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# Structural characteristics of extra-membrane domains and guanidine hydrochloride-induced denaturation of photosystem 2 core antenna complexes CP43 and CP47

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## Abstract

The structural characteristics of the extra-membrane domains and guanidine hydrochloride-induced denaturation of photosystem 2 (PS2) core antenna complexes CP43 and CP47 were investigated using fluorescence emission and circular dichroism (CD) spectra. The extra-membrane domains of CP43 and CP47 possessed a certain degree of secondary and tertiary structure and not a complete random coil conformation. The tertiary structure and the chlorophyll (Chl) *a* microenvironment of CP47 were more sensitive to guanidine hydrochloride (GuHCl) than that of CP43. Changes in energy transfer from β-carotene to Chl *a* corresponded well to changes in the tertiary structure while their correlation with changes in the secondary structure was rather poor. Unlike most of water-soluble proteins, both CP43 and CP47 are partly resistant to denaturation induced by guanidine hydrochloride (GuHCl); the denaturation of CP43 or CP47 is not a two-state process. Those features most probably reflect their character as intrinsic membrane proteins.

*Additional key words*: aromatic amino acid; chlorophyll; circular dichroism; CP43; CP47; denaturation; energy transfer; extra-membrane domain; fluorescence; guanidine hydrochloride; β-carotene.

## Introduction

CP43 and CP47, the core antenna subunits of photosystem 2 (PS2), are encoded by the *psb*C and *psb*B genes, respectively, in the chloroplast genome of higher plants and green algae, and in the genomic DNA of cyanobacteria (Bricker 1990, Barber *et al.* 2000, Bricker and Frankel 2002). Both CP43 and CP47 have six trans-membrane α-helices, which are separated by five extrinsic loop domains (Bricker and Frankel 2002). The large loop domain of CP43 contains two long and three short helices. In the case of CP47, the lumenal domain contains two long and four short helices and three β-sheets (Ferreira *et al.* 2004). Nevertheless, due to the insufficient resolution of this PS2 structure, additional structural characteristics of these CP43 and CP47 domains remain to be elucidated.

CP43 and CP47 bind chlorophyll (Chl) *a* and β-carotene (β-Car), but not Chl *b* (Bricker 1990, Barber *et al.* 2000, Bricker and Frankel 2002). The PS2 crystal structure shows that there are 14 Chl *a* and 4 β-Car molecules in CP43 and 16 Chl *a* and 2 β-Car molecules in CP47 (Ferreira *et al.* 2004). CP43 and CP47 can accept excitation energy that is harvested by the light-harvesting complex 2 (LHC2) and then transfer it directly to the PS2 reaction centre (RC) (Bricker 1990).

The process of protein denaturation is of great importance, for example, during the insertion and translocation of proteins into and through phospholipid membrane (Sathish *et al.* 2002). Unfortunately, compared to small water-soluble proteins, very little denaturation data are available for membrane proteins. Guanidine hydrochloride (GuHCl) is the most powerful among commonly used protein denaturants (Nozaki 1972), which can form

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*Abbreviations*: CD – circular dichroism; Chl – chlorophyll; GuHCl – guanidine hydrochloride; LHC2 – light-harvesting complex 2; PS – photosystem; RC – reaction centre; Trp – tryptophan; Tyr – tyrosine; UV – ultraviolet; β-Car – β-carotene; $\lambda_{max}$ – the fluores cence emission maximum.
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multiple hydrogen bonds with protein (Robinson and Jencks 1965, Makhatadze and Privalov 1992, Bhuyan 2002). GuHCl contributes to the denaturation of proteins through a hydrophobic effect involving the side chains. By favouring exposure of the non-polar group to the solvent, the hydrophobic interactions would be broken, thus leading to the denaturation of proteins (Lois Tiffany and Krimm 1973). GuHCl offers many advantages over other denaturants such as acids, heat, or detergent in studies of water-soluble proteins. For example, the product is better defined because the degree of denaturation is maximized; the denaturation more likely approaches a two-state mechanism (Pace 1986). Though GuHCl has been widely used in studying water-soluble proteins, it has been seldom used in work with membrane proteins. In the present study, by analyzing the spectral characteristics of native CP43 and CP47 and their changes induced by GuHCl, we have investigated the structural and denaturation characteristics of CP43 and CP47, especially their extra-membrane domains. The denaturation differences between water-soluble proteins and membrane proteins are also evaluated.

