Light stability of the isolated CP43 studied by infrared and terahertz spectroscopy

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Abstract

Here, the light-induced changes of the core antenna complex chlorophyll protein 43 (CP43) isolated from PSII of spinach were assessed using infrared and terahertz (THz) spectroscopy. We found that the collective vibrational modes at low frequencies and the secondary structures of CP43 were altered upon illumination. The helix changed more than any other secondary structure. CP43 is more easily damaged by illumination than PSII. Infrared and THz spectroscopy have different functions in protein studies. THz spectroscopy has potential in evaluating the stability of proteins.

Additional key words: amide I band; conformation; degradation; photoinhibition.

Introduction

The photosynthetic proteins CP43 and CP47 in PSII form an internal antenna system. The CP43 and CP47 harvest light and transfer excitons to PSII reaction centers (Barber *et al.* 2000, Bricker and Frankel 2002). CP43 complexes interact with the reaction centers at D_1 positions and interact with 13 chlorophyll (Chl) *a* pigments and three β-carotenes as shown by crystallographic data (Guskov *et al.* 2009, Umena *et al.* 2011, Hall *et al.* 2016). D_1 and CP43 provide ligands to the inorganic Mn_4CaO_5 center that are required for the oxidation of H_2O (Liu *et al.* 2011).

Upon high light illumination, the pigments in the PSII membrane suffered by impairment (Telfer *et al.* 1991, Bumann and Oesterhelt 1995), proteins such as D_1 were degraded and photosynthetic activity was lost (Aro *et al.* 1990, Shipton and Barber 1991, Pokorska and Romanowska 2007). CP43 and CP47 in PSII membrane were damaged during strong illumination (Salter *et al.* 1992). When PSII membranes from spinach were subjected to light of 5,000 μmol(photon) m^{-2} s^{-1} to an aerobic environment, the degradation of both D_1 and D_2 proteins occurred. CP43 was similarly degraded to three products of low molecular mass (Yamamoto and Akasaka 1995). CP43 was lost when PSII membranes were illuminated upon alkaline Tris treatment. This loss of CP43 occurred due to its cross-linking with CP47 and D_1 (Mori *et al.* 1995). In these researches, CP43 is in the interior of PSII. A study on the isolated CP43 would be more significant because it can delete the effects of other components in PSII.

Terahertz (THz) radiation ranges from 0.1 to 10 THz which is between far infrared and microwave rays on the electromagnetic spectrum. It bridges the gap between electronics and photonics and exhibits unique properties (Chen *et al.* 2013). Biomolecules exhibit collective vibrational modes in the THz range (Yoneyama *et al.* 2008). THz spectroscopy can provide information on both phase and amplitude, so it can be used to study the collective vibrational modes of biomolecules (Tani *et al.* 1997, Zhou *et al.* 2019). THz spectroscopy can accurately measure the flexibility of proteins and can differentiate between proteins, conformations, and wild type *vs.* mutants (Markelz *et al.* 2002, Mernea *et al.* 2015). THz spectroscopy has seldom been used on the study of photosynthetic membrane proteins.

In order to further investigate the light-induced changes of the isolated CP43 and photoinhibition mechanism, infrared spectroscopy, SDS-PAGE, and THz spectroscopy were used in this study. The results showed that the collective vibrational modes at low frequencies and the secondary structure of CP43 changed upon illumination. CP43 is more easily damaged by illumination than PSII is. The structure damage and degradation of CP43 should be related to the photoinhibition. THz spectroscopy is a good complement to the study of photosynthetic membrane proteins.

Materials and methods

CP43 purification: Spinach (*Spinacia oleracea* L.) was grown in half Hoagland solution under conditions of controlled light [400 μmol(photon) m^{-2} s^{-1}, 12-h photoperiod], temperature (27°C light, 18°C dark) and humidity (60%). PSII particles were isolated from spinach according to Kuwabara and Murata (1982). Core complexes were extracted as described by Ghanotakis *et al.* (1989). The purification of CP43 followed the protocols of Shan *et al.* (2001). The core complexes were first treated with 0.8 mol L^{-1} Tris buffer (pH 8.0) for 15 min to a final Chl concentration of 0.1 mg mL^{-1} and centrifuged at 40,000 × *g* for 10 min. The procedure was performed twice to remove completely the 33 kDa extrinsic protein. The pellet was resuspended in 50 mmol L^{-1} 2-(N-morphine) ethane-sulfonic acid (MES) (*Sigma-Aldrich*, USA), 150 mmol L^{-1} NaCl, and 400 mmol L^{-1} sucrose (pH 6.0) at Chl concentration of 1.5 mg mL^{-1}, and incubated with an equal volume of 20 mmol L^{-1} Bis-Tris, 4 mol mL^{-1} LiClO$_4$, and 15% L-dodecyl maltoside (DM) (*Sigma-Aldrich*, USA) buffer (pH 6.0) for 20 mmol L^{-1} Bis-Tris (pH 6.0) for 2 h and then loaded onto a DEAE-*Fractogel TSK 650S* anion-exchange column (1.6 × 12 cm) that was equilibrated with 20 mmol L^{-1} Bis-Tris and 0.05% DM (pH 6.0) at a flow rate of 0.5 ml min^{-1}. CP43 was the first fraction that did not bind to the column and was eluted. The preparations were stored at –196°C. All of the above procedures were performed at 4°C in the dark.

