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Monitoring fluorescence of individual chromophores in peridinin–chlorophyll–protein complex using single molecule spectroscopy

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Abstract

Single molecule spectroscopy experiments are reported for native peridinin–chlorophyll a–protein (PCP) complexes, and three reconstituted light-harvesting systems, where an N-terminal construct of native PCP from *Amphidinium carterae* has been reconstituted with chlorophyll (Chl) mixtures: with Chl a, with Chl b and with both Chl a and Chl b. Using laser excitation into peridinin (Per) absorption band we take advantage of sub-picosecond energy transfer from Per to Chl that is order of magnitude faster than the Förster energy transfer between the Chl molecules to independently populate each Chl in the complex. The results indicate that reconstituted PCP complexes contain only two Chl molecules, so that they are spectroscopically equivalent to monomers of native-trimeric-PCP and do not aggregate further. Through removal of ensemble averaging we are able to observe for single reconstituted PCP complexes two clear steps in fluorescence intensity timetraces attributed to subsequent bleaching of the two Chl molecules. Importantly, the bleaching of the first Chl affects neither the energy nor the intensity of the emission of the second one. Since in strongly interacting systems Chl is a very efficient quencher of the fluorescence, this behavior implies that the two fluorescing Chls within a PCP monomer interact very weakly with each other which makes it possible to independently monitor the fluorescence of each individual chromophore in the complex. We apply this property, which distinguishes PCP from other light-harvesting systems, to measure the distribution of the energy splitting between two chemically identical Chl a molecules contained in the PCP monomer that reaches 280 cm^{-1}. In agreement with this interpretation, stepwise bleaching of fluorescence is also observed for native PCP complexes, which contain six Chls. Most PCP complexes reconstituted with both Chl a and Chl b show two emission lines, whose wavelengths correspond to the fluorescence of Chl a and Chl b. This is a clear proof that these two different chromophores are present in a single PCP monomer. Single molecule fluorescence studies of PCP complexes, both native and artificially reconstituted with chlorophyll mixtures, provide new and detailed information necessary to fully understand the energy transfer in this unique light-harvesting system.
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Keywords: Light-harvesting complexes; Fluorescence; Single molecule spectroscopy; Chromophore interaction

The water-soluble peridinin–chlorophyll *a*–protein (PCP) complex from dinoflagellate *Amphidinium (A.) carterae* represents a unique example in a large family of light-harvesting antennas. While most of the antennas collect light energy predominantly by chlorophyll (Chl) or bacteriochlorophyll (BChl) molecules, the main pigment of PCP is a carotenoid, peridinin (Per) [1], which absorbs in a spectral region not available to the Chls, ranging from 450 to 550 nm. Besides Per, the system in its native form contains also Chl *a* molecules. The structure of native PCP, which has been

Abbreviations: Chl, chlorophyll; Per, peridinin; PCP, peridinin–chlorophyll a–protein; APC, allophycocyanin; LH2, light-harvesting complex 2; SMS, single molecule spectroscopy; A, *Amphidinium*

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determined with 2 Å resolution [2], reveals a trimer of protein subunits (Fig. 1)[1]. A single monomer of PCP contains two Chl *a* molecules (marked in green) and eight Per (marked in red) organized in two clusters. Each Per molecule is in van der Waals contact with the tetrapyrrole ring of the Chl *a* of the same cluster. The center-to-center distance between the two Chls *a* within a monomer is 17.4 Å, while the distance between two Chls bound to different monomers ranges from 40 to 54 Å [2]. The pigments are embedded in the hydrophobic cavity formed by the protein.

The exceptional properties of PCP along with the detailed knowledge about the structure of the complex have sparked in recent years considerable interest in this system [3–13]. Most research has been aimed at understanding the exact mechanism of energy transfer between Per and Chl [3–6] by studying the spectroscopic properties of PCP ensembles. The quantum efficiency of the energy transfer reaches up to 100% [4] and involves an intramolecular charge transfer state [11,13]. In addition, circular dichroism experiments accompanied with theoretical calculations have suggested relatively weak dipole–dipole coupling (about 10 cm^{-1}) between Chls within a single monomer [7,8]. It has also been speculated that Chls in native PCP are isoenergetic due to similar chemical and electronic surroundings [7], although this point raises some controversy [9].

The advancement in single molecule detection has provided valuable insight into the optical properties of protein–pigment systems that are severely obscured by ensemble averaging [14–20]. In particular, single molecule spectroscopy (SMS) has been useful for unraveling details about the energy transfer in phycoerytrocyanin [20], chromophore–chromophore interactions in allophycocyanin (APC) [19] and the influence of structural changes of the surroundings on the fluorescence properties of the bacterial light-harvesting complex 2 (LH2) [15]. However, in the case of APC [19] and LH2 [14,18], the fluorescing state has an excitonic character due to strong coupling between 2 and 18 chromophores, respectively. Such a strong excitonic coupling between the chromophores effectively diminishes the possibility of using single chromophore emission as a sensitive probe of the protein surroundings. From this perspective, the PCP complex, which is thought to feature weak coupling between fluorescing Chl molecules, offers an appealing alternative for monitoring the interaction between a truly single chromophore with its local surroundings, without any significant influence of chromophore–chromophore interactions.