## Materials and methods

**Purification of CP43 and CP47**: PS2-enriched membrane was prepared from spinach as described by Kuwabara and Murata (1982). The oxygen evolving core complex (OECC) was isolated as discussed by Ghanotakis *et al*. (1989). CP43 and CP47 were then purified according to the method of Alfonso *et al.* (1994) with some modifications (Shan *et al.* 2001).

**GuHCl treatment:** The purified CP43 or CP47 was suspended in 20 mM Bis-Tris and 0.05 % DM (pH 6.0). The final concentrations of CP43 and CP47 were 3 g Chl *a* $m^{-3}$. The spectra were obtained after CP43 and CP47 had been incubated in solutions with various concentrations of GuHCl in the dark at room temperature for 6 h.

**Fluorescence and circular dichroism (CD) measurements**: Fluorescence spectra were measured by an *F-4500* fluorescence spectrophotometer (*Hitachi*, Tokyo, Japan). Both the excitation and emission band passes were 5.0 nm, and the scan rate was 2 nm $s^{-1}$ with a time constant of 2 s. CD spectra were recorded at room temperature with a *Jasco J-715* spectropolarimeter (*Jasco*, Tokyo, Japan) at a scanning speed of 1.66 nm $s^{-1}$, a bandwidth of 2 nm, a response time of 1 s, and an accumulation of 4 times in a 1-mm-pathlength cell. CD spectra were measured in the far-UV region (190–250 nm) and the red region (640–720 nm). The calculation of the secondary structure contents of the native protein was performed with the software program package *Dicroprot 2000* (release *1.0.4*) from the Internet (http://dicroprot-pbil.ibcp.fr). The evaluations were performed using the *Selcon3* program developed by Sreerama and Woody (1993).

## Results

**Fluorescence spectra** provide a sensitive means of characterizing the conformation and function of proteins (Dubey and Jagannadham 2003). CP43 and CP47 are two kinds of membrane protein with pigments, so they can be followed by a number of fluorescence signals, including fluorescence of aromatic amino acids and pigments (Booth *et al.* 2001). The ultraviolet (UV) emission spectra of CP43 and CP47 mainly reflect the presence of aromatic amino acids, Trp and Tyr (Burstein *et al.* 1973). For CP43, there are 5 tryptophans (Trps) and 3 tyrosines (Tyrs) in the trans-membrane domains, and 12 Trps and 9 Tyrs in the extra-membrane domains; for CP47, there are 5 Trps and 1 Tyr in the trans-membrane domains, and 10 Trps and 16 Tyrs in the extra-membrane domains (Barber *et al.* 2000). The 280-nm irradiation can excite both Trp and Tyr residues, whereas that with 295 nm radiation exclusively excites Trp residues (Eftink and Shastry 1997, Shutova *et al.* 2001). Fig. 1 and Table 1 show that upon excitation at 280 nm, the fluorescence emission maxima of CP43 and CP47 were at 323 and 324 nm, respectively. Upon excitation at 295 nm, the two fluorescence emission maxima of CP43 were at 324 and 346 nm, whereas those of CP47 were at 321 and 339 nm. After incubation with GuHCl, whether upon excitation at 280 or 295 nm, the fluorescence emission maxima of both CP43 and CP47 were red-shifted. Although the fluorescence maximum of CP47 was red-shifted more readily than that of CP43, after the treatment with 7 M GuHCl, both proteins had nearly the same fluorescence maximum, namely at 354 nm. As to the intrinsic fluorescence intensities of CP43 and CP47, whether upon excitation at 280 or 295 nm, they both increased at first but decreased later with the increase of GuHCl concentration.

We also measured fluorescence emission spectra excited by 480 nm radiation absorbed mostly by β-Car. The spectra of both CP43 and CP47 upon excitation at 480 nm (Fig. 2) were similar in shape to the spectra obtained upon excitation at 436 nm (data not shown). This indicated that the photon energy absorbed by β-Car could be effectively transferred to Chl *a*, and the integrity of the CP43 and CP47 preparation was maintained to facilitate this energy transfer. Upon excitation at 480 nm, the fluorescence intensity of both CP43 and CP47 gradually decreased when they were treated with GuHCl. When GuHCl concentration reached 6 M for CP43 and 5 M for CP47, the fluorescence maximum nearly dis-

appeared. While the fluorescence maximum of CP43 remained the same during the GuHCl treatment of CP43, in the case of CP47 the maximum was shifted from 693 to 685 nm already in the presence of 1–2 M GuHCl (Fig. 2).