White light illumination: The light treatment of 5,000 μmol(photon) m^{-2} s^{-1} was conducted with a halogen lamp (*CDD-1000, Bela Design*, China), which shows an emission of 260–2,500 nm and an emission of 400–760 nm in the visible light range. Light was filtered through a flat glass bottle containing CuSO$_4$ solution to absorb heat from the light source. The CP43 sample was placed in a vessel, the temperature of which was controlled by circulating cold water. The concentration of the CP43 sample was 0.2 mg(Chl *a*) mL^{-1}. During the light treatment, the sample was stirred continually.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE was performed as described by Qu *et al.* (2007). The polyacrylamide concentrations of the separation and stacking gels were 15 and 4%, respectively. The gel was stained with *Coomassie Brilliant Blue R-250*.

Fourier transformed-infrared (FT-IR) spectroscopy: CP43 or PSII samples were washed in D$_2$O, and the sample concentration was approximately 2 g(Chl *a*) L^{-1}. For FT-IR measurements, the sample was first dripped on a CaF$_2$ disk and then dried into a semi-hydrated film in a vacuum. Spectra were obtained using a FT-IR spectrometer (*Nicolet Magna 550 Series II, Midac Co.*, USA). Data treatment and band decomposition of the original amide I have been described by Taneva *et al.* (1995).

Terahertz time-domain spectroscopy (THz-TDS): Concentration of the CP43 sample used was 2 g(Chl *a*) L^{-1}. Samples of 50 μl were mixed with approximately 88 mg of spectrophotometric-grade polyethylene powder, which is close to transparent in the THz region. Mixtures were then pressed into disks of ~ 1.5 mm in thickness and 10 mm in diameter. A vacuum cell was used to delete the water vapor signal. For each measurement, data from the polyethylene powder instead of the CP43 sample were used as a reference waveform.

THz-TDS measurements were employed (Shi and Wang 2005). A mode-locked Ti:sapphire laser (*Mai Tai, Spectra Physics*, USA) was used to generate THz radiation from a piece of GaAs (110) wafer by optical rectification. THz waves were passed through samples and directed onto ZnTe (110) crystals for electrooptical sampling. All the acquired samples and reference waveforms were converted to their Fourier transforms $E_r(\omega)$ and $E_s(\omega)$. $E_r(\omega)$ is the electrical field strengths of the signal passing through an empty aperture and $E_s(\omega)$ is that propagating through the sample. The transmission spectra were obtained from the $E_s(\omega)/E_r(\omega)$ ratio.

Statistical analysis: All experiments were repeated at least three times. The relative changes [%] of α-helix or β-sheet are expressed as mean ± standard deviation (SD). The analyses were done with *SPSS 20.0* (*IBM*, Chicago, USA).

Results

Infrared spectra of CP43: The secondary structures of membrane proteins can be determined successfully using infrared spectroscopy. The vibration of amide I at 1,700–1,600 cm^{-1} occurs due to C=O vibrations in the polypeptide, and the frequency of which changes according to H bonding and transition dipole coupling, both of which can be used to study protein structure (De Las Rivas and Barber 1997, Hutchison *et al.* 1998, Hu *et al.* 2005).

