpretations in terms of changes of their environment or type of bonding to the protein are given. The intensity of the differential features in the amide I and amide II spectral region allows the exclusion of the eventuality of large protein conformational changes occurring upon primary charge separation.

Fourier transform infrared spectroscopy *Photosynthesis* *Photosystem I* *P700* *Chlorophyll* *Pheophytin*

1. INTRODUCTION

In photosynthesis of green plants and blue-green algae, electron transport from water to $NADP^+$ requires the cooperation of two photosystems: PS I and PS II, both located in the photosynthetic membrane. The oxido-reduction potentials and the nature of the electron carriers differ considerably for PS I and PS II. PS I, which performs electron transport from plastocyanin to ferredoxin:$NADP^+$ reductase, incorporates as primary electron donor a Chl *a* monomer or dimer: P700 (named after its visible absorption maximum). From P700 an electron is transferred to early acceptors, and eventually to iron-sulfur proteins F_X, F_A and F_B [1]. PS II, which performs electron transport from the oxygen-evolving complex to a pool of quinone molecules linking the two photosystems via the cytochrome b_6-*f* complex, also has a Chl *a* monomer or dimer as primary electron donor (P680). From P680 an electron is transferred to a Pheo *a* molecule, and further on to a primary, then a secondary plastoquinone molecule. $P680^+$ is rereduced by secondary electron donors that link PS II to the oxygen-evolving complex [2]. With regard to the redox components it includes and the redox potential it spans, the acceptor side of PS II resembles the well-characterized RC of purple photosynthetic bacteria [3]. Moreover, some homology has been observed between the polypeptide sequences of PS II and those of purple bacteria RCs [4].

RCs have been isolated and purified for a

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Abbreviations: FTIR, Fourier transform infrared; PS, photosystem; Chl, chlorophyll; Pheo (BPheo), pheophytin (bacteriopheophytin); RC, reaction center; I, bacteriopheophytin intermediary electron acceptor

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number of bacteria, and successful crystallization of the RC of *Rhodopseudomonas viridis* has led to a high resolution X-ray structure [5]. Unfortunately, in the case of green plants, no isolation procedure has ever led to a PS I or II RC totally depleted of its antenna Chls, and still retaining its essential function: efficient separation and stabilization of charges. However, a number of procedures have been reported that allow membranes to be enriched in or depleted of PS I or II and lead to fractions containing as low as ~40 antenna Chls per P700 or per P680, while retaining the function of the photosystems [6].

Amongst the spectroscopic techniques used to study the primary photochemical reactions in green plants, ESR, optical absorption and linear dichroism have given precise information on the redox states and the orientation of the pigments and electron carriers involved [7,8], but little on the changes of interaction that occur between these molecules and the protein during the first steps of charge separation and charge migration. FTIR difference spectroscopy, which allows small changes in the vibration of molecular bonds to be monitored on a high but constant background absorption, is in principle able to yield such information. Moreover, due to the non-specificity of IR spectroscopy, one has access to all bonds of the pigments, redox carriers, polypeptides and lipids, whose IR absorption changes during the process that is monitored.

In a previous work, we have performed light-induced FTIR difference spectroscopy on reconstituted RCs of the purple photosynthetic bacteria *Rps. sphaeroides, Rps. capsulata* and *Rps. viridis*. We have detected light-induced molecular changes associated with the primary electron donor oxidation in the three species [9], and with the BPheo intermediary electron acceptor (I) reduction in *Rps. viridis* [10]. We have also obtained FTIR difference spectra on intact chromatophore membranes. Although the background absorption by components other than the RCs is much larger, the difference spectra were quite similar [9,10]. This has prompted us to investigate, using the same technique, the well-characterized states that can be photoaccumulated on thylakoid subfragments enriched in either PS I or II, but still retaining a significant number of antenna Chl molecules. Here, light-induced FTIR difference spectra associated (i) with the accumulation of the photooxidized primary donor in PS I particles, and (ii) with the accumulation of the photoreduced Pheo intermediary electron acceptor in PS II-enriched particles are presented.