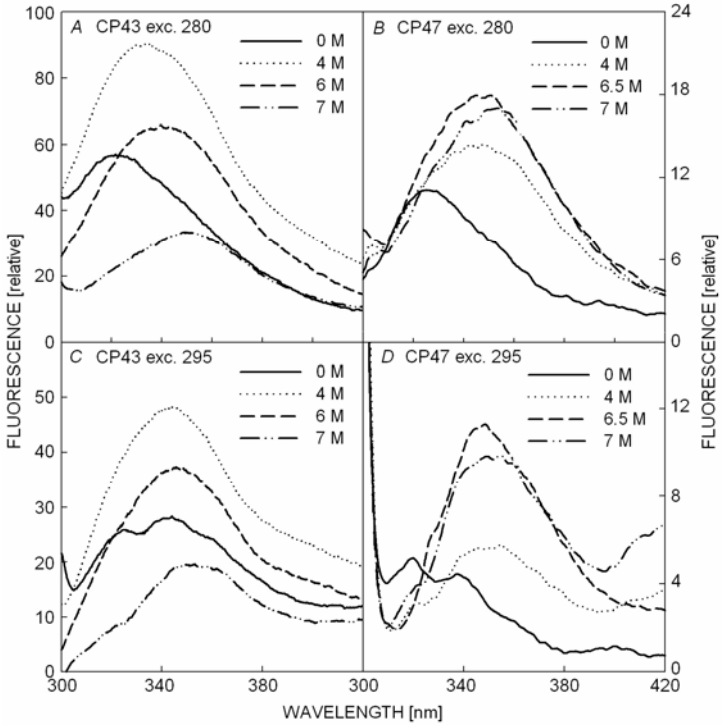


Fig 1. Fluorescence emission spectra of CP43 (*A, C*) and CP47 (*B, D*) excited at 280 nm (*A, B*) and 295 nm (*C, D*) after treatments with different concentrations of GuHCl. The spectra were measured after samples had been incubated in solutions with different concentrations of GuHCl in the dark at room temperature (25 °C) for 6 h. Final concentration of chlorophyll in the CP43 and CP47 preparations was 3 g $m^{-3}$.

Table 1. The emission fluorescence maxima [nm] and intensities [relative; in parentheses] of CP43 and CP47 when excited at 280 and 295 nm after incubation with different concentrations of GuHCl.

| Sample | Excitation wavelength [nm] | GuHCl [M] 0 | 4 | 6 | 6.5 | 7 |
|---|---|---|---|---|---|---|
| CP43 | 280 | 323 (54.8) | 334 (90.2) | 341 (65.1) | – | 354 (33.2) |
| | 295 | 324 (26.7 | 346 (48.1) | 348 (37.0) | – | 354 (19.3) |
| | | 346 (27.6) | – | – | – | – |
| CP47 | 280 | 324 (11.4) | 347 (14.3) | 348 (15.0) | 349 (17.8) | 354 (17.0) |
| | 295 | 321 (5.07) | 350 (5.7) | 350 (7.5) | 350 (11.0) | 354 (9.8) |
| | | 339 (4.4) | – | | – | – |

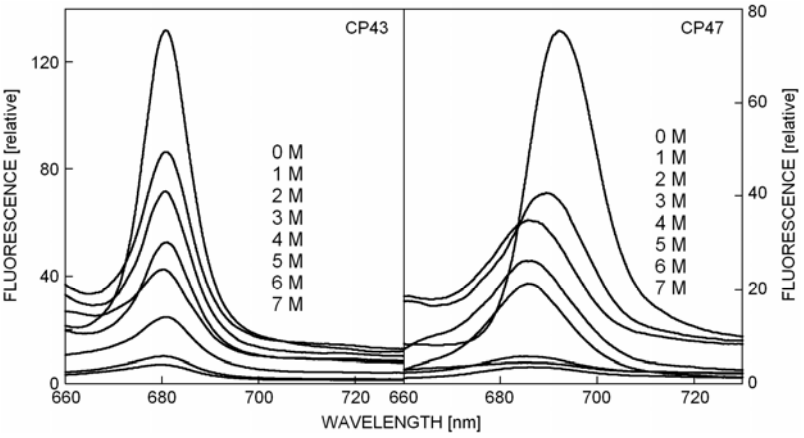


Fig. 2. The 77 K fluorescence emission spectra of CP43 and CP47 excited at 480 nm after treatment with different concentrations of GuHCl. The GuHCl concentration from top to bottom was 0–7 M, respectively. For other details see legend of Fig. 1.