The secondary structures could be identified through assessment of the overlapping sub-bands in the amide I region *via* second derivative analysis. Fig. 1*B* shows that the peak at 1,655 cm^{-1} is mediated by the α-helix, whilst the peaks at 1,637 and 1,626 cm^{-1} are due to the β-sheet (De Las Rivas and Barber 1997). Fig. 1*A* shows that the FT-IR spectra of CP43 changed under illumination. In order to more distinctly present the changes of secondary structures, absorption difference spectra for the amide I band were obtained (Fig. 1*C*). It shows that, under illumination, the α-helix content decreased with time, while the β-sheet content began increasing slightly from 5 min. Fig. 1 also indicates that the helix changed more than any other secondary structure. The percentage contents of CP43 secondary structures were obtained *via* Fourier deconvolution. The helix contents of CP43 decreased more than that of PSII, and the β-sheet contents of CP43 increased more than that of PSII when illuminated for the same time (Fig. 2). It implies that CP43 is more easily damaged by illumination than the PSII complex.
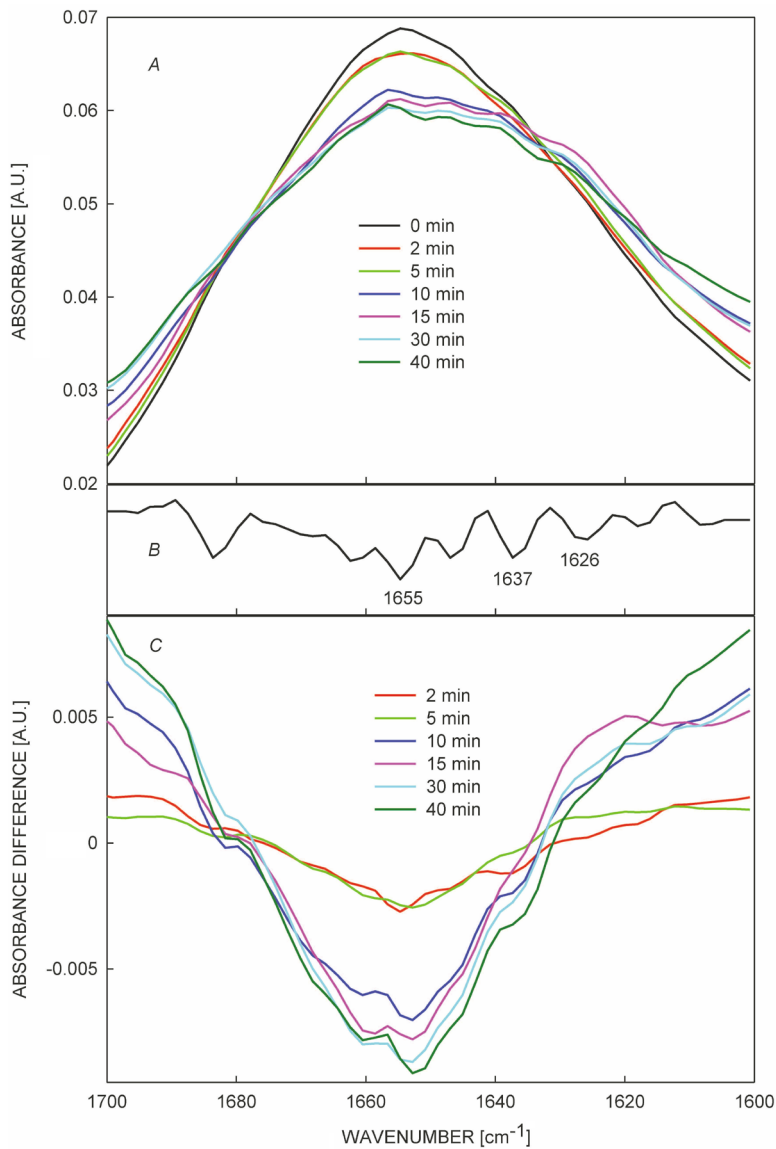
Fig. 1. Infrared absorption spectra of chlorophyll protein 43 (CP43). (*A*) Fourier transform-infrared (FT-IR) spectra of the amide I (1,700–1,600 cm^{-1}) band of CP43 in D_2O following normalization when illuminated at 5,000 μmol(photon) m^{-2} s^{-1}. (*B*) Second derivative of the amide I band of CP43 measured in D_2O. (*C*) Absorption difference spectra of the amide I band (the infrared spectra of illuminated samples minus that of control sample at 0 min of illumination after area normalization).
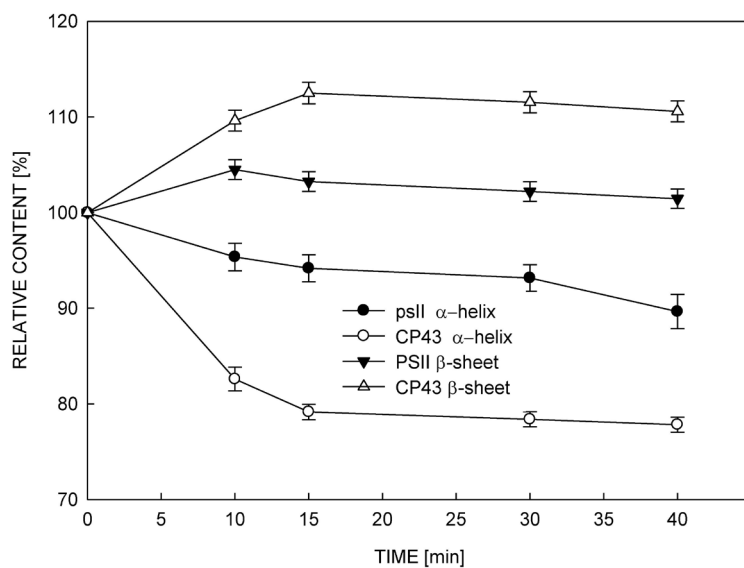