The present study concerns room temperature SMS of the light-harvesting PCP complex, which has so far been investigated exclusively on the ensemble level. In order to observe fluorescence from single PCP complexes, we apply an excitation scheme, which takes advantage of the very efficient energy transfer between Per and Chl [11]: instead of exciting Chl directly, the energy of the laser is tuned into the absorption band of Per (more than 120 nm or 4000 cm^{-1} above the Chl emission). Since the energy transfer between Per and Chl in PCP is more than an order of magnitude faster than the Förster energy transfer between the Chl, we expect the former to dominate the fluorescence properties of the system. The experiments have been carried out on native PCP from *A. carterae*, as well as on three reconstituted systems, where Chl *a* and Chl *b* were incorporated into the N-terminal half of native PCP. The fluorescence spectra of individual native PCP, as well as of PCP reconstituted with Chl *a* or Chl *b*, feature single and relatively narrow emission lines with a typical linewidth of about 300 cm^{-1}. On the other hand, for most of PCP complexes reconstituted with *both* Chl *a* and Chl *b* we observe two spectrally separated emission lines. In agreement with the ensemble spectrum, the average splitting between the two fluorescence lines is equal to 500 cm^{-1}, implying that these artificial complexes contain *two different* Chl molecules.

In the fluorescence trajectories measured for PCP complexes reconstituted with either Chl *a* or Chl *b* we clearly detect two intensity steps, which we attribute to subsequent bleaching of the two Chls comprising the complex. Importantly, the bleaching of the first Chl does not affect either the fluorescence intensity or the emission energy of the remaining one. We conclude therefore, that in a clear contrast to all other light-harvesting systems, the two *fluorescing* Chls within a PCP monomer feature extremely weak dipole–dipole interactions. We apply this property, which enables one to independently trace the fluorescence of individual Chls, to demonstrate that

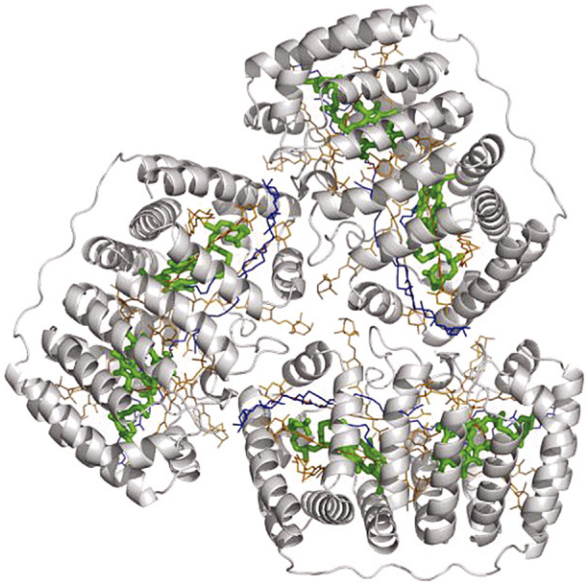

Fig. 1. Structure of native, trimeric PCP complex. The protein is shown as a grey ribbon. The pigments are represented as green (Chl *a*) and red (Per) sticks, while blue sticks correspond to six integral lipid molecules. Each monomer contains two Chl *a* molecules and eight Per molecules grouped into two nearly symmetric units.

[1] The term 'monomer' is used here in a topological context, referring to large PCP from A. carterae from which the X-ray structure has been solved. Large PCP is a 32 kDa protein, it originates from a gene duplication and binds two Chl/Per clusters. For historical reasons, this species is generally referred to as the PCP monomer. With respect to the large PCP monomer, the 16-kDa small PCP discovered subsequently, as well as the generated N-PCP, are topologically "half-mers": they carry only a single Chl/Per cluster and dimerize to species that are homologous to the monomer of large PCP.

Chls within PCP monomers are energetically distinguishable. In fact, in the case of single PCP complexes reconstituted with either Chl *a* or Chl *b*, the splitting reaches 280 cm^{-1}. The stepwise bleaching of the Chls is also present in the fluorescence trajectories obtained for the native complex, although, due to larger number of Chls, the actual number of intensity steps cannot be accurately determined. The SMS results of the PCP complex, both native and reconstituted, provide new and detailed information important for understanding the mechanisms of energy transfer in this system. In addition, they show that PCP is indeed a perfectly suitable system for investigating chromophore–chromophore and protein–chromophore interactions on a single chromophore level.

1. Materials and methods

1.1. Native and reconstituted PCP complexes

Native PCP from *A. carterae* was purified according to Hofmann et al. [2]. Reconstitution of PCP [21] followed the protocol of Polivka et al. [22]. Briefly, 625 µl N-domain apoprotein of PCP (0.32 mg ml^{-1}) was combined with 225 µl of buffer (pH 7.6) containing 25 mM Tris and 10 mM KCl. Following the addition of Per (12 µmol) and Chl *a* (or *b*) (3 µmol) in 150 µl ethanol, the mixture was incubated at 4 °C for 48 h. For PCP reconstituted with both Chl *a* and Chl *b* 1.5 µmol of each pigment were used. The crude reconstitution product was first purified on a small Sephadex G-25 (PD-10) column equilibrated with Tris buffer (5 mM, pH 7.6) containing 2 mM KCl. Then it was bound to a column of DEAE Trisacryl (Sigma, Darmstadt) and removed with the same buffer containing 0.1 M NaCl. Finally the product was desalted on Sephadex G-25 (PD-10) (Biosciences, Uppsala) and equilibrated with Tris buffer (5 mM, pH 7.6). Details are reported elsewhere [21].

1.2. Ensemble spectroscopy

Absorption measurements on the ensembles were performed with a Cary 50 Cone spectrometer (Varian), and fluorescence was measured using an F900 fluorimeter (Edinburgh Analitical Instruments). The solution was in each case placed in a quartz quvette (Hellma). The fluorescence spectra of the PCP ensembles were obtained at the excitation wavelength of 514 nm.