Table 2. The contents of the protein secondary structures [%] in the whole protein and in the extra-membrane and intra-membrane domains of CP43 and CP47.

| Sample | Whole protein | | | | Extra-membrane domains | | | | Intra-membrane domains |
|---|---|---|---|---|---|---|---|---|---|
| CP43 | 52 | 10 | 10 | 72 | 34 | 14 | 14 | 62 | 100 |
| CP47 | 44 | 14 | 11 | 69 | 25 | 19 | 15 | 59 | 100 |

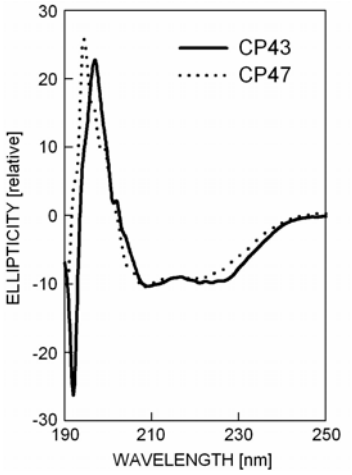


Fig. 3. The CD spectra of CP43 and CP47 in the far-ultraviolet region (190–250 nm) measured at room temperature. For other details see legend of Fig. 1.

**CD spectra**: In the far-UV region, CP43 and CP47 exhibited two negative peaks near 208 and 222 nm with the signal a little more pronounced in magnitude at the former wavelength, and the spectra changed from positive to negative at around 191–193 nm (Fig. 3). Table 2 shows the secondary structure contents of CP43 and CP47, which were derived from the CD spectral analysis. The contribution of various types of the secondary structures to the overall structure of the extra-membrane domains was calculated based on the fact that CP43 has 126 and 473 amide residues in the trans-membrane domains and the whole protein, respectively, whereas CP47 has 126 and 509 residues, respectively (Barber *et al.* 2000). Upon treatment with GuHCl, the magnitude of the CD peaks of CP43 and CP47 both decreased gradually. When the GuHCl concentration reached 7 M, most of the CD activity still remained; for example, at 222 nm, 65 % of the CD magnitude of CP43 and 54 % of CP47 remained (Fig. 4*A*,*C*).

In the red region, the CD spectrum for native CP43 contained a doublet signal with a negative peak at 685 nm and a positive peak at 667 nm, whereas that of native CP47 showed a doublet with negative and positive signals at 680 and 666 nm, respectively. The CD magnitude of CP47 was much larger than that of CP43 despite the fact that they had the same Chl *a* contents. The doublet signal of both CP43 and CP47 decreased after GuHCl treatment. When the GuHCl concentration reached 7 M, the CD activity of CP43 and CP47 in the red region still remained 35 and 36 %, respectively (Fig. 4*B*,*D*).

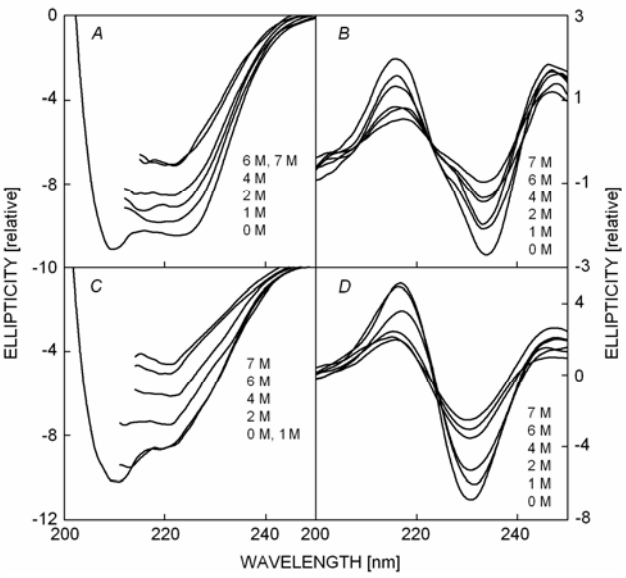


Fig. 4. The CD spectra of CP43 (*A, B*) and CP47 (*C, D*) after treatments with different concentrations of GuHCl measured in the far-ultraviolet region between 200 and 250 nm (*A, C*) or in the red region between 650 and 700 nm (*B, D*). The GuHCl concentration from bottom to top was 0, 1, 2, 4, 6, and 7 M, respectively. For other details see legend of Fig. 1.