Fig. 2. Relative changes [%] of the α-helix and β-sheet content in PSII and chlorophyll protein 43 (CP43) with illumination time. Relative changes [%] = $C_t \times C_n \times 100$. C_t is the α-helix or β-sheet percentage content for the treated samples, and C_n is the α-helix or β-sheet percentage content for the native sample. The data represented were mean values ± standard deviations from three experiments.

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In addition, as seen in Fig. 1, the decreased extent of the CP43 helix content was really greater than the increased extent of the β-sheet content.

Degradation of CP43: The SDS-PAGE results indicated that CP43 apparently did not change when illuminated for 10 min (Fig. 3). At 30 min, it began to degrade, and it degraded most at 60 min. When CP43 degraded, no new bands appeared below the band of CP43 itself, implying that the degradation of CP43 was nonspecific. The various peptides resulting from the degradation were not enough to be visible on the gel or left the gel completely.

THz spectra of CP43: The response time of CP43 was slower than that of polyethylene (Fig. 4). The response time of the illuminated samples was shorter than that of
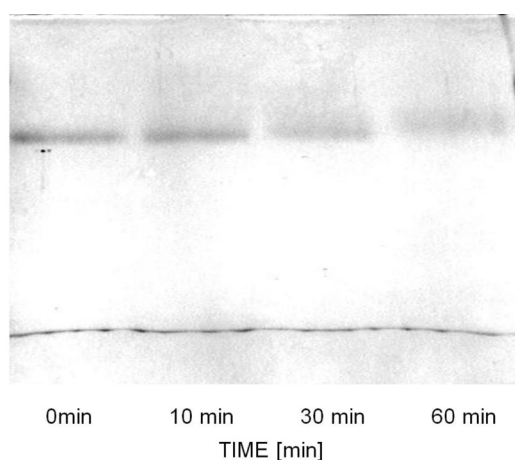

Fig. 3. Effects of illumination on the degradation of chlorophyll protein 43 (CP43). CP43 samples were illuminated in white light at 5,000 μmol(photon) m^{-2} s^{-1} for 10, 30, and 60 min, respectively.

the intact one, and it was the shortest when illuminated for 30 min.

The time-domain THz waveforms shown in Fig. 4 were further transitioned into frequency-domain amplitudes (transmission spectra) using fast Fourier transform (Fig. 5). The transmission spectrum of CP43 showed no major changes upon illumination for 5 min. Under illumination for 10 and 30 min, the left half of the spectrum shifted to a lower frequency, and a peak at 1.81 THz appeared.

The absorbance of the samples is given by $E_s(\omega)/E_r(\omega) = T(\omega)\ exp\ [-\alpha d + i\ (n\omega d/c)]$, where $T(\omega)$ is the loss of reflection on sample surface, n is the sample index, c is the speed of light in vacuum, d is the sample thickness, α is the power absorption coefficient, and αd represents the absorption of the sample. For 5 min of illumination, the absorption spectra were close to the intact curve, only with intensity being lower than that of the intact curve between 1.2 and 2.1 THz (Fig. 6). After 10 min of illumination, the spectra changed much more than the intact curve. Between 0 and 1.0 THz, the curve presented an increasing trend with increasing frequency, and the absorbance intensity was lower than that of the intact curve. Between 2.0 and 2.3 THz, a peak at 2.18 THz appeared, instead of the decreasing trend for the intact curve. On the whole, the absorbance of CP43 at 10 min became lower at low frequency and higher at high frequency than that of the intact curve, presenting an increasing trend. The spectra for 30 min of illumination showed no major changes relative to the spectra obtained after 10 min of illumination.