1.3. Single molecule spectroscopy

In order to achieve the concentrations appropriate for single molecule detection, the PCP solution of about 0.2 OD at the Per-related absorption (~450 nm) was further diluted by five to six orders of magnitude in a Tris–EDTA buffer solution (Fluka 93302, pH=7.4). The sample was then carefully dispersed on a coverslip surface. In a final step, the coverslip was glued to another glass plate in order to prevent the sample from drying and too rapid oxidation.

Single molecule spectroscopy experiments were performed using a modified scanning confocal microscope (ZEISS LSM 410). High spatial resolution and detection efficiency was achieved with a high numerical aperture oil-immersion objective (ZEISS 40×1.3 NA oil). The excitation energy of a continuous-wave Nd:YAG laser was 532 nm (as marked in Fig. 2a), which corresponds to the absorption of Per, not of Chl. Indeed, in this spectral region the direct absorption of Chls is less than 10%. In other words, instead of tuning the excitation into the vibronic band of the chromophore, a typical approach in single molecule experiments, we adjust laser energy into the absorption band of Per and take advantage of the very efficient energy transfer from Per to Chls [1,4,23]. The fluorescence emissions of Chl *a* and Chl *b*, which occur around 670 nm and 650 nm, respectively, make it easy to spectrally isolate the signal of single PCP complexes, by using appropriate filters and dichroic mirrors. The excitation power measured after the microscope objective was about 10 µW.

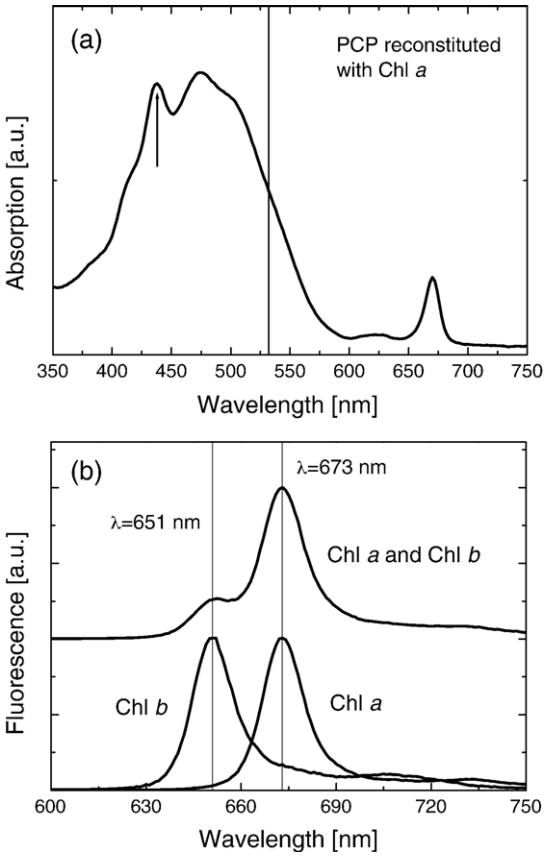

Fig. 2. Ensemble characterization of reconstituted PCP complexes. (a) Room temperature absorption of PCP reconstituted with Chl *a*. The arrow marks the Soret band, while the vertical line corresponds to the excitation energy of 532 nm, used in single molecule experiments. (b) Fluorescence spectra excited at 514 nm obtained for PCP reconstituted with Chl *a*, with Chl *b*, and with Chl *a* and Chl *b*. Note the presence of two emission lines for the latter PCP complex.

The configuration of the experimental setup enabled us to scan a 30 µm×30 µm large area of the sample. The fluorescence images of single PCP complexes were collected using an avalanche photodiode (EG and G SPCM-AQR-141). After the microscope, the detection path was split into two beams of equal intensity. One beam was guided to the avalanche photodiode, while the other was dispersed using an Amici-prism and the spectrally-resolved fluorescence signal was detected with a Peltier-cooled, back-illuminated CCD camera (Princeton Instruments, EEV 1300/100-EMB-chip). The integration time used for the spectra acquisition was typically 0.3 s, and the spectral resolution was about 1.5 nm.

2. Results and discussion

2.1. Ensemble characterization

The absorption of PCP ensemble reconstituted with Chl *a* (Fig. 2a) is dominated by a broad and intense band (from 350 nm to 550 nm) associated predominantly with the absorption of Per [1]. The main contribution from Chl, besides the Q_Y band seen around 670 nm, is through Soret band (marked by an arrow) at 437 nm. The vertical line at 532 nm marks the excitation energy used in SMS experiments. We note that the absorption spectrum obtained for the PCP reconstituted with Chl *a* is almost identical to the one measured previously

for the native PCP complex from *A. carterae* [7]. The fluorescence spectra measured for PCP reconstituted with either Chl *a* or Chl *b* (Fig. 2b) feature strong emission lines at 673 nm and 651 nm, respectively (marked by vertical lines). Interestingly, in the case of PCP complexes where both Chls were added during the reconstitution, we observe two well-separated lines at the two above energies corresponding to Chl *a* and Chl *b* emissions. However, on the basis of the ensemble experiments it is impossible to conclusively determine whether the complexes contain identical Chl molecules (Chl *a* and Chl *a*, or Chl *b* and Chl *b*) or if there are two different Chls present in some of them. This question can be answered using SMS techniques.