2. MATERIALS AND METHODS

Spinach PS II-enriched particles were prepared according to [11] with the modifications in [12,13]; they contained approx. 250–300 Chl molecules per PS II. Pea PS I particles containing approx. 100–150 Chls per PS I were prepared according to [14]. Suspensions of these were deposited on CaF_2 windows and air-dried overnight at room temperature in the dark. For PS I particles, the artificial electron acceptor methyl viologen was added to the suspensions (70 μM) before drying. The absorbance of the films thus prepared ranged from 1 to 2 *A* units at the red absorption maximum. Infrared microcells were formed by adding on the films about 10 μl of different solutions (0–10 mM Na ascorbate in 5 mM Tris buffer for PS I particles, 100–200 mM Na dithionite in 150 mM Na borate buffer, pH 9.5, for PS II particles), a thin spacer (3.5 μm thick mylar or 6.5 μm thick teflon) and another CaF_2 window. These microcells could be thermostatically controlled by a flow of nitrogen gas.

The principles of FTIR difference spectroscopy are described elsewhere [9,10,15–17]. FTIR absorbance spectra were taken before, during and after illumination with white, red ($\lambda > 665$ nm) or orange ($\lambda > 530$ nm) actinic light, on a Nicolet 60SX FTIR spectrophotometer equipped with an MCT-A detector.

3. RESULTS AND DISCUSSION

3.1. *Photooxidation of the primary electron donor in PS I particles*

Fig.1 shows FTIR difference spectra of a film of PS I particles covered with 10 mM Na ascorbate solution. Fig.1A shows the difference between two absorbance spectra taken in the dark, indicating the noise level. Fig.1B shows the 'light-minus-dark' spectrum of the same sample: it was obtained by subtracting the absorbance spectrum of the sample taken just before illumination from one taken during continuous illumination with actinic