The dynamics of conformations happens over picosecond intervals, and that leads to collective vibrational mode changes which can be measured under THz frequencies (Markelz *et al.* 2002). In this study, the THz time-domain waveforms, transmission and absorbance spectra of CP43 all changed, which indicates that the collective vibrational mode of CP43 changed upon illumination and implies that THz spectroscopy has potential in evaluating the stability of proteins.
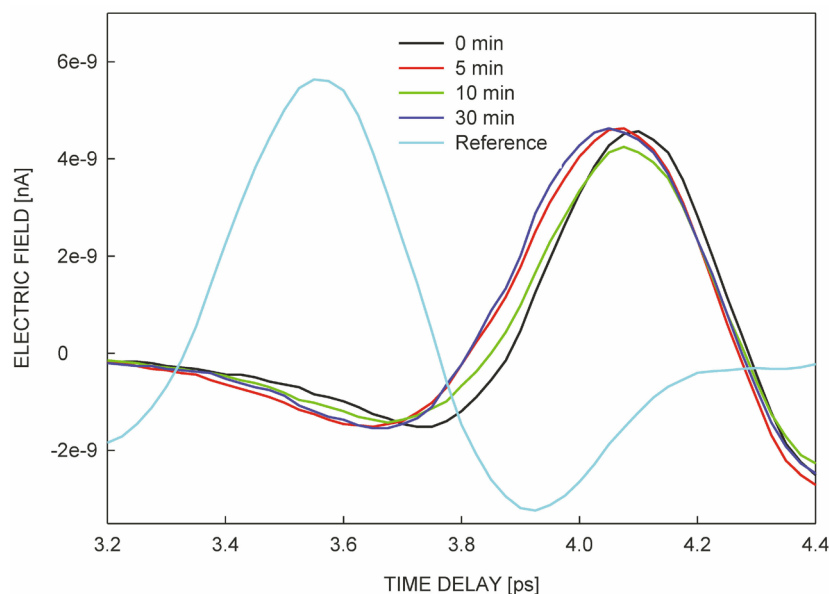

Fig. 4. The time-domain terahertz (THz) waveforms of chlorophyll protein 43 (CP43). Illumination intensity was at 5,000 μmol(photon) m^{-2} s^{-1}. Illumination times were 0, 5, 10, and 30 min, respectively. Reference curve was obtained from polyethylene.
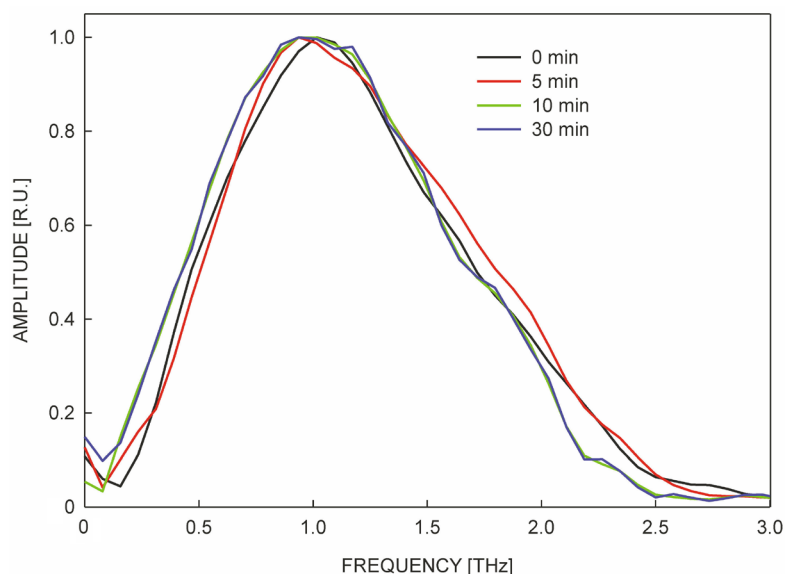
Fig. 5. Terahertz (THz) transmission spectra of chlorophyll protein 43 (CP43). The light intensity and illumination time are shown in Fig. 4. Reference curve was obtained from polyethylene.
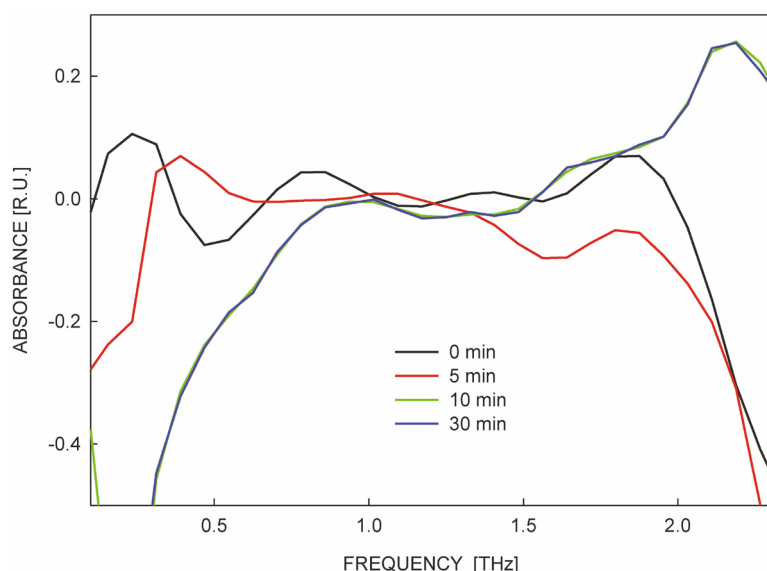