2.2. Characterization of single native PCP complexes

A summary of SMS results obtained for native PCP complexes is given in Fig. 3. Bright spots seen in Fig. 3a correspond to fluorescence emission of single complexes, each containing six Chl *a* molecules [2]. The images of the molecules are round and they feature almost no blinking during the scan. The extraordinarily bright spot visible on the left side of the image is most probably a higher aggregate of native PCP. The fluorescence spectrum of a PCP complex consists of a single intense line, as shown in Fig. 3b. A single Gaussian fit, represented with a solid line, reproduces well the lineshape of the fluorescence emission. In Fig. 3c the histogram of the emission energies of over 80 single native PCP complexes is displayed. Although the wavelength of the fluorescence exhibits some variation from complex to complex, the maximum of the overall distribution ($\lambda = 673.5$ nm) corresponds to the energy measured at ambient temperature for PCP ensemble. This correlation indicates that the photo-physical properties of single PCP complexes are not affected by the surface and/or preparation procedure in any significant way. In Fig. 3d we show a histogram of the bleaching time obtained for over 150 single native PCP complexes. We find that the fluorescence of PCP is remarkably stable and, apart from some occasional blinking, the signal could frequently be observed for tens of seconds. The solid line in Fig. 3d represents a single exponential fit, which yields the average survival time of native PCP fluorescence to be around 29 s. A qualitatively similar behavior has been observed also for the reconstituted PCP complexes.

2.3. Complexes reconstituted with Chl a and/or Chl b

In contrast to the native PCP, which is predominantly a trimer, the majority of the reconstituted PCP complexes is

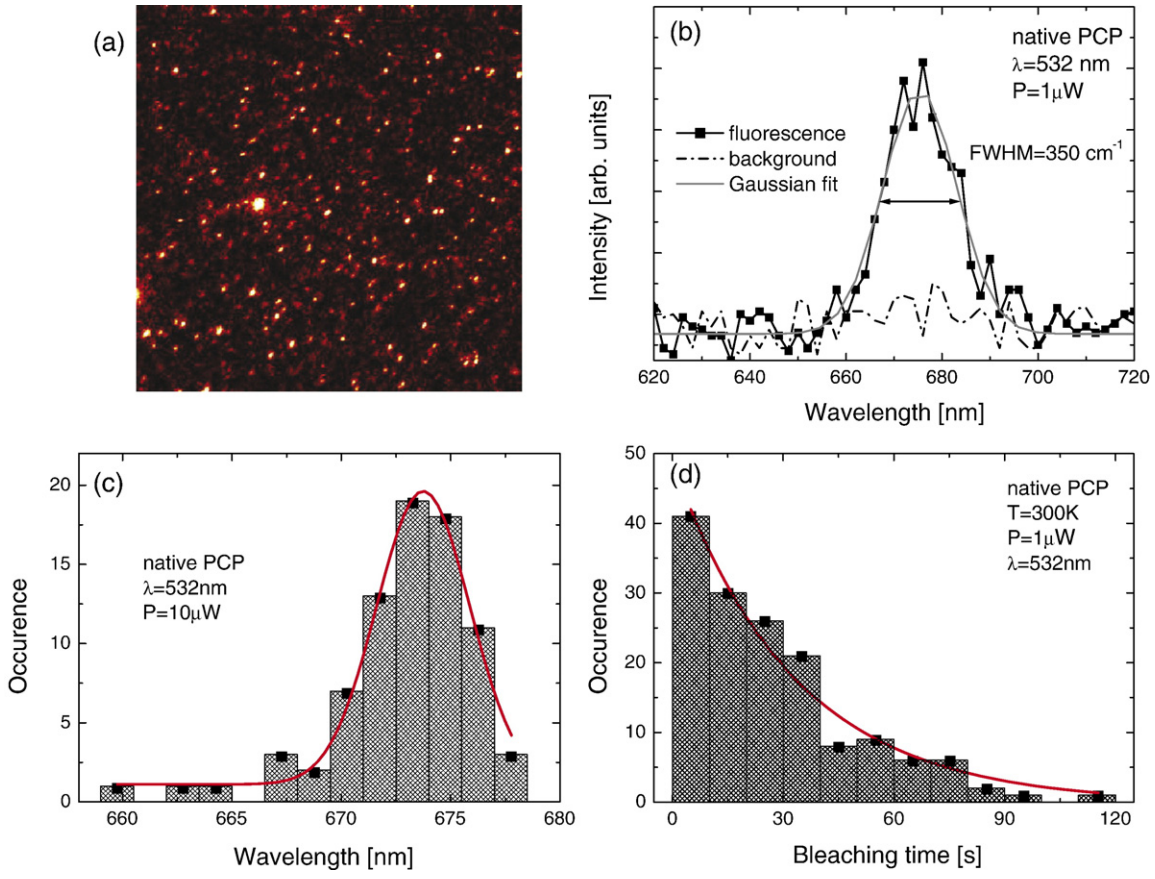

Fig. 3. Summary of SMS results obtained for native PCP: (a) 30 μm by 30 μm image showing the fluorescence of single PCP complexes excited at 532 nm. (b) Fluorescence spectrum of a single native PCP complex (points). Solid line represents the Gaussian fit, for comparison the background is also displayed. (c) Histogram of fluorescence emission wavelengths measured for over 150 single native PCP complexes. The solid line is the ensemble spectrum. (d) Histogram of bleaching times obtained for over 150 single PCP complexes. The solid line is an exponential fit to the data.