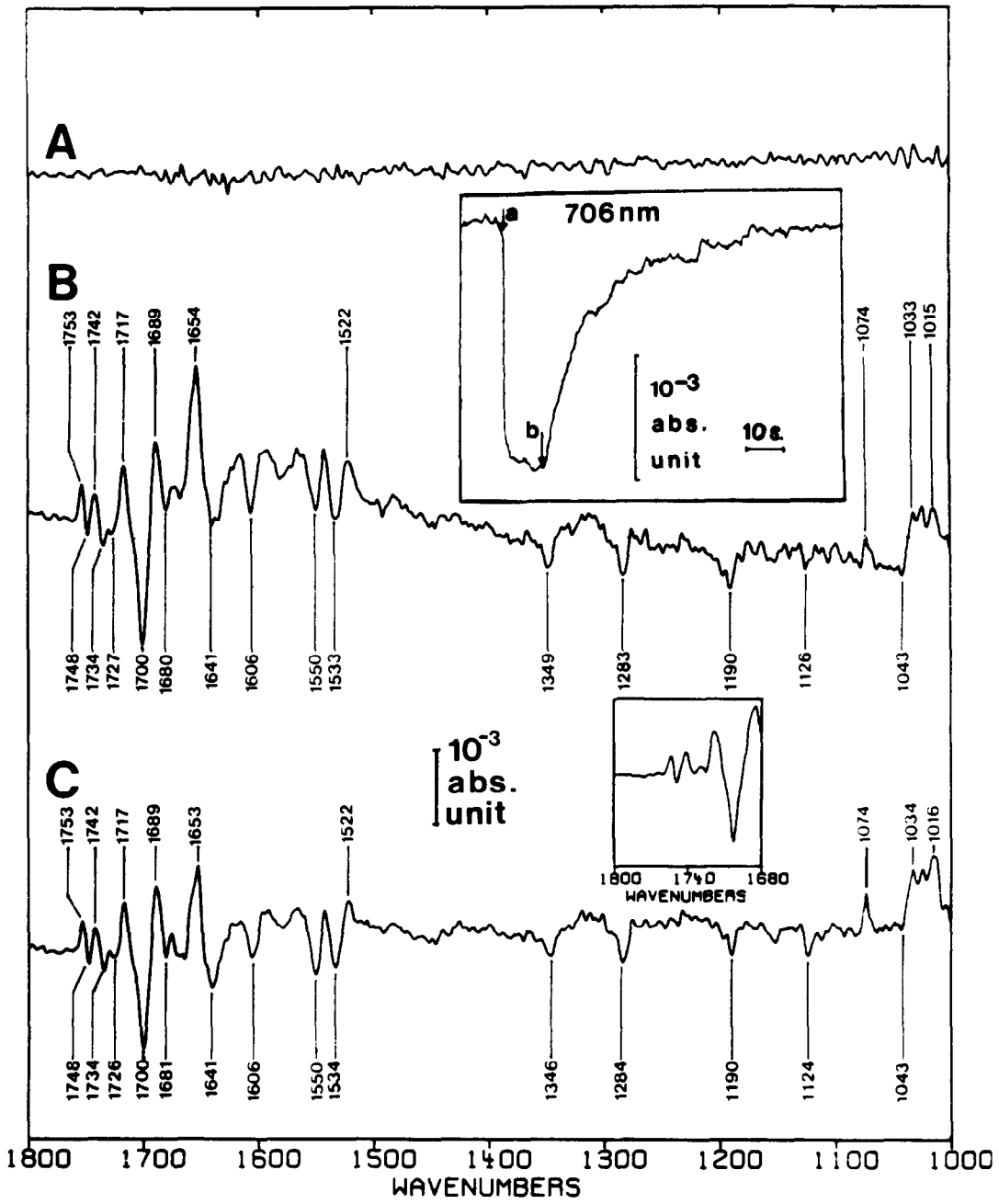


Fig.1. Light-induced FTIR difference spectra of a film of PS I particles air-dried with 70 μM methyl viologen and hydrated with 5 mM Tris buffer. (A) Difference between two single-beam spectra taken before illumination. (B) Difference spectrum ('light-minus-dark') obtained from spectra recorded during and before illumination. (C) Difference spectrum obtained from spectra recorded after and before illumination. Upper inset: kinetics of the bleaching of the primary donor absorption measured on the same sample at 706 nm on a laboratory-built spectrophotometer: (a) beginning of illumination; (b) end of illumination. Lower inset: light-minus-dark FTIR spectrum of a film of intact spinach thylakoid membranes, in the spectral range 1680–1800 cm^{-1}. For all measurements the temperature was 22°C. For all FTIR measurements the resolution was 4 cm^{-1}. 512 interferogram scans were added (accumulation time: 92 s). The frequencies are quoted within 1 cm^{-1}.

light (530 nm < λ < 1100 nm). One can see that even in the region of strong water and amide absorption, between 1500 and 1700 cm^{-1}, significant molecular changes can be detected. Fig.1C shows the difference between the absorbance spectrum taken immediately after illumination and one taken just before. The upper inset shows the kinetics of bleaching of the primary donor absorption measured at 706 nm on the same film. The experimental conditions were the same as those used for FTIR measurements, except that illumination was performed with blue-green actinic light (400 nm < λ < 650 nm). The half-time of the decay is ~9 s. Under the same conditions, an absorbance increase was observed at 820 nm, its amplitude being ~15% of the absorbance change at 706 nm (not shown). This demonstrates that the oxidized primary donor $P700^+$ can be accumulated in these hydrated films upon illumination with continuous actinic light [18].

Under our experimental conditions, reduction of the iron-sulfur complexes should not accompany the photooxidation of P700. To assess the possible contribution of the iron-sulfur centers in the FTIR difference spectra, the alternate electron acceptor methyl viologen was omitted. The films prepared in this way, however, yielded the same FTIR light-induced difference bands, thus demonstrating the dominant contribution of the primary electron donor P700 in the spectra shown in fig.1B,C.