Fig. 6. Terahertz (THz) absorption spectra of chlorophyll protein 43 (CP43). The light intensity and illumination time are shown in Fig. 4.

Discussion

It is known that structure of the general protein, especially the nonphotosynthetic protein, is stable against high light. However, the results here showed that CP43, as a photosynthetic core antenna protein, is sensitive to high illumination (Figs. 1–3).

This occurs because of the association of proteins, lipids, and pigments with $^1O_2$. $^1O_2$ causes destruction, degradation, and pigment bleaching of PSII and its components, highlighting its importance in the attenuated PSII activity (Ohad *et al*. 1992, Aro *et al*. 1993, Andersson and Aro 2001, Krieger-Liszkay 2004). The formation of $^1O_2$ occurs due to triplet-triplet energy transfer from triplet Chl or triple carbonyl to O_2, which happens both in the PSII antenna and reaction center. In the PSII antenna complex, triple Chl is formed through photosensitization (Pospíšil 2016).

Additionally, the carotene in CP43 has the capability to quench triplet Chl. To maintain an effective quenching effect, β-carotene must be properly distanced and oriented from Chls (Pospíšil 2016). Under strong illumination, structural changes to CP43 change the distance and orientation between Chl *a* and β-carotene, and then decrease the quenching effect and increase the $^1O_2$ formation.

For green higher plants, excessive energy can be dissipated as heat by the reported xanthophyll cycle. The xanthophyll in PSII possesses the lowest singlet excited state, below that of ^{1}Chl* (Frank *et al*. 2000, Müller *et al*. 2001). Therefore, it may directly deexcite ^{1}Chl* and decrease the production of $^1O_2$. However, there are no xanthophylls in CP43, and this results in the greater sensitivity to illumination than that of PSII (Fig. 2).

Photoinhibition of PSII is a phenomenon that occurs

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in all oxygenic photosynthetic organisms under high irradiance (Ohad *et al.* 1992). Despite light being the driving force of PSII, high light additionally switches off electron transport and destroys PSII structures (Aro *et al.* 1993). Although photoinhibition has been studied for a long time, there is no consensus regarding its mechanism. Upon illumination, the structure damage and degradation of CP43 should be related to the photoinhibition.

Low-frequency modes are associated with the collective motion of the biomolecules. The results presented here show that the changing trend of infrared spectra is not always in agreement with that of THz spectra. THz spectra are complex and difficult to interpret, and the secondary structure of proteins is not as amenable to analysis by this approach. Infrared and THz spectroscopy have different functions in the study of proteins. The infrared amide I band in the 1,700–1,600 cm^{-1} region can characterize secondary structures, whilst THz spectroscopy is appropriate for the visualization of protein collective vibrational modes. In addition, the photosynthetic proteins usually contain pigments, which are easily bleached by visible light, while THz radiation is located from 0.1 to 10 THz and has no such photobleaching effects. Therefore, THz spectroscopy is indeed a good complement to the study on photosynthetic membrane proteins.

Under illumination, the THz absorbance spectrum of CP43 shows an increasing trend compared with that of the intact curve (Fig. 6*B*). Can this increasing trend be considered an indicator of the denaturation of biological molecules? It remains to be studied further.

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