expected to contain only two Chls, thus resembling the spectroscopic properties of a native PCP monomer. This is due to the surface specific features on the C-terminal domain that promote trimerization [2]; this domain is absent in the reconstituted protein. Recently, the crystal structure of PCP refolded with Chl *a* has been elucidated (Schulte, Hiller and Hoffman, unpublished results) and it has been found to indeed form monomers in the crystal. Fig. 4 shows examples of the fluorescence spectra of three single PCP complexes reconstituted with Chl *a*, with Chl *b*, and with Chl *a* and *b*. As expected from the ensemble characterization (see Fig. 2), in the former two cases the fluorescence spectrum consists of a single narrow line, as shown in Fig. 4a and b. Similarly to the native PCP, the

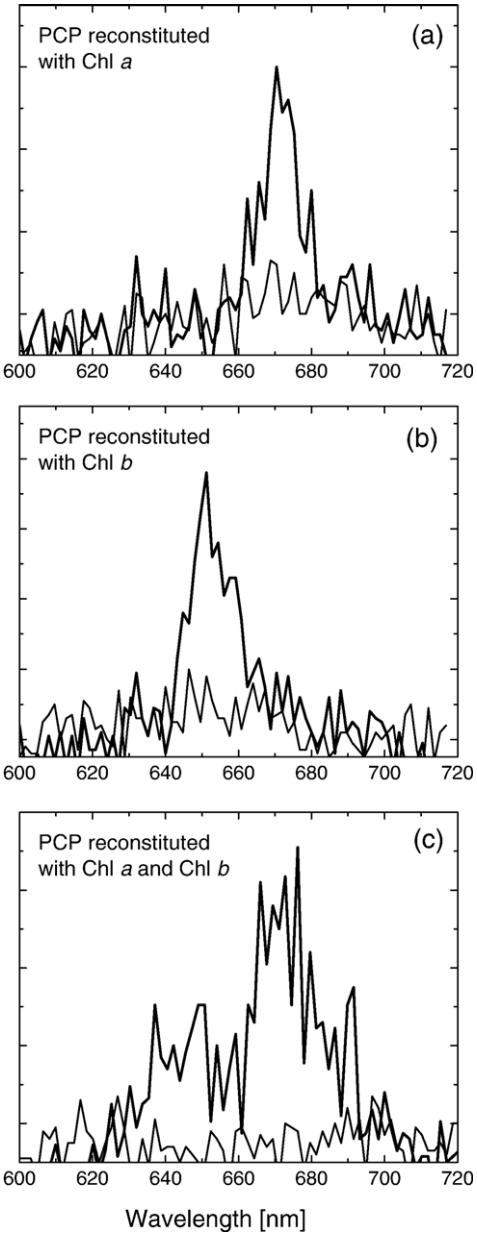

Fig. 4. Representative fluorescence spectra of single PCP complexes reconstituted with: (a) Chl *a*, (b) Chl *b*, and (c) Chl *a* and Chl *b*. In all cases the excitation wavelength and the power is equal to 532 nm and 10 μW, respectively. The bottom curves are the background.

distributions of the central wavelength measured for over 150 complexes reflect quite well the fluorescence spectra of the respective ensemble (data not shown).

Remarkably, the emission of single PCP complexes reconstituted with *both* Chl *a* and Chl *b* is in most cases (~60%) composed of two lines at around 670 nm and 650 nm (Fig. 4c). This implies that, as a result of reconstitution, single PCP complexes contain two different chromophores: Chl *a* and Chl *b*. Such a conclusion can only be proved through a SMS experiment. The simultaneous observation of two emission lines is a direct consequence of the excitation approach where the Chl are excited via sub-picosecond energy transfer from Per molecules. This strategy reduces the impact of Förster energy transfer between the Chl, which is in the order to tens of picoseconds. Obviously for a system, where the energy transfer between Chl *b* and Chl *a* molecule were a dominant process, one would expect fast thermalization from high-energy Chl *b* to low-energy Chl *a*. In such a case the fluorescence emission would originate almost exclusively from Chl *a* and demonstration of the presence of two different Chl within the PCP monomer would require more sophisticated analysis. However, as found recently (S. Mackowski, S. Wörmke, T. H. P. Brotosudarmo, C. Jung, R. G. Hiller, H. Scheer, C. Bräuchle, unpublished results), the energy transfer in Chl *a*/*b*–N-PCP complex occurs not only from Chl *b* to Chl *a* but also in the less energetically preferred reversed direction with a comparable rate. The presence of the bilateral energy transfer, which is partially responsible for simultaneous observation of both Chl *a* and Chl *b* fluorescence, is due to the energy separation between Chl *a* and *b* being small relative to the broadening of absorption and emission lines.

We observe some variation of the relative intensity ratio between Chl *a*- and Chl *b*-related fluorescence between complexes. These changes could originate from at least two sources. On the one hand, we have no control of the orientation of the PCP complexes on the surface. Therefore, one would expect differently oriented transition dipole moments, which would change from one complex to the other. Such an effect could clearly be responsible for variations in the relative intensity of the fluorescence emission. On the other hand, due to relatively long acquisition time of our single molecule experiment (0.3 s), we are not sensitive to dynamics that might occur on a shorter timescale. For instance, the bleaching of one of the Chl molecules during the acquisition time would diminish the overall integrated fluorescence intensity of this line.

In the case of PCP reconstituted with both Chl *a* and Chl *b*, apart from fluorescence spectra consisting of two spectrally resolved lines, we also observe the spectra featuring only a single line. We attribute these to PCP complexes containing either two identical chromophores or the PCP complexes with only single chromophore, either Chl *a* or Chl *b*. Since the reconstitution procedure is random in nature, formation of such complexes is expected.