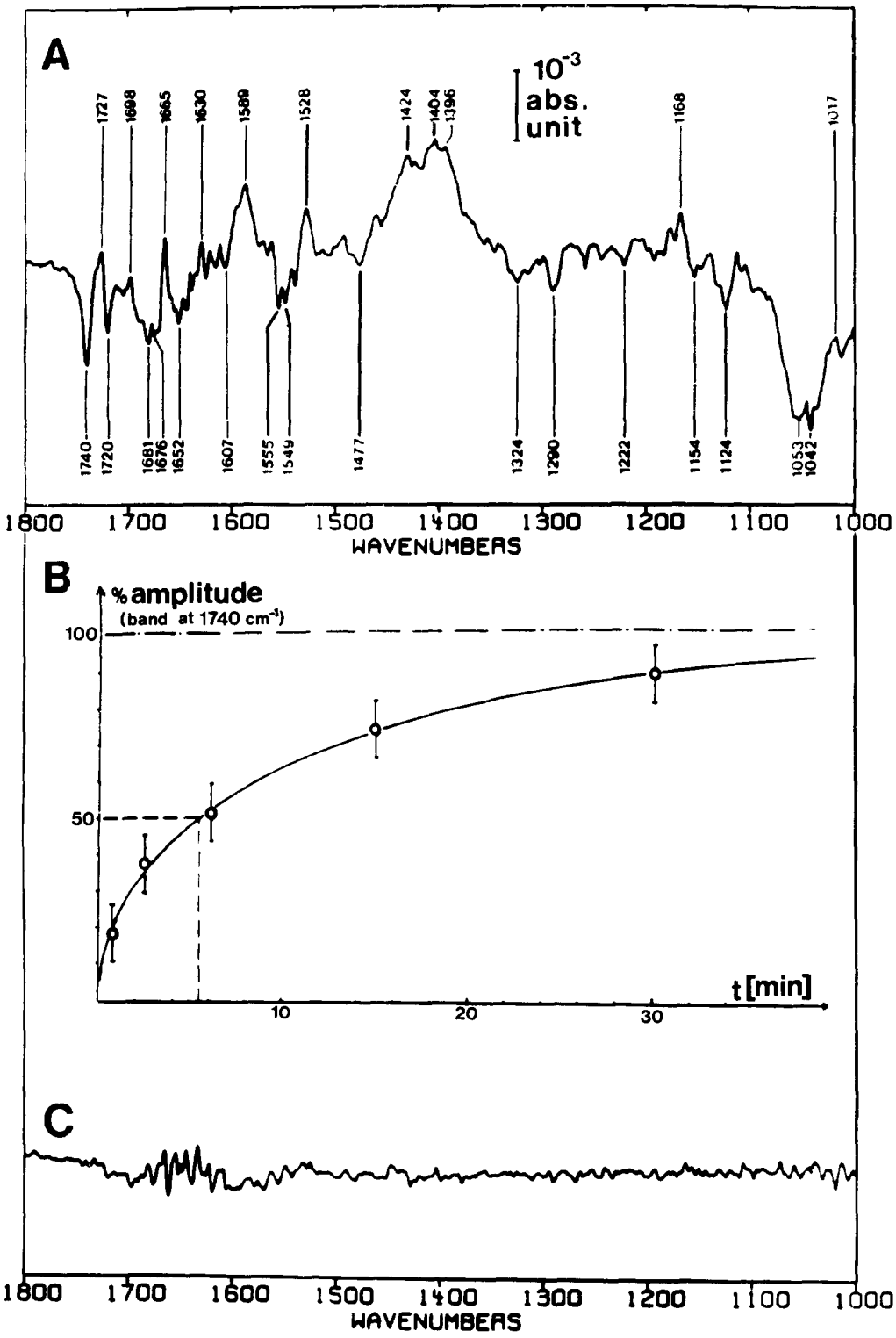
The most prominent features in the spectra in fig.1B,C are observed in the region between 1600 and 1760 cm^{-1}. In this region, carbonyl groups from Chl molecules (two ester C=O and one keto C=O are present in each Chl *a* molecule), as well as from proteins (peptide and side chain C=O groups), are apt to yield differential absorption features. The largest negative band in the spectra, the one at 1700 cm^{-1}, could correspond to a decrease in absorption of the 9-keto carbonyl of the Chl *a* molecule(s) constituting the primary donor. This decrease might be connected to the appearance of the somewhat weaker absorbing positive band at 1689 cm^{-1} in the spectra shown in fig.1B,C. A possible interpretation for this differential feature would be a change in binding type for the 9-keto C=O, due to a different ligation to the protein and/or to the influence of a charge in its vicinity. Previous modelling studies of P700 photooxidation have incorporated a keto-enol tautomerization (F.K. Fong, personal communication) or the involvement of a Schiff base [19,20]. However, without detailed infrared studies of these models and the IR changes occurring upon their one-electron oxidation, discussion of our results in relation to them is not justified.