## Discussion

**The structure of the extra-membrane domains of CP43 and CP47**: Proteins lacking Trp will only show Tyr fluorescence, which occurs at about 304 nm upon excitation at 280 nm. In contrast, the fluorescence of proteins with both Trp and Tyr will be dominated by the contribution from Trp residues upon excitation at 280 nm because Trp has a higher molar extinction coefficient and fluorescence quantum yield than Tyr and can serve as an energy transfer acceptor for Tyr (Oikawa *et al.* 1985, Barrow *et al.* 1992, Eftink and Shastry 1997). The fluorescence emission maximum ($\lambda_{max}$) of Trp is sensitive to the polarity of the microenvironment around the indole

side chain (Eftink and Shastry 1997). As a result, the emission spectra of Trp residues can reflect the polarity of their surrounding environment and their location in proteins (Lakowicz 1983). According to Burstein *et al.* (1973), Eftink and Shastry (1997), and Isaev-Ivanov *et al.* (2000), there are three main classes of Trp residues in proteins, in which the $\lambda_{max}$ and location are different: class I ($\lambda_{max}$ =320–332 nm) includes Trps located in the inner low-polar regions of protein molecules, and classes II ($\lambda_{max}$ = 340–342 nm) and III ($\lambda_{max}$ = 350–353 nm) include exposed Trp residues. The class II Trp residues are on the surface of native protein molecules and those of class III in the unfolded denatured ones; they differ only in the extent of perturbation of their electronic structures by water molecules.

Based on the above theory, it was suggested that the fluorescence emission maximum at 324 nm for CP43 and 321 nm for CP47 when excited at 295 nm must derive from the Trps located in the trans-membrane domains; while the emission maximum at 346 nm for CP43 and 339 nm for CP47 must derive from the Trps located in the extra-membrane domains. In addition, the fact that the longer emission maximum band was at 346 nm for CP43 and 339 nm for CP47 but not 350–353 nm proved that the extra-membrane domains of CP43 or CP47 do possess a certain degree of tertiary structure and not a complete random coil conformation. This result is in agreement with data of Ferreira *et al.* (2004). In contrast with CP43, the longer emission maximum band of CP47 was blue-shifted, indicating that the Trps of CP43 were more exposed to solvent than the Trps of CP47, and further implying that the extra-membrane domains of CP47 seem to have a more compact tertiary structure than those of CP43 in spite of nearly the same secondary structure contents between them (Table 2).

CD is a powerful technique for the structural analysis of biological systems, which can provide insight into the molecular architecture of the photosynthetic antenna system (Garab *et al.* 1991). The characteristics of the CD spectra in the far-UV region of CP43 and CP47 (Fig. 3) suggested that CP43 and CP47, in the native states, seem to belong to the α+β class of protein (separate α-helix and β-sheet regions) (Manavalan and Johnson 1983, Greenfield 1996, Dubey and Jagannadham 2003). The β-sheet must exist in the extra-membrane domains of CP43 and CP47 because the trans-membrane domains of CP43 and CP47 are formed by α-helixes only (Bricker 1990, Barber *et al.* 2000, Bricker and Frankel 2002). These results were also proved by the secondary structure analysis results obtained by the computer program (Table 2). So, here, the data of Ferreira *et al.* were not only confirmed but also additional details were added.