2.4. Interaction between chlorophylls within a PCP monomer

In Fig. 5a we show a sequence of 25 fluorescence spectra measured subsequently for a single PCP complex reconstituted

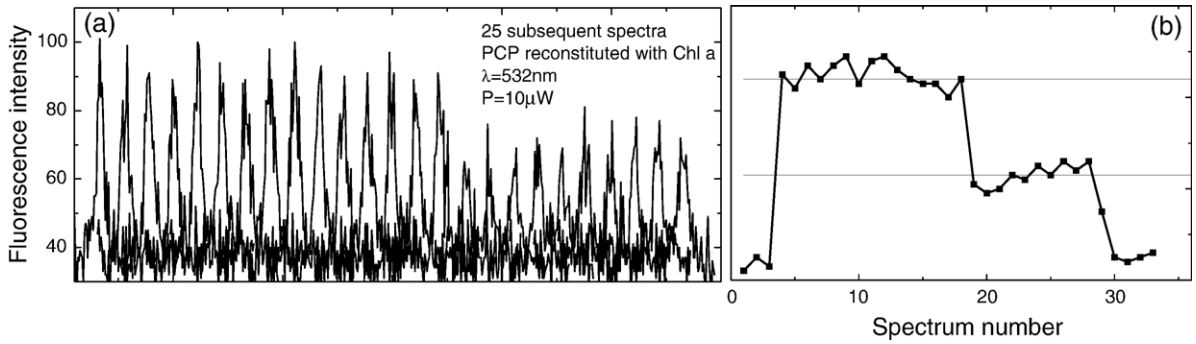

Fig. 5. Representative fluorescence intensity trace measured for a single PCP complex reconstituted with Chl *a*. (a) Sequence of fluorescence spectra measured for a single complex. The averaging time is 0.3 s per spectrum. (b) Corresponding time trace of the fluorescence intensity. Two well-defined levels of the intensity could be identified, as indicated by horizontal lines.

with Chl *a*. The acquisition time of a single spectrum was 0.3 s. An intensity trace obtained for this complex by fitting every spectrum with a single Gaussian line is displayed in Fig. 5b. The trajectory features two clear intensity steps, a behavior observed for approximately 70% of over 150 single PCP complexes reconstituted with either Chl *a* or Chl *b*. It is to some degree surprising that the fluorescence intensity measured consecutively with the acquisition time of 0.3 s features such a regular bleaching behavior. We believe that limited blinking of individual Chl molecules during the measurement could be due to extremely efficient Per quenching of Chl triplet state in PCP [6,7]. In addition to the emission intensity we also measure the fluorescence spectrum of the complex. As an example, in Fig. 6 we show the fluorescence spectra of two single PCP complexes reconstituted with Chl *a*, which exhibit two-step bleaching of the emission intensity, similar to the situation discussed above. The spectra were taken in the sequence indicated by the numbers, and the stepwise drop of the intensity is presented in the central graph of each set. Clearly, in both cases the drop in fluorescence intensity is precisely correlated with pronounced changes in the fluorescence spectrum. Indeed, it appears as each of the two steps seen in the fluorescence