According to the known frequency range of the Chl *a* ester carbonyl absorption [21–23], the two small but very reproducible differential features at 1748/1753 cm^{-1} and 1734/1742 cm^{-1} in fig.1B could be due to a shift to higher frequencies of the 7c-propionic acid and the 10a-carbomethoxy ester groups, respectively. This interpretation has been proposed for FTIR difference spectra of the primary electron donor photooxidation in bacterial RCs and chromatophores [9]. The positive band at 1717 cm^{-1} (also observed at 1711–1714 cm^{-1} in the spectra in [9]) lies between the domains of keto and ester C=O stretching vibrations, and cannot be ascribed precisely to either carbonyl group. An alternative interpretation for this band would involve carbonyl side chains of amino acid residues. In this case, however, they would not be easily accessible, since deuteration of the film for 16 h at room temperature did not lead to changes in the light-induced difference spectra (not shown).

The large positive band at 1654 cm^{-1} still lies within the range where a hydrogen-bonded keto group may absorb. However, an alternative interpretation would be a change in the amide I absorption. As $\Delta A/A$ does not exceed 10^{-3} at the amide I band, and assuming 1000–2000 peptide bonds per P700, such a change would affect at most one or two peptide bonds per PS I RC.

The most characteristic FTIR differential signals observed on PS I particles can also be detected on intact thylakoid membranes, especially in the car-

Fig.2. (A) Light-minus-dark spectrum of a film of PS II particles prereduced with 150 mM Na dithionite in Na borate buffer. (B) 'Back-reaction' curve showing the percentage of the signal that can be reinduced by light after a 'dark' relaxation period. The amplitude is that of the band at 1740 cm^{-1}. (C) Light-minus-dark spectrum of a similar sample hydrated with pure Na borate buffer (no dithionite). For all experiments, measuring conditions were as in fig.1.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bonyl stretching frequency region (fig.1, lower inset), notably the differential features at 1748/1753 and 1734/1741 cm^{-1}, and the large negative band at 1700 cm^{-1}. This result demonstrates that primary processes of green plant photosynthesis can be investigated by FTIR difference spectroscopy even in intact thylakoids.

3.2. *Photoreduction of the intermediary electron acceptor in PS II*

Dithionite is known to reduce the electron acceptors of PS II up to the primary quinone. Illumination of this sample at temperatures $\geqslant -40°C$, using actinic light (665 nm $< \lambda <$ 1100 nm), leads to a trapped P680-Pheo$^-$ configuration, as shown by ESR [2]. This state reacts back in a few minutes at room temperature and a few tens of minutes at $-40°C$.