**The changes of the apo-protein of CP43 and CP47 when treated with GuHCl**: The aromatic amino acid fluorescence can reflect the tertiary structure changes of proteins and so provides a valuable structural probe for the study of protein unfolding (Kelly and Price 1997, Bian *et al.* 2000, Tanaka *et al.* 2002). Basically, with the unfolding of a protein, the Tyr and Trp residues in the inner low-polarity regions would be exposed to a polar environment gradually, and the fluorescence emission maximum would suffer a red shift (Dubey and Jagannadham 2003). After GuHCl treatment, the native proteins of CP43 and CP47 were unfolded, as could be seen from the red-shifted fluorescence emission maximum when excited at 280 or 295 nm (Fig. 1 and Table 1). In comparison to CP43, the fluorescence maximum of CP47 was more easily red-shifted, demonstrating that the tertiary structure of CP47 is more sensitive to GuHCl treatment. The reason may be related to the fact that there are more lipids in CP43 than in CP47 (Tremolieres *et al.* 1994) because GuHCl binds mainly with polar groups of a protein by multiple hydrogen bonds (Makhatadze and Privalov 1992).

When the GuHCl concentration reached 7 M, the same fluorescence emission maximum, namely 354 nm, which is the fluorescence maximum of Trp when completely exposed to water, was observed for CP43 and CP47 (Fig. 1 and Table 1), indicating the Trps in CP43 and CP47 were completely exposed to water, and further implying the tertiary structure of CP43 and CP47 was completely impaired.

The far-UV CD spectrum is widely used to quantitatively assess the overall secondary structure content of a protein (Greenfield 1996, Kelly and Price 1997), and the ellipticity at 222 nm can be used to estimate the α-helix content (Barrow *et al.* 1992). Upon treatment with GuHCl, the secondary structures of both CP43 and CP47 were disturbed, as could be seen from the decrease of CD activity in the far-UV region (Fig. 4*A*,*C*). In addition, the gradual decrease also implied that the denaturation of CP43 or CP47 is not a two-state process as that of water-soluble proteins. When GuHCl concentration reached 7 M, most of the CD activity in the far-UV region still remained (Fig. 4*A*,*C*), reflecting that the secondary structures of CP43 and CP47 were only partially impaired by GuHCl, which was also different from water-soluble proteins. The resistance to denaturation by GuHCl may be a common feature of intrinsic membrane proteins, and the reason for this may be the hydrophobic nature of the lipid-associated sequence (Oikawa *et al.* 1985).

**The energy transfer in CP43 and CP47 and its changes induced by GuHCl**: The energy absorbed by β-Car can be transferred to Chl *a* in CP43 and CP47. The fluorescence intensity upon excitation at 480 nm reflects the energy transfer from β-Car to Chl *a*. So the decrease of fluorescence intensity upon excitation at 480 nm after GuHCl treatment (Fig. 2) indicated the loss of the energy transfer. When GuHCl concentration reached 6 M for CP43 and 5 M for CP47, the energy transfer from β-Car to Chl *a* disappeared because the fluorescence disappeared (Fig. 2). Considering the fact that when treated

with 7 M GuHCl, the tertiary structures of CP43 and CP47 were completely impaired, while most of the secondary structure of CP43 and CP47 remained, it was concluded that changes in energy transfer from β-Car to Chl *a* corresponded well to changes in the tertiary structure while their correlation with changes in the secondary structure was rather poor.

The fluorescence of CP43 and CP47 upon excitation at 480 nm is essentially the Chl *a* fluorescence. The Chl *a* fluorescence is very sensitive to the change of Chl *a* microenvironment. Upon excitation at 480 nm, the fluorescence peak of CP47 shifted to the blue region, whereas that of CP43 had no obvious shift (Fig. 2). This indicated that GuHCl disturbed the Chl *a* microenvironment of CP47 more readily than that of CP43.

The CD spectrum in the red region is very sensitive to the microenvironment of the chromophore, including the position, orientation, and distance between the chromophores. The doublet CD signals of CP43 and CP47 in the red region are mainly ascribed to the excitonic interaction between Chl *a* molecules, which is one of the energy-transfer modes between Chl *a* molecules in the antenna complexes (van Grondelle 1985, van Grondelle *et al.* 1994). The CD magnitude of CP47 was much larger than that of CP43 (Fig. 4*B*,*D*), reflecting that Chl *a* molecules in CP47 are in a more aggregated state and have a stronger interaction with the protein matrix than those in CP43. These characteristics are favourable for energy transfer. After treatment with GuHCl, the doublet in the red region of both CP43 and CP47 decreased (Fig. 4*B*,*D*), indicating the destruction of Chl *a* excitonic interaction and further implying the decrease of the energy transfer between Chl *a* molecules.

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