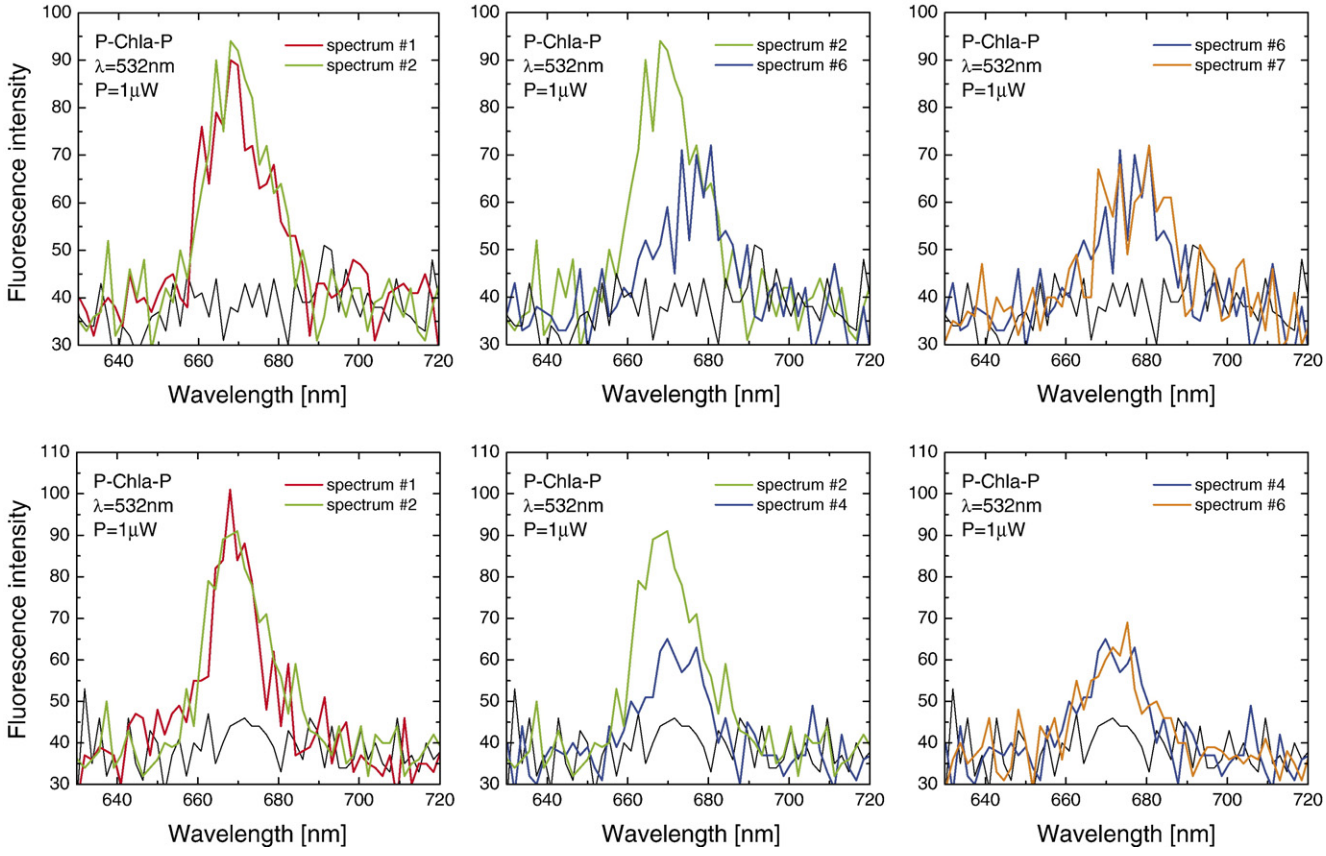

Fig. 6. Relevant parts of fluorescence spectra sequences measured for two different PCP complexes reconstituted with Chl *a*. The upper row shows a complex with large splitting between the chlorophyll emissions, while in the bottom row the splitting is relatively small. The spectra in each case are numbered according to the sequence they were taken. The graphs in the middle display the drop in the fluorescence intensity.

intensity trajectory is due to removal of an individual chromophore that contributes to the emission. Therefore, the combination of intensity and spectral information allows us to unambiguously attribute the stepwise decrease of the fluorescence intensity to subsequent bleaching of two *distinguishable* Chl *a* molecules within the PCP monomer. Importantly, as displayed in Fig. 6, the bleaching of one of the Chl molecules *does affect* neither the energy nor the intensity of the fluorescence attributed to the second Chl. We note that, similarly as in the case of PCP reconstituted with both Chl *a* and Chl *b*, the observation of two Chl *a* molecules in a single monomer is possible when exciting into the Per absorption band. Indeed, the Förster energy transfer between Chl a molecules is about 10 ps [8], which is still significantly longer than the time characteristic for the energy transfer from Per to Chl.

A key conclusion that can be drawn from these results concerns the coupling between the Chls within a PCP monomer. The observation of subsequent bleaching of the two Chl *a* molecules in reconstituted PCP complexes, together with the insensitivity of the fluorescence emission of the second Chl to the bleaching of the first one, demonstrates weak dipole–dipole coupling between the Chls within a PCP monomer. First of all, it has been shown that oxidized BChl molecules are very efficient quenchers of the fluorescence in LH1 [24] and LH2 [16] complexes. Controlled chemical oxidation, which induces only slight (~2%) changes in the absorption of LH1, leads already to 50% reduction of the fluorescence [24]. Strongly interacting Chl *a* molecules should also exhibit very similar behavior: upon bleaching of one Chl molecule, the fluorescence intensity of the second Chl should be dramatically reduced. In fact, one could even expect that for a pair of strongly interacting Chls the fluorescence intensity would feature only a single step, attributable to an excitonic complex formed by these two molecules. Such a situation has been observed for another water-soluble photosynthetic complex, APC [19], which in its trimeric form contains three pairs of strongly coupled (~100 cm^{-1}) open-chain tetrapyrrole chromophores. As a result, the fluorescence trajectory measured for this complex features three intensity steps [19]. An even more extreme case has been demonstrated for LH2, where the fluorescence originates from an exciton formed by 18 strongly coupled BChl molecules, separated by only 9 Å. The bleaching of the complex takes place in a single step [16,17]. Interestingly, a single LH2 complex, in addition to these strongly coupled BChls, which are entirely responsible for the excitonic character of its fluorescence, contains also a ring of 9 monomeric BChl molecules, separated by 21 Å. It has been concluded, based on low-temperature single molecule excitation spectroscopy, that these 9 BChls do not form an exciton, i.e. they should be weakly coupled [18,25]. However, due to the extremely fast energy transfer from these monomeric BChl molecules to the strongly coupled ring of 18 BChl, they show no fluorescence. Altogether, the fact that fluorescence of PCP originates from weakly coupled Chl molecules distinguishes this light-harvesting complex from most of other antenna systems. This unusual property of the PCP complex reflects presumably the qualitatively different light harvesting strategy, which facilitates carotenoids rather than Chls as major absorbing pigments [2].

The spectra displayed in Fig. 6 demonstrate that two Chl *a* molecules within reconstituted PCP complex can feature significant energy separation. Indeed, although the emitting chromophores as well as their immediate environment are chemically identical, the energy difference between the two emission lines could reach 280 cm^{-1}. This result shows that, in agreement to the findings based on the ensemble spectroscopy [9], the two Chls in PCP can be energetically distinguishable due to moderate differences in their immediate surroundings, which might, in turn, represent local minima in the folding energy landscape of the holoprotein that do not equilibrate during the measurement. In Fig. 7 we present the distribution of the energy splitting between the two Chl *a* molecules obtained for over 120 complexes. The vast majority of the complexes feature measurable splitting, frequently comparable with the linewidth of the fluorescence emission. Importantly, this effect can only be observed for a system where due to weak interaction between the chromophores it is possible to independently monitor their fluorescence.