Fig.2A shows the light-minus-dark FTIR difference spectrum of a film of PS II particles prereduced with 150 mM Na dithionite (from now on referred to as the 'Pheo$^-$ spectrum'). Fig.2B shows the amplitude of the band at 1740 cm^{-1} of Pheo$^-$ spectra taken at various times after a previous illumination. The half-time of the decay is 300–350 s at room temperature. Fig.2C represents a control experiment in which only dithionite was omitted. The absence of signal demonstrates (i) that the signals in the spectrum of fig.2A are inherent to Pheo reduction and (ii) the absence of PS I activity in these particles.

In a previous work, light-induced FTIR difference spectra of the photoreduction of I in *Rps. viridis* RCs and chromatophores were presented [10]. These 'I$^-$' spectra can be compared to those obtained here, although one has to be careful when comparing a BPheo *b* molecule to a Pheo *a*. However, several similarities can be pointed out, especially in the 1600–1750 cm^{-1} region, where stretching vibrations of the carbonyl groups are expected to contribute [21,22]. In *Rps. viridis* I$^-$ spectra, two negative bands at 1746 and 1732 cm^{-1} were tentatively attributed to a decrease in absorption of both 7c-propionic and 10a-carbomethoxy ester C=O vibrations of the BPheo molecule, respectively. The Pheo$^-$ spectrum of fig.2A also shows two negative bands, at 1740 and 1720 cm^{-1}, which can be similarly attributed to a decrease in absorbance of the 7c-propionic and 10a-carbomethoxy ester C=O groups of the Pheo molecule, respectively, upon its photoreduction. An alternative interpretation for these bands could be in terms of a deprotonation of amino acid carboxyl side chain groups. In this case, the broad positive band centered at 1589 cm^{-1} could be interpreted in terms of the appearing ionized carboxyl group. A similar interpretation had been proposed for the band at 1593 cm^{-1} in *Rps. viridis* I$^-$ spectra [10].

In the remainder of the spectrum, a large number of bands appear at reproducible frequencies, but small variations in their relative amplitudes were observed from sample to sample. The largest bands which appear at reproducible frequencies are those labelled in fig.2A. Since the signal-to-noise ratio, background amplitude and maximum band amplitude are quite comparable to those observed in [9,10] and in fig.1B,C, the slightly larger variations in the Pheo$^-$ signals could be due to differences in the state trapped under our experimental conditions, especially in the redox state of the primary and secondary quinone acceptors. Further experiments carried out at cryogenic temperatures, where the redox state of the quinones is better defined [24], will be necessary to determine more precisely the contribution of these acceptors in the difference spectra. At present, the complexity of the spectrum in the region between 1600 and 1700 cm^{-1} does not allow one to attribute one of the bands therein to a change in the 9-keto C=O absorption upon photoreduction, as was the case for I$^-$ spectra of *Rps. viridis*. The dominant features are the negative clusters around 1681/1676 and 1652 cm^{-1} and the positive bands at 1665 and 1630 cm^{-1}.

As in the case of the 'P700$^+$' spectra discussed above, a contribution of the peptide backbone cannot be definitely discarded, but if there is indeed such a contribution, it can only involve one or two peptide bonds, as seen from the small amplitude of the ΔA signal in the amide I and II region.

4. CONCLUSIONS

It appears from this work and [9,10] that the lack of large protein conformational changes is a general concept in the primary reactions of both plant and bacterial photosynthesis. Moreover, this study demonstrates that small but specific molecular changes occurring at the pigments and

their binding sites upon primary charge separation and stabilization in PS I and II are clearly identified by FTIR difference spectroscopy. In this respect, studies involving model compounds and isotope labeling will be necessary to assign each band in the difference spectra to specific molecular bonds in the pigment molecules and the proteins. Alteration of the vibrations of these bonds, which occurs upon separation and stabilization of charges, and which is monitored by light-induced FTIR difference spectroscopy, should thus allow a better description at the molecular level of the primary processes of green plant photosynthesis.

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