2.5. Intensity steps for native PCP complexes

Similar to the behavior observed for PCP reconstituted with Chl *a*, fluorescence trajectories measured for single native PCP complexes, which contain six Chl a molecules, also exhibit intensity steps (Fig. 8). The fluorescence spectra detected for five intensity steps as well as the fluorescence intensity measured for every spectrum are displayed in Fig. 8a and b, respectively. Clearly, the fluorescence intensity decreases with time and features stepwise behavior. As shown in Fig. 8c, the intensities measured as a function of time group themselves into several subsets. Due to three times higher number of emitting Chls, an estimation of the exact number of intensity steps for individual native PCP complexes is more difficult compared to the straightforward case

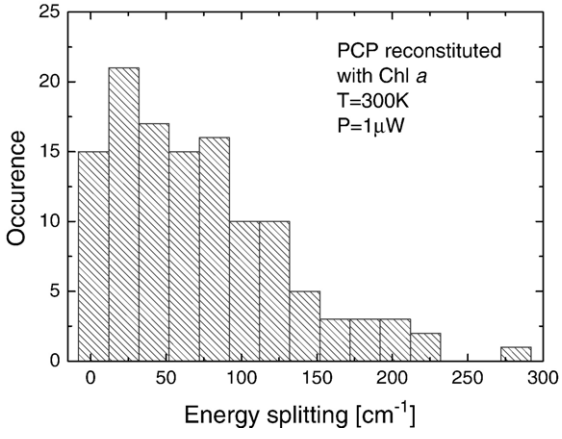

Fig. 7. Statistical distribution of the energy splitting determined for over 120 single PCP complexes reconstituted with Chl *a*.

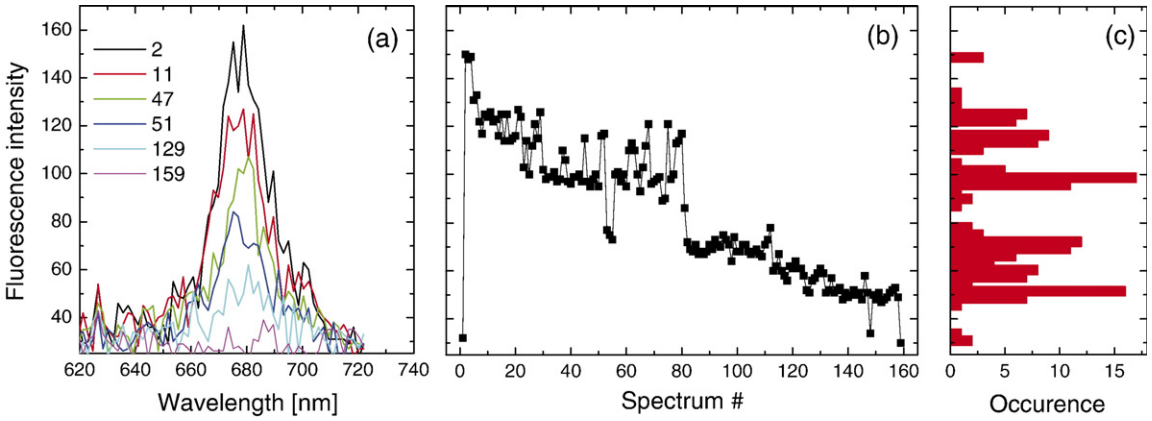

Fig. 8. Fluorescence intensity trace measured for a single native PCP complex. (a) Five spectra measured for this complex at various times of the trace, as described. (b) The whole intensity trace, each spectrum corresponds to 0.3 s. (c) Intensity histogram. The background level is about 30 counts.

of monomeric PCP. Nevertheless, for most of single native PCP complexes the actual number of observed intensity steps varies between four and six. There were again no significant spectral shifts of the fluorescence emission during the measurement (Fig. 8a), which indicates that the bleaching event of one of the chromophores does not influence the spectroscopic characteristics of the remaining fluorescing pigments. This implies weak interaction between Chl within monomers as well as between monomers within a PCP trimer. We note that there are also some native PCP complexes (less than 10%), which exhibit only two-step bleaching. We attribute this behavior to monomeric PCP, some amount of which has been suggested to coexist with the trimeric form of native PCP [2,7]. The results of fluorescence spectroscopy obtained for single native PCP complexes reinforce further the conclusions drawn from experiments performed on the much simpler reconstituted PCP complexes.

3. Conclusions

In conclusion, we have studied single light-harvesting PCP complexes, both native trimers and monomer-like ones, reconstituted with Chl *a*, Chl *b*, or with Chl *a* and Chl *b*, using an approach of SMS, which utilizes energy transfer as an excitation channel. Analysis of the fluorescence intensity traces demonstrate, in contrast to all other light-harvesting complexes, extremely weak dipole–dipole interaction between the Chls responsible for the fluorescence emission. Moreover, through independent fluorescence monitoring of each Chl, we conclude that the Chls in PCP are energetically distinguishable and the splitting can reach 280 cm^{-1}. On the other hand, the two emission lines seen in the fluorescence spectra of single PCP complexes reconstituted with both Chl *a* and Chl *b* are split by 500 cm^{-1}, which indicates the presence of two different Chl molecules in a single PCP monomer. Our findings, based on SMS experiments of PCP complexes, provide new and detailed information necessary to fully understand the energy transfer in this unique light-harvesting system. We also envision that this complex, which offers a possibility to independently monitor the fluorescence of individual Chl molecules, is an ideal system for future studies regarding very vital problem of chromophore–chromophore and protein–chromophore interactions